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Starter Distillate-Volume 4 #86 7/12/74

VOLUME 4

GRAS MONOGRAPH SERIES

STARTER DISTILLATE

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prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION AND WELFARE

JULY 12, 1974

This publication was prepared under Contract Number FDA 72-100 with the Public Health Service, Food and Drug Administration, Department of Health, Education, and Welfare

prepared by

Tracor Jitco, Inc.

May, 1942 Research Bulletin 301

Diacetyl and Acetylmethylcarbinol Production in the Manufacture of Unsalted Butter

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Diacetyl and Acetylmethylcarbinol Production in the Manufacture of Unsalted Butter[1]

By T. I. Hedrick and B. W. Hammer

High-flavored unsalted butter has increased greatly in commercial importance during recent years. This type of butter is churned for table use from either sweet or neutralized sour cream that has been ripened to a relatively high acidity after pasteurization and addition of butter culture. It is distinct from the unsalted butter used in ice cream mixes and certain other products; such butter is made without butter culture from cream having a low acidity at churning.

Without added salt it is necessary that the butter have a high flavor if it is to satisfy consumers. Accordingly, the problems with unsalted table butter differ from those with salted butter. Absence of salt permits development of acidities during cream ripening and holding of the butter which would be very objectionable with salted butter because of the probability of serious chemical defects appearing. Development of acidity in the cream favors production of flavor and also limits various types of bacterial deterioration.

Since diacetyl is such an important contributant to the desirable flavor and aroma of butter, the amount of diacetyl can be used as an index of flavor development in cream or butter. The common assumption that diacetyl is formed by the biological oxidation of acetylmethylcarbinol suggests the determination of acetylmethylcarbinol also. While there may be other compounds, for example volatile acids and esters of fatty acids, contributing to butter flavor, information with reference to them has not advanced to the point where analyses seem advisable.

The data reported herein deal (a) with effects of acidity, added citric acid, agitation, ripening temperature and different butter cultures on diacetyl and acetylmethylcarbinol production in cream ripened under laboratory conditions and also under semi-commercial conditions; and (b), in the case of butter

[1]Project 127 of the Iowa Agricultural Experiment Station. The studies were financed in part through a grant from the Iowa State Brand Creameries Inc., of Mason City, Iowa.

0188

made under semi-commercial conditions, with effects of various factors on flavor, with ratios of diacetyl and acetylmethylcarbinol contents of ripened cream and the corresponding butter and with changes in diacetyl and acetylmethylcarbinol contents of butter during holding under different conditions.

HISTORICAL

Studies of various investigators have established the importance of diacetyl as a flavor constituent of butter and have indicated various factors that influence its production in butter cultures by the normal butter culture organisms. The literature along these lines has been repeatedly reviewed and is not considered here.

The manufacture of butter without salt results in a product that from the standpoint of deterioration is definitely different from the usual salted butter. In unsalted butter objectionable chemical changes are less likely to occur than in salted butter; for example, fishiness is unusual in unsalted butter (5), and when it develops excessive amounts of copper often are involved. Without the restraining effect of salt, unsalted butter is relatively susceptible to growth of various organisms, including certain types that have a deteriorating action; however, with organisms that are not relatively acid tolerant, the acid present in the fresh butter, or produced in it during holding, may have a distinct inhibitory influence.

Minster (7) considered the presence of a few lactic acid organisms in unsalted butter as beneficial, since they tended to keep down growth of more objectionable species.

In unsalted butter held at 69.8°F., Nelson and Hammer (9) found large increases in the numbers of butter culture organisms with both plate and microscopic procedures. Striking evidence of the growth was the appearance of long chains of streptococci in the butter. Organisms other than streptococci also showed extensive growth. In unsalted butter held at −4°F., there were conspicuous decreases in the microscopic counts, the individual samples showing decreases from 7 to 98 percent (average 74%); the largest decreases occurred with samples having the highest counts when they were placed at −4°F. and the smallest decreases with samples having the lowest initial counts.

Slatter and Hammer (14) noted that in unsalted butter made from sweet cream with butter culture there commonly was a striking production of acetylmethylcarbinol plus diacetyl when the butter was held at temperatures permitting activity of the butter culture organisms; also, the pH of the butter markedly decreased. Production of the carbinol plus diacetyl varied

widely with different lots of butter under the same conditions. The largest production occurred at the higher holding temperatures and in samples which developed the lowest pH values. Production usually was greater with a large addition of butter culture to the cream than with a small addition, and in some cases it was increased conspicuously by adding citric-acid-fermenting streptococci along with the usual butter culture. Occasionally the carbinol plus diacetyl decreased during the first few days of holding; this decrease sometimes was followed by an increase. Also, a striking production of the carbinol plus diacetyl sometimes was followed by a decrease.

Wiley (16) reported that at 40° or 65°F. deterioration of unsalted butter through bacterial action was not delayed by the presence of acid in the butter. At 0° or 14°F. the greatest deterioration was observed in butter from cream ripened to pH 5 (about 0.4% acid); butter from cream acidified to the same point by addition of pure lactic acid did not deteriorate to the same extent. Fat of the butter from the ripened cream showed notable oxidation after cold storage, but that from the acidified cream did not. In a later study Wiley (17) found that acidity, butter culture organisms, salt and low pasteurization temperatures each favored oxidation of the fat of butter during cold storage, while diacetyl and acetylmethylcarbinol did not. He concluded that the results indicate the presence in ripened pasteurized cream and in unripened raw cream of a fat-oxidizing enzyme which is most active at low pH values (about 5) and high salt concentrations.

In studies on changes in diacetyl and acetylmethylcarbinol contents of butter at various temperatures, Prill and Hammer (13) found that with salted butter which was frozen or at 36° to 45°F. only slight changes occurred; even at 70°F. the contents of the compounds were largely retained for considerable periods. With unsalted butter at −10° to 0°F. the changes were relatively slight, but at 36° to 45°F. or at 70°F. significant changes occurred, both increases and decreases being involved.

GENERAL PROCEDURE

Trials were carried out both in the bacteriological laboratory and in the butter manufacturing plant of the Iowa State College, although even in the latter case the trials were on a relatively small scale (semi-commercial). The general methods employed in the two types of trials are as follows:

LABORATORY TRIALS

The cream was pasteurized in the regular operations of the butter plant, a 300-gallon vat and 150°F. for 30 minutes being employed. The butterfat contents ranged from 32 to 37

0188

percent. The sweet cream had an initial acidity of 0.20 percent or less; after pasteurization it was neutralized (at about 120°F.) to approximately 0.10 percent acid with sodium sesqui-carbonate. The sour cream had an initial acidity of 0.40 to 0.50 percent; before pasteurization this was reduced (at about 90°F.) to 0.25 percent with "Alkali Special" and after pasteurization the reduction was continued (at about 120°F.) to 0.15 percent with sodium sesqui-carbonate. The cream was cooled to 36°F., and a portion was removed to a container that had been thoroughly steamed. Five percent butter culture by weight was added, and then 800-gram portions of the mixture were put into sterile quart jars having glass tops. The portions were warmed to the desired ripening temperatures and incubated at such temperatures until the desired acidities had developed. In order to avoid agitation of the 800-gram portions during the ripening, which would influence the diacetyl contents by incorporating air, a 250-gram portion of the mixture, in a pint bottle, was held at each of the ripening temperatures and used to follow the increase in acid. When the desired acidity was reached in a 250-gram portion, 300 grams were removed from the 800-gram portion and the various analyses made. The remaining 500 grams were churned in a small laboratory churn, but the difficulty of controlling the operation resulted in such irregularities in the retention of buttermilk in the butter that the data are not considered in detail.

SEMI-COMMERCIAL TRIALS

The cream was pasteurized in the regular operations of the butter laboratory, either in a 300-gallon vat at 150°F. for 30 minutes or with a vacreator (2) using a temperature of 198°F. and a final vacuum of about 28 inches. Fat contents, acidities and neutralization procedures were essentially the same as with the cream employed in the laboratory trials. The pasteurized cream was cooled to 36°F.

For each churning 150 pounds of cream was transferred to a small vat, and 5 percent butter culture by weight was added. Usually the mixture of cream and culture was heated to the ripening temperature and held over night, but in trials on effect of temperature the ripening period varied. The cream increased in temperature (sometimes as much as 10°F.) during the ripening. When the ripening was complete the temperature was lowered for churning to approximately 48°F., and a sample was taken for the various analyses.

Each lot of cream was churned in a small single roll churn having a normal capacity of about 60 pounds of butter. The cream was churned until the butter granules were about the

size of peas. After draining the buttermilk, the granules were rinsed with water until the water from the drain did not have a milky appearance. Two washings with water about 5°F. colder than the buttermilk were employed. The free moisture was drained, and an attempt was made to incorporate approximately 17 percent moisture in the finished butter.

Several 1-pound samples of butter were taken from each churning and wrapped in parchment paper. These were held under different conditions for various periods and then examined.

ANALYTICAL METHODS

ACID AND pH DETERMINATIONS

Acid determinations were made by titrating 9 grams of cream without dilution, using 0.1 normal sodium hydroxide and phenolphthalein; results were calculated as lactic acid to the nearest 0.01 percent. pH determinations were made with a potentiometer, a modified quinhydrone electrode and a calomel half-cell; values were calculated to the nearest 0.01 of a pH unit.

DIACETYL AND ACETYLMETHYLCARBINOL DETERMINATIONS

Cream and butter were analyzed for diacetyl and acetylmethylcarbinol by the colorimetric method of Prill and Hammer (10), with a few minor modifications used by Hoecker and Hammer (4). Also, the color intensities were determined with a Klett-Summerson photoelectric colorimeter. Results were recorded as parts per million (p.p.m.) of diacetyl or acetylmethylcarbinol. Actually the p.p.m. of acetylmethylcarbinol represent acetylmethylcarbinol plus diacetyl, but since the amount of diacetyl is insignificant compared to the amount of carbinol, this procedure did not materially affect the acetylmethylcarbinol values.

EXPERIMENTAL

EFFECTS OF VARIOUS FACTORS ON DIACETYL AND ACETYLMETHYLCARBINOL CONTENTS OF RIPENED CREAM

EFFECT OF RIPENING CREAM TO VARIOUS ACIDITIES

LABORATORY TRIALS

Results of 36 trials on sweet cream ripened to various acidities at temperatures from 50° to 70°F. are given in table 1. Each trial included either three or four acidities; in six trials a mixture of cream and culture also was held at 32°F. for a normal ripening period.

0188

As the acidity of the cream increased, the diacetyl content increased in 29 trials (81%), and the acetylmethylcarbinol content increased in 32 trials (89%). Most of the increases were significant, but some of them were very small. Both diacetyl and acetylmethylcarbinol increases were small in the pH range of 6.5 to 5.5.

The diacetyl content decreased in two trials (6%). One of the decreases was small, from 0.57 to 0.44 p.p.m.; the other was larger, from 0.46 to 0.19 p.p.m., and may have been caused by an abnormal culture. The acetylmethylcarbinol content also decreased in two trials (6%), the decrease being large in each case; one of the decreases was in a trial that showed a decrease in diacetyl content.

An increase in diacetyl content followed by a decrease occurred in three trials (8%). One of the decreases was small, being 0.15 p.p.m.; and one was rather large, being 0.46 p.p.m. In the three trials corresponding changes in acetylmethylcarbinol did not occur, but in another trial (3%) the carbinol content increased and then decreased very slightly, from 29.8 to 29.0 p.p.m.

There was an increase in diacetyl content, then a decrease and finally an increase in two trials (6%) and a similar series of changes in carbinol in one trial (3%). The decreases were small in all cases, and in one of the changes in diacetyl the final increase was large.

The diacetyl contents of the six lots of cream plus butter culture held during a normal ripening period at 32°F. ranged from 0.12 to 0.28 p.p.m. with an average of 0.22 p.p.m.; the acetylmethylcarbinol contents varied from 3.5 to 16.5 p.p.m. and averaged 10.6 p.p.m.

Table 2 gives results of three trials on neutralized sour cream ripened to various acidities at temperatures from 58° to 62°F. Three acidities were used in each of two trials, and four acidities were employed in one trial; in each trial a mixture of cream and culture was held at 32°F. for the normal ripening period.

An increase in acidity resulted in an increase in diacetyl and also in acetylmethylcarbinol in each case. The increases in diacetyl regularly were conspicuous, and in two of the trials the increases in acetylmethylcarbinol were large.

The diacetyl and acetylmethycarbinol contents of the three lots of neutralized sour cream plus butter culture held at 32°F. generally were higher than those of sweet cream plus culture, the average diacetyl content being 0.42 p.p.m. (range from 0.35 to 0.55 p.p.m.) compared to 0.22 p.p.m. for the sweet cream and the average carbinol content being 22.1 p.p.m. (range from 20.9 to 23.3 p.p.m.) compared to 10.6 p.p.m. for the sweet cream.

This relationship probably is explained by the development of the two compounds during the souring of the cream.

SEMI-COMMERCIAL TRIALS

Results of 10 trials on cream (seven on sweet cream, three on sour neutralized cream) ripened to various acidities at temperatures from 50° to 55°F. are presented in table 3. Three acidities were employed in three of the trials with sweet cream and two acidities were used in the remaining trials. In two trials with sour cream, determinations were made on the cream just after pasteurization and again after the addition of culture.

With an increase in acidity there was an increase in diacetyl in nine trials (90%) and in acetylmethylcarbinol in seven trials (70%). Some of the increases were small. The diacetyl decreased in one trial (10%), the drop being from 1.88 to 1.49 p.p.m. with a change in acidity from 0.37 to 0.49 percent. The acetylmethylcarbinol decreased in three trials (30%), in one instance after a striking increase. Two of these trials involved sour cream in which the citric acid may have been completely fermented; the cream had relatively high diacetyl and acetylmethylcarbinol contents before the addition of butter culture, containing 0.21 p.p.m. diacetyl and 21.7 p.p.m. acetylmethylcarbinol in one trial and 0.56 and 29.8 p.p.m., respectively, in the other. With no citric acid present reduction of diacetyl and acetylmethylcarbinol by the citric-acid-fermenting organisms in butter cultures would be expected.

EFFECT OF ADDED CITRIC ACID

In the trials involving addition of citric acid to the cream, the acid was added as a sterile aqueous solution at the time of inoculating the butter culture.

LABORATORY TRIALS

Fourteen trials were carried out in which sweet cream was ripened with and without added citric acid to about the same acidity at temperatures from 54° to 60°F.; the amount of citric acid used was 0.05 percent except in one trial where it was 0.10 percent. Table 4 gives the results.

Addition of citric acid resulted in a higher diacetyl content in all the trials (100%) and in a higher acetylmethylcarbinol content in 12 trials (86%). With both diacetyl and acetylmethylcarbinol some of the increases were very small.

Eight trials were carried out in which neutralized sour cream was ripened with and without citric acid to about the same acidity at temperatures from 58° to 63°F.; in six of the trials three quantities of citric acid were used and in two trials only one quantity was employed. The results are given in table 5.

In six trials (75%) added citric acid resulted in an increase

0188

in the diacetyl content that, in the four trials involving three amounts of acid, was roughly in proportion to the amount added. In one trial (12.5%) 0.05 and 0.10 percent added citric acid did not increase the diacetyl content but 0.15 percent did, and in one trial (12.5%) 0.10 and 0.15 percent added acid gave the same diacetyl content. The added citric acid regularly increased the acetylmethylcarbinol content, and in the trials in which three amounts were used the increase was roughly in proportion to the amount added.

SEMI-COMMERCIAL TRIALS

Data on seven trials (four on sweet cream and three on neutralized sour cream) in which cream was ripened with and without added citric acid (0.05%) to about the same acidity at temperatures from 50° to 54°F. are given in table 6.

The added citric acid increased the diacetyl content in six comparisons (86%). The increases varied widely, with three of them being 0.20 p.p.m. or less. The one decrease in diacetyl content was very small, being 0.13 p.p.m. Added citric acid increased the acetylmethylcarbinol content in three comparisons (43%), the increases ranging from 12.8 to 34.8 p.p.m. In one trial (14%) the carbinol content was the same with and without added citric acid. In the three comparisons (43%) in which addition of the acid resulted in a decrease in the carbinol content, the decreases ranged from 4.0 to 15.8 p.p.m.

EFFECT OF AGITATION DURING RIPENING

LABORATORY TRIALS

In nine trials (seven with sweet cream and two with neutralized sour cream) cream was ripened with and without agitation at 60°, 63° or 70° F. Cream held at 60° or 63° F. was agitated three times during the ripening and that held at 70°F. was agitated every hour for 3 or 4 hours. Table 7 gives the data.

In each of the trials the diacetyl and acetylmethylcarbinol contents of the agitated cream were much higher than those of the unagitated cream. Essentially the same acidity developed with and without agitation.

The effect of agitation with added citric acid was studied in six trials (four with sweet cream and two with neutralized sour cream). One-tenth percent citric acid was used, and the ripening temperature was 60°, 63° or 70°F. The results are presented in table 8.

As without addition of citric acid, agitation greatly increased both the diacetyl and acetylmethylcarbinol contents. It had no appreciable effect on the acid development.

SEMI-COMMERCIAL TRIALS

Five trials were carried out (three with sweet cream and two with neutralized sour cream). The cream with the butter culture added was placed in the vats late in the afternoon and ripened over night. The next morning, approximately 3 hours before the ripening was complete, the cream was agitated by revolving the coils for one-half hour. Since the vats were only one-third full, the agitation was relatively vigorous. The data are given in table 9.

As in the laboratory trials the diacetyl and acetylmethylcarbinol contents were greatly increased by the agitation. The acid development was not appreciably influenced.

EFFECT OF RIPENING TEMPERATURE

LABORATORY TRIALS

In 18 trials sweet cream was ripened at 50°, 60° and 70°F. For each trial cream containing culture was warmed to these temperatures and ripened in constant temperature incubators to approximately the same acidity. The ripening periods varied from 5 to 45 hours, depending on the temperature and on the degree of acidity desired. Determinations of diacetyl and acetylmethylcarbinol were made soon after the desired acidity was reached. Table 10 presents the data.

The highest diacetyl content developed at 50°F. and the lowest diacetyl content at 70°F. in 14 (78%) of the trials;[2] the same relationship occurred with acetylmethylcarbinol in 11 trials (61%). Both diacetyl and acetylmethylcarbinol production were highest at 50°F. and lowest at 60°F. in two trials (11%). The highest diacetyl content was produced at 60°F. and the lowest at 70°F. in one trial (6%), and the same relationship occurred with the carbinol content in five trials (28%). Diacetyl production was highest at 70°F. and lowest at 60°F. in one trial (6%). Variations in acetylmethylcarbinol production at the three ripening temperatures were small and definitely less on a percentage basis than variations in diacetyl production.

SEMI-COMMERCIAL TRIALS

Since it was impossible to control the ripening temperature adequately in the small vats, cream containing culture was warmed to the desired temperature and then drawn into 10-gallon cans. The cans were held in a refrigerator at about the desired temperature until the desired acidity was reached. The temperature comparisons regularly involved cream ripened at 62° and 70°F. to about the same acidity; 55°F. was used only once because it required such an extended ripening period.

[2]Equal diacetyl and acetylmethylcarbinol values are not considered separately but are fitted into the general statements.

On reaching the desired acidity, cream ripened at 70°F. was cooled by placing the cans in ice water and stirring; after reaching 45°F. the cream was held over night at 36° to 40°F. The next morning the cream was warmed to 48°F. and churned. The cream ripened at 62°F. reached the desired acidity after ripening over night. The cans were placed in ice water, cooled to 48°F. and the cream then churned. Four trials were carried out, three with sweet cream and one with neutralized sour cream. Table 11 presents the data.

Diacetyl production in the cream ripened at 62°F. was larger than in cream ripened at 70°F. in two trials (50%) and smaller in two trials (50%). Acetylmethylcarbinol production showed similar variations. The differences in production of diacetyl and acetylmethylcarbinol in a comparison commonly were small, but in one trial involving a relatively low acidity the differences were large. Ripening at 55°F. gave slightly lower diacetyl and acetylmethylcarbinol contents in the one trial in which it was used.

In three trials a special vat of cream was ripened along with the cream used for the temperature comparisons; the treatment of the cream consisted of adding 0.1 percent citric acid and agitating during the ripening over night to an acidity of 0.45 percent. Diacetyl and acetylmethylcarbinol production in this cream was much larger in each trial than in the cream ripened at either 70°F. or 62°F.

EFFECT OF DIFFERENT BUTTER CULTURES

In various trials, both laboratory and semi-commercial, different butter cultures were employed to ripen cream, which sometimes was sweet and sometimes was sour. In certain trials the cultures came from plants receiving commercial cultures from the same laboratory, and in other trials they came from plants receiving commercial cultures from different laboratories. The cultures from the plants were transferred, usually twice, to have them in an active condition before they were inoculated into the cream. In each trial an attempt was made to have the different cultures develop essentially the same acidity in the cream, and the general conditions of ripening were kept uniform except that the incubation periods necessarily varied because of differences in the rates of acid development by the cultures.

The diacetyl and acetylmethylcarbinol contents developed by the different cultures varied rather widely in some trials and were rather uniform in others. When the same cultures were used in several trials they did not always rank the same on the basis of production of diacetyl or acetylmethylcarbinol, and the variations were not explainable on the basis of the slight differences in acidities of the ripened cream. Also, with the

same cultures being used in several trials, all of them sometimes showed relatively high values in a trial and sometimes relatively low values.

RELATIONSHIP OF DIACETYL AND ACETYLMETHYLCARBINOL CONTENTS OF RIPENED CREAM

In general, as the diacetyl increased during the ripening of cream the acetylmethylcarbinol also increased, but in some instances an increase in diacetyl was accompanied by a decrease in the carbinol. Commonly, increases in diacetyl and acetylmethylcarbinol were in the same general proportion, but again there were exceptions to this relationship. The occasional decreases in diacetyl often were accompanied by increases in acetylmethylcarbinol, and when there was no change in diacetyl there often was an increase in the carbinol.

EFFECT OF VARIOUS RIPENING PROCEDURES ON FLAVOR OF UNSALTED BUTTER

Much of the unsalted butter made under semi-commercial conditions from cream ripened with various procedures was scored for flavor after 1 week at 36° to 40°F.; after 1 month at 36° to 40°F.; and after 6 months at −10° to 0°F. Usually two judges[3] scored the butter without knowledge of its identity, but in a few instances only one judge was available. Since the scoring of unsalted butter is not well standardized, comparative scores rather than absolute scores are given.

EFFECT OF RIPENING ACIDITY

AFTER 1 WEEK AT 36° TO 40°F.

Ripening the cream to the higher of the two acidites used in each trial gave butter which scored 0.25 to 0.75 of a point higher than that from the cream ripened to the lower acidity in five (71%) of the seven trials. There was no difference in score in one trial (14%) in which the acidities of the two lots of cream differed only 0.04 percent (0.31 and 0.35%); both lots of butter were criticized as tallowy and this off-flavor probably tended to mask the desirable flavor. With the higher acidity the butter scored 0.25 of a point lower in one trial (14%) in which the difference in acidities of the two lots of cream was small although both were relatively high (0.48 and 0.51%).

In the two trials in which the lots of cream had the greatest differences in acidity, 0.08 percent (0.38 and 0.46%) and 0.12 percent (0.37 and 0.49%), variations in the scores of the butter

[3]Dr. N. E. Fabricius and Dr. H. C. Olson did much of the scoring.

0782

were the largest, being 0.75 of a point higher in each case with the higher cream acidity.

AFTER 1 MONTH AT 36° TO 40°F.

With the higher acidity the butter scored 0.25 to 0.5 of a point higher in three trials (43%). No difference in score was noted in two trials (29%). With the higher acidity the butter scored 0.5 of a point lower in two trials (29%). After the holding the scores were 0.25 to 1.5 points lower than after only 1 week at 36° to 40°F. except in one instance in which the score of the butter made from the higher acid cream increased 0.25 point.

AFTER 6 MONTHS AT −10° TO 0°F.

Ripening to the higher acidity gave butter which scored from 0.25 to 0.75 of a point higher in all the trials (100%). Following the holding the scores usually were 0.25 to 2.5 points lower than after 1 week at 36° to 40°F., but there were increases in score (0.5 to 1.0 point) in three instances, one involving butter from the lower acid cream and two involving butter from the higher acid cream.

EFFECT OF ADDED CITRIC ACID

AFTER 1 WEEK AT 36° TO 40°F.

Addition of citric acid to the cream before ripening resulted in a score increase of 0.25 to 0.5 of a point in four (57%) of the seven trials. No difference in score was noted in three trials (43%); in two of these sour cream was used and various off-flavors tended to mask the desirable flavor, while in the other addition of citric acid resulted in such a slight flavor improvement that no higher score was given.

AFTER 1 MONTH AT 36° TO 40°F.

With added citric acid there was a score improvement of 0.25 to 0.75 of a point in six trials (86%). There was no difference in score in one trial (14%). Following the holding the scores commonly were 0.25 to 2.0 points lower than after only 1 week at 36° to 40°F., but with three lots of butter, one made without acid added to the cream and two made with added acid, the scores increased from 0.25 to 0.75 of a point.

AFTER 6 MONTHS AT −10° TO 0°F.

Addition of citric acid resulted in a score increase of 0.5 to 0.75 of a point in six trials (86%). It resulted in a score decrease of 0.5 of a point in one trial (14%). After the holding the butter usually scored 0.5 to 2.25 points lower than after 1 week at 36° to 40°F., although in three instances, one involving butter made without acid added to the cream and two

involving butter made with added acid, the scores increased from 0.5 to 1.25 points.

EFFECT OF AGITATION DURING RIPENING

AFTER 1 WEEK AT 36° TO 40°F.

Agitation of the cream during ripening gave a score increase of 0.25 to 0.75 of a point in three (60%) of the five trials. There was no difference in score in two trials (40%); this may have been due to off-flavors in the butter which tended to mask the desirable flavor.

AFTER 1 MONTH AT 36° TO 40°F.

With agitation of the cream there was a score increase of 0.5 to 0.75 of a point in two trials (40%). No difference in score occurred in two trials (40%); there seemed to be a tendency for some of the butter from agitated cream to have a slight oxidized flavor. Agitation resulted in a score decrease of 0.25 of a point in one trial (20%). After the holding the butter commonly scored 0.25 to 1.5 points lower than after only 1 week at 36° to 40°F., but in two instances, one involving butter from unagitated cream and one involving butter from agitated cream, there were score increases; these were 0.25 and 0.75 of a point.

AFTER 6 MONTHS AT −10° TO 0°F.

Agitation of the cream resulted in a score increase in four trials (80%); three of the increases were 1.0 point, and the other was 0.25 of a point. There was no difference in score in one trial (20%). Following the holding the scores were 0.25 to 2.0 points lower than after 1 week at 36° to 40°F.

EFFECT OF RIPENING TEMPERATURE

AFTER 1 WEEK AT 36° TO 40°F.

Ripening of the cream at 62°F., rather than at 70°F., gave a score increase of 0.25 to 0.75 of a point in two (50%) of the four trials. There was no difference in score in one trial (25%). Ripening at 62°F. resulted in a score decrease of 0.5 of a point in one trial (25%).

AFTER 1 MONTH AT 36° TO 40°F.

With ripening at 62°F. there was a score increase of 0.5 to 0.75 of a point in each of the three trials (100%). Following the holding the butter scored from 0.25 to 0.75 of a point lower than after only 1 week at 36° to 40°F. except in two cases involving butter made from cream ripened at 62°F.; in one of

these cases there was no change in score while in the other there was a score increase of 0.25 of a point.

AFTER 6 MONTHS AT −10° TO 0°F.

Ripening at 62°F. gave a score increase of 0.5 of a point in each of two trials (50%). It gave a score decrease of 0.75 and 1.0 point in two trials (50%). After the holding the scores were 1.0 to 2.75 points lower than after 1 week at 36° to 40°F.

GENERAL RESULTS

The general effects of the various cream ripening procedures on the flavor of the unsalted butter are shown in the following summary:

Factor studied	Butter held: 1 week 36° to 40°F.	1 month 36° to 40°F.	6 months −10° to 0°F.
	No. of trials showing changes		
Ripening acidity			
Higher acidity gave higher score	5	3	7
Higher acidity gave same score	1	2	0
Higher acidity gave lower score	1	2	0
Total	7	7	7
Addition of citric acid to cream			
Citric acid gave higher score	4	6	6
Citric acid gave same score	3	1	0
Citric acid gave lower score	0	0	1
Total	7	7	7
Agitation during ripening of cream			
Agitation gave higher score	3	2	1
Agitation gave same score	2	2	1
Agitation gave lower score	0	1	0
Total	5	5	5
Ripening temperature, 62° and 70°F.			
62°F. gave higher score	2	3	2
62°F. gave same score	1	0	0
62°F. gave lower score	1	0	2
Total	4	3	4

The data in the summary, together with the actual differences in scores of comparative lots of butter, were analyzed

with the usual statistical methods, and the results used in the interpretations. In some of the comparisons a particular ripening procedure evidently was beneficial from the standpoint of the score of the butter under certain holding conditions, while in others it was not.

The higher acidity in the cream was especially useful with the butter held 6 months at −10° to 0°F. and was of some value with the butter held 1 week at 36° to 40°F.; it was of little value with the butter held 1 month at 36° to 40°F., presumably because during this holding there was an opportunity for extensive growth of the butter culture organisms. Addition of citric acid apparently had a desirable effect under all the holding conditions, as would be expected since this compound is a source of flavoring constituents of butter. Agitation had its most desirable effect with the butter held 6 months at −10° to 0°F. and its least desirable effect with the butter held 1 month at 36° to 40°F. Ripening at 62°F., rather than 70°F., evidently had no desirable effect with the butter held 6 months at −10° to 0°F. or 1 week at 36° to 40°F., but was definitely beneficial with the butter held 1 month at 36° to 40°F.

RATIOS OF DIACETYL AND ACETYLMETHYLCARBINOL CONTENTS OF THE RIPENED CREAM AND OF THE CORRESPONDING BUTTER

With 74 semi-commercial churnings (some not included in tables) the minimum, maximum and average ratios of the diacetyl and the acetylmethylcarbinol contents of the ripened cream and of the corresponding butter are as follows:

	Ratio of content of cream to content of butter		
	Minimum	Maximum	Average
Diacetyl	1:0.142	1:0.709	1:0.352
Acetylmethylcarbinol	1:0.059	1:0.683	1:0.217

The average ratio was considerably higher with diacetyl than with acetylmethylcarbinol; the less significant minimum and maximum ratios also were higher with diacetyl.

When the data are divided into groups on the basis of the factor whose effect on the diacetyl and acetylmethylcarbinol contents of ripening cream was being studied, the following values are obtained:

Factor studied	No. trials	Ratio of content of cream to content of butter		
		Minimum	Maximum	Average
Ripening acidity	23			
Diacetyl		1:0.164	1:0.652	1:0.390
Acetylmethylcarbinol		1:0.065	1:0.385	1:0.215
Added citric acid	14			
Diacetyl		1:0.142	1:0.547	1:0.328
Acetylmethylcarbinol		1:0.105	1:0.625	1:0.215
Agitation	10			
Diacetyl		1:0.165	1:0.395	1:0.300
Acetylmethylcarbinol		1:0.059	1:0.297	1:0.179
Ripening temperature	12			
Diacetyl		1:0.206	1:0.709	1:0.388
Acetylmethylcarbinol		1:0.107	1:0.683	1:0.244
Different butter cultures	15			
Diacetyl		1:0.210	1:0.486	1:0.322
Acetylmethylcarbinol		1:0.152	1:0.391	1:0.227

With each of the groups the average ratio was considerably higher with diacetyl than with acetylmethylcarbinol. There was reasonably close agreement between the values for the different groups; the lowest average ratio for both diacetyl and the carbinol occurred when the effect of agitation was investigated. The minimum and maximum ratios also were higher with diacetyl than with the carbinol except in the case of the maximum values when the effect of added citric acid was studied, also, there was little difference between the minimum values for diacetyl and acetylmethylcarbinol when the effect of added citric acid was investigated and little difference between the maximum values when the effect of ripening temperature was studied.

As already indicated the difficulty in controlling the churning of small volumes of cream in the laboratory resulted in such irregularities in the retention of buttermilk in the butter that the data are not considered in detail. This irregularity is suggested by the following ratios of the diacetyl and acetylmethylcarbinol contents of the cream and of the butter with 309 laboratory churnings (some not included in tables):

	Ratio of content of cream to content of butter		
	Minimum	Maximum	Average
Diacetyl	1:0.111	1:2.080	1:0.725
Acetylmethylcarbinol	1:0.054	1:0.961	1:0.339

The average ratios are considerably higher than with the 74 semi-commercial churnings; the maximum ratios also are higher while the minimum ratios are slightly lower. The values regularly were higher for diacetyl than for the carbinol.

CHANGES IN DIACETYL AND ACETYLMETHYLCARBINOL CONTENTS DURING HOLDING OF BUTTER

As would be expected from the variations in the ratios of diacetyl and acetylmethylcarbinol contents of ripened cream and of the corresponding butter, the diacetyl and acetylmethylcarbinol contents of a series of lots of butter sometimes did not follow the same order as the contents of the lots of cream from which the butter was churned. The irregularities were of all possible types. In some cases the diacetyl or acetylmethylcarbinol content of a lot of butter was lower than that of an earlier lot in the series although the content of the cream was higher. In other cases a value for diacetyl or acetylmethylcarbinol was lower than the values on the lots preceding and following it in the series, even when there was no drop in the value for the cream. Still other types of variations occurred. Because of these irregularities the diacetyl and acetylmethylcarbinol contents of the lots of butter are not given in detail. The diacetyl contents of the semi-commercial churnings of butter ranged from 0.16 to 1.46 p.p.m., while the acetylmethylcarbinol contents varied from 2.9 to 24.0 p.p.m. The highest values for both diacetyl and acetylmethylcarbinol were obtained on a lot of butter made from cream ripened with agitation after addition of 0.1 percent citric acid. Considerably higher values for diacetyl and acetylmethylcarbinol were obtained on butter from the laboratory churnings, but these undoubtedly were influenced by a high retention of buttermilk.

Changes in the diacetyl and acetylmethylcarbinol contents of butter during holding are of importance because of their probable relationship to development or disappearance of flavor. Changes in the contents of some of the semi-commercial churnings during 1 week at 36° to 40°F., during 3 days at 60°F. plus 4 days at 36° to 40°F. and during 1 month at 36° to 40°F. are shown in the following summary:

Compound and type of change	Holding conditions: 1 week 36° to 40° F.	3 days 60° F. plus 4 days 40° F.	1 month 36° to 40° F.
	No. of samples showing changes		
Diacetyl			
Increases total	47	45	39
0.10 p.p.m. or less	11	5	6
.11 to .20 p.p.m.	9	7	6
.21 to .30 p.p.m.	17	6	7
.31 to .92 p.p.m.	10	27	20
No change total	2	0	0
Decreases total	7	11	4
0.10 p.p.m. or less	2	6	1
.11 to .20 p.p.m.	2	1	2
.21 to .30 p.p.m.	0	0	1
.31 to .78 p.p.m.	3	4	0
Acetylmethylcarbinol			
Increases total	38	37	29
1.0 p.p.m. or less	7	3	4
1.1 to 3.0 p.p.m.	21	9	12
3.1 to 7.0 p.p.m.	9	11	11
7.0 to 23.0 p.p.m.	1	14	2
No change total	0	0	1
Decreases total	18	18	11
1.0 p.p.m. or less	5	4	2
1.1 to 3.0 p.p.m.	11	6	9
3.1 to 7.0 p.p.m.	2	4	3
7.1 to 11.5 p.p.m.	0	4	0

Under each set of holding conditions, both increases and decreases occurred in the diacetyl contents and also in the acetylmethylcarbinol contents; while some of the changes were too small to be of significance, others were relatively large. With each compound under each set of conditions the increases were more numerous than the decreases.

Because of the possibility of ripening butter to increase the diacetyl and acetylmethylcarbinol contents and improve the flavor, just as cream is ripened, the diacetyl and acetylmethylcarbinol contents of butter held 3 days at 60° F. plus 4 days at

36° to 40° F. and of butter held 1 week at 36° to 40° were compared. The results are given in the following summary:

Diacetyl	No. trials	Acetylmethylcarbinol	No. trials
Higher values with some holding at 60° F.			
Increases total	42	Increases total	40
0.10 p.p.m. or less	19	1.0 p.p.m. or less	8
.11 to .20 p.p.m.	10	1.1 to 3.0 p.p.m.	12
.21 to .30 p.p.m.	8	3.1 to 18.2 p.p.m.	20
.31 to .69 p.p.m.	5		
Same value with some holding at 60° F.			
Total	1	Total	4
Lower values with some holding at 60° F.			
Decreases total	13	Decreases total	11
0.10 p.p.m. or less	7	1.0 p.p.m. or less	0
.11 to .20 p.p.m.	3	1.1 to 3.0 p.p.m.	4
.21 to .30 p.p.m.	0	3.1 to 12.8 p.p.m.	7
.31 to .64 p.p.m.	3		

The summary shows that the short holding at 60°F. resulte in a higher diacetyl content in 42 (75%) of the 56 trials, n change in one trial (2%) and a decrease in 13 trials (23%. The acetylmethylcarbinol content was higher in 40 (73%) o the 55 trials, the same in 4 trials (7%) and lower in 11 tria (20%).

DISCUSSION

Since production of diacetyl and acetylmethylcarbinol i ripening cream is brought about by action of the butter cultur organisms and is influenced by a number of factors, some var ation in the results of comparable trials would be expecte just as with many other biological processes. Differences in th activities of the various butter cultures and in the citric aci contents of the cream, especially sour cream where some o the citric acid probably is destroyed during the souring, ma have been factors in the irregularities.

The general effects of an increase in the cream acidity, add tion of citric acid to the cream and agitation of the cream du ing ripening in producing higher diacetyl and acetylmethy

carbinol contents in ripening cream are in agreement with the results of various studies on butter cultures. Michaelian, Farmer and Hammer (6) noted that in a butter culture most of the acetylmethylcarbinol plus diacetyl was produced relatively late in the ripening. They also reported that butter culture organisms could destroy acetylmethylcarbinol plus diacetyl, with high acidities tending to retard the destruction and neutralization tending to accelerate it. Hammer (3) indicated the importance of citric acid as a source of volatile acids in butter cultures, and the significance of this compound from the standpoint of development of flavoring materials in butter cultures has been emphasized by other investigators. In butter cultures that were aerated by shaking, Prill and Hammer (11) obtained increases in diacetyl on holding. Virtanen (15) observed that if the ripening process was conducted in thin layers, the production of diacetyl and acetylmethylcarbinol was especially active. A large increase in production of diacetyl and acetylmethylcarbinol in butter cultures by aeration under pressure was reported by Brewer *et al.* (1).

Variations in the results obtained with different butter cultures are in agreement with commercial experience; also, they emphasize the necessity of proper selection of the cultures to be used in manufacture of unsalted butter.

The general effects of certain cream-ripening procedures in increasing the diacetyl and acetylmethylcarbinol contents of cream and also the score of butter held under certain conditions indicate the importance of diacetyl as a flavor constituent of butter. Irregularities between diacetyl contents of the cream and scores of the butter involve the degree of retention of diacetyl in the butter and also overshadowing of the desirable effect of diacetyl through off-flavors of various types in the butter; presumably the off-flavors involve both bacteriological and chemical changes.

Variations in the ratios of the diacetyl and acetylmethylcarbinol contents of the cream and of the corresponding butter suggest that significant differences occur in the churning process, even when an attempt is made to carry it out on a uniform basis. Differences probably include not only the degree of retention of buttermilk in the butter but the rates of diacetyl and acetylmethylcarbinol production during the churning process (12) and the loss of these materials during the washing of butter (8). Hoecker and Hammer (4) also reported variations in the degree of retention of diacetyl and acetylmethylcarbinol during churning.

Increases and decreases in the diacetyl and acetylmethylcarbinol contents of butter under various holding conditions emphasize that changes in these compounds go on in butter just as they do in a butter culture. The increases presumably

play a part in the flavor development that unsalted butter made with butter culture so commonly shows, especially for a short period immediately after it is made, while the decreases may explain the rapid loss of flavor that this type of butter sometimes undergoes. It may be that with unsalted butter a short ripening period on a rather standardized basis for the butter itself would be advantageous from the standpoint of flavor development (14).

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0188

TABLE 1.—DIACETYL (Ac₂) AND ACETYLMETHYLCARBINOL (Amc) CONTENTS OF SWEET CREAM RIPENED TO VARIOUS ACIDITIES.

Laboratory trials

Trial no.	Cream: Ripening temp.	% acid	pH	Ac₂ p.p.m.	Amc p.p.m.	Trial no.	Cream: Ripening temp.	% acid	pH	Ac₂ p.p.m.	Amc p.p.m.
1	70° F.	0.37	5.12	0.81	35.0	8	70° F.	0.35	5.54	0.52	33.8
	70	.44	4.80	1.09	42.0		70	.40	5.32	.57	38.3
	70	.51	4.67	1.35	52.1		70	.44	5.00	.72	45.4
2	70	.33	5.54	.46	30.0	9	65	.42	5.02	.90	36.8
	70	.38	5.43	.59	31.1		65	.47	4.81	1.05	39.4
	70	.41	5.14	.74	40.7		65	.51	4.77	1.05	42.4
							65	.60	4.73	1.75	55.4
3	70	.35	5.00	.27	21.7	10	65	.30	5.47	.71	19.4
	70	.38	5.50	.31	25.0		65	.32	5.23	.74	17.3
	70	.42	5.45	[illegible]	[illegible]		65	.40	5.15	.72	12.0
							65	.44	5.00	.75	12.0
4	70	.34	5.71	.42	30.0	11	65	.46	4.77	1.14	60.0
	70	.40	5.45	1.00	41.3		65	.50	4.75	1.39	61.2
	70	.44	5.33	.93	[illegible]		65	.53	4.71	2.78	68.6
							65	.58	4.67	2.32	85.8
5	70	.29	5.63	.48	37.0	12	60	.34	5.61	.40	23.7
	70	.39	5.43	.70	38.3		60	.38	5.55	.47	25.9
	70	.41	5.31	.91	42.4		60	.42	5.50	.52	27.3
6	70	.35	5.21	.73	34.8	13	60	.37	5.57	.57	26.8
	70	.42	5.18	1.05	47.7		60	.49	5.12	.56	29.8
	70	.49	4.46	1.07	50.4		60	.54	4.98	.41	29.0
7	70	.41	5.40	.31	25.0	14	60	.33	5.79	.58	32.8
	70	.50	5.12	.37	38.0		60	.39	5.45	.50	44.0
	70	.53	4.95	.44	49.0		60	.45	5.28	.60	47.8

Trial no.	Cream: Ripening temp.	% acid	pH	Ac₂ p.p.m.	Amc p.p.m.	Trial no.	Cream: Ripening temp.	% acid	pH	Ac₂ p.p.m.	Amc p.p.m.
15	60	.38	5.22	1.40	34.0	25	55	.27	5.65	.59	21.6
	60	.44	5.05	1.43	38.1		55	.35	5.33	.69	24.9
	60	.49	4.84	1.70	54.4		55	.40	5.03	.72	26.9
							55	.44	4.82	.84	34.0
16	60	.35	5.12	1.02	36.3	26	52	.18	6.43	.20	9.0
	60	.40	4.89	1.52	41.4		55	.29	5.53	.62	29.2
	60	.52	4.70	1.77	53.5		55	.35	5.38	.71	31.2
							55	.43	5.21	.75	31.2
							55	.48	4.92	.84	32.4
17	60	.35	5.48	.62	28.0	27	52	.10	6.51	.23	3.5
	60	.41	5.29	.84	28.3		55	.30	5.66	.90	34.5
	60	.46	5.01	.98	32.6		55	.42	4.97	1.11	37.7
							55	.48	4.84	1.20	40.7
							55	.52	4.78	1.84	52.3
18	60	.30	5.42	.84	44.4	28	52	.20	6.30	.21	9.4
	60	.40	5.25	.85	44.0		55	.41	5.15	1.13	39.9
	60	.48	5.00	.97	48.4		55	.50	4.85	1.72	51.3
							55	.55	4.75	2.02	60.6
							55	.60	4.60	2.86	81.0
19	60	.32	5.78	.60	41.4	29	52	.18	6.57	.12	14.1
	60	.39	5.52	.70	45.4		55	.30	5.85	.46	2.5
	60	.44	5.38	.74	52.1		55	.34	5.81	.27	2.6
							55	.42	5.67	.24	2.3
							55	.45	5.56	.10	2.2
20	52	.14	6.40	.24	16.5	30	55	.28	5.80	.60	25.0
	55	.28	5.49	.50	19.0		55	.33	5.61	.45	25.9
	55	.34	5.34	.52	13.1		55	.38	5.42	.72	30.0
	55	.39	5.21	.80	15.2		55	.43	5.10	.74	35.0
	55	.44	5.00	.90	15.5						
21	52	.19	5.62	.27	10.0	31	55	.35	5.17	.57	21.9
	55	.41	5.11	.57	31.4		55	.45	5.05	.67	22.4
	55	.44	5.05	.97	31.4		55	.51	4.87	.91	23.9
	55	.51	4.86	1.03	39.5		55	.56	4.74	1.12	35.8
	55	.54	4.80	1.17	40.8						
22	55	.44	4.01	1.44	59.1	32	55	.35	5.72	.46	45.4
	55	.49	4.79	1.54	60.6		55	.39	5.50	.50	46.0
	55	.53	4.77	1.73	69.7		55	.43	5.35	.60	47.0
	55	.58	4.73	2.00	74.1		55	.46	5.30	.61	50.4

TABLE 1. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Ame) CONTENTS OF SWEET CREAM RIPENED TO VARIOUS ACIDITIES—Continued.

Trial no.	Laboratory trials					Trial no.	Laboratory trials				
	Cream						Cream				
	Ripening temp.	% acid	pH	Ac_2 p.p.m.	Ame p.p.m.		Ripening temp.	% acid	pH	Ac_2 p.p.m.	Ame p.p.m.
31	50°F.	0.33	5.12	0.89	42.9	34	50°F.	0.37	5.13	1.12	35.6
	50	.39	5.42	.90	41.0		50	.45	4.99	1.14	38.6
	50	.46	5.17	1.12	45.7		50	.50	4.85	1.85	58.8
32	50	.40	5.36	.60	27.4	35	50	.33	5.68	.55	25.8
	50	.51	5.04	.82	32.2		50	.39	5.52	.62	27.9
	50	.58	4.90	1.04	35.2		50	.43	5.46	.64	30.5
33	50	.33	5.66	.79	40.2	36	50	.40	4.96	1.52	37.8
	50	.40	5.40	.85	42.7		50	.44	4.84	1.63	46.6
	50	.48	5.20	1.00	44.8		50	.50	4.72	2.04	50.7

TABLE [illegible]. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Ame) CONTENTS OF LOTS OF NEUTRALIZED SOUR CREAM RIPENED TO VARIOUS ACIDITIES.

Trial no.	Laboratory trials										
	Cream										
	Ripening temp.	% acid	pH	Ac_2 p.p.m.	Ame p.p.m.	Ripening temp.	% acid	pH	Ac_2 p.p.m.	Ame p.p.m.
1	50°F.	0.33	5.36	0.79	41.1	50°F.	0.33	5.36	0.79	41.1
	50	.39	5.25	.85	42.5	50	.39	5.25	.85	42.5
	50	.45	5.14	1.12	45.9	50	.45	5.14	1.12	45.9
2	50	.33	5.55	.55	25.8	50	.33	5.55	.55	25.8
	50	.39	5.52	.62	27.9	50	.39	5.52	.62	27.9
	50	.43	5.46	.64	30.5	50	.43	5.46	.64	30.5
3	50	.40	4.96	1.52	37.8	50	.40	4.96	1.52	37.8
	50	.44	4.84	1.63	46.6	50	.44	4.84	1.63	46.6
	50	.50	4.72	2.04	50.7	50	.50	4.72	2.04	50.7

TABLE 1. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Ame) CONTENTS OF SWEET CREAM RIPENED TO VARIOUS ACIDITIES—Continued.

Trial no.	Laboratory trials: Cream: Ripening temp.	% acid	pH	Ac_2 p.p.m.	Ame p.p.m.	Trial no.	Laboratory trials: Cream: Ripening temp.	% acid	pH	Ac_2 p.p.m.	Ame p.p.m.
31	50°F.	0.33	5.62	0.89	42.0	34	50°F.	0.37	5.13	1.12	35.6
	50	.39	5.42	.90	41.0		50	.45	4.99	1.14	38.6
	50	.46	5.17	1.12	45.7		50	.50	4.85	1.85	56.8
32	50	.40	5.16	.60	27.4	35	50	.33	5.68	.55	25.8
	50	.51	5.04	.82	32.2		50	.39	5.52	.62	27.9
	50	.58	4.90	1.04	35.2		50	.43	5.43	.61	30.5
33	50	.33	5.06	.59	40.2	36	50	.40	4.96	1.52	37.2
	50	.40	5.40	.85	42.7		50	.41	4.84	1.63	46.6
	50	.48	5.20	1.00	44.8		50	.50	4.72	2.03	55.7

TABLE 1. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Ame) CONTENTS OF SLIGHTLY ACIDIFIED SOUR CREAM RIPENED TO VARIOUS ACIDITIES.

Trial no.	Laboratory trials: Cream: Ripening temp.	% acid	pH	Ac_2 p.p.m.	Ame p.p.m.
1	72°F.	0.15	5.56	1.14	35.3
	72	.22	5.39	1.15	35.5
	72	.28	5.26	1.15	35.5
	72	.32	5.14	1.15	35.5
2	72	.14	5.63	.13	25.9
	72	.21	5.54	1.03	35.5
	72	.27	5.44	1.03	35.5
	72	.31	5.35	1.03	35.5
3	72	.14	5.60	1.03	25.9
	72	.19	5.49	1.03	35.5
	72	.26	5.39	1.03	35.5
	72	.29	5.29	1.03	35.5

TABLE 3. DIACETYL (Ac_2) AND ACETYLMETHLYCARBINOL (Amc) CONTENTS OF SWEET (Swt.) OR NEUTRALIZED SOUR (Sr.) CREAM RIPENED TO VARIOUS ACIDITIES.

Semi-commercial trials

Trial no.	Cream					
	Type of cream	Ripening temp.	% acid	pH	Ac_2 p. p. m.	Amc p. p. m.
1	Swt.	53°F.	0.36	5.39	0.44	11.8
	Swt.	53	.42	5.23	.48	11.9
	Swt.	53	.49	4.91	.54	15.3
2	Swt.	53	.35	5.34	.43	17.9
	Swt.	53	.40	5.26	.60	24.0
	Swt.	53	.45	5.17	.72	38.5
3	Swt.	53	.35	5.32	.46	20.4
	Swt.	53	.42	5.12	.68	25.7
	Swt.	53	.48	4.95	.97	30.1
4	Swt.	50	.48	4.79	4.23	37.4
	Swt.	50	.52	4.73	4.80	101.4
5	Swt.	52	.48	4.90	2.84	87.4
	Swt.	52	.51	4.85	3.78	62.2
6	Swt.	54	.35	5.23	1.19	28.9
	Swt.	54	.40	5.15	1.23	32.5
7	Swt.	55	.38	4.98	1.24	33.8
	Swt.	55	.46	4.85	2.15	32.8
8	Sr.	51	.31	5.35	1.55	62.0
	Sr.	51	.35	5.26	1.95	72.4
9	Sr.	after past.	.23	6.52	.21	21.7
	Sr.	plus culture	.26	6.47	.28	34.0
	Sr.	54	.32	5.92	.67	31.1
	Sr.	54	.38	5.69	.76	31.7
10	Sr.	after past.	.17	6.58	.16	29.8
	Sr.	plus culture	.18	6.52	.62	35.0
	Sr.	52	.37	5.29	1.88	61.7
	Sr.	52	.49	4.98	1.49	28.8

TABLE 4. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Amc) CONTENTS OF SWEET CREAM RIPENED WITH AND WITHOUT ADDED CITRIC ACID TO ABOUT THE SAME ACIDITIES.

Laboratory trials

Trial no.	Cream					
	% citric acid added	Ripening temp.	% acid	pH	Ac_2 p. p. m.	Amc p. p. m.
1	0.00	55°F.	0.36	5.06	0.90	34.5
	.05	55	.35	5.15	1.01	32.6
2	.00	55	.42	4.97	1.11	37.7
	.05	55	.40	5.02	1.45	36.5
3	.00	55	.48	4.85	1.20	46.7
	.05	55	.46	4.91	1.49	49.2
4	.00	55	.52	4.78	1.84	52.3
	.05	55	.50	4.81	2.52	55.2
5	.00	55	.30	5.85	.47	12.5
	.05	55	.32	5.73	.98	18.5
6	.00	55	.34	5.81	.27	2.5
	.05	55	.35	5.61	.98	13.1
7	.00	55	.36	5.67	.24	2.4
	.05	55	.36	5.61	.63	5.2
8	.00	55	.37	5.56	.18	2.2
	.05	55	.37	5.51	.56	2.7
9	.00	55	.56	4.74	1.19	35.8
	.05	55	.57	4.72	1.27	44.4
10	.00	55	.39	5.19	.75	35.0
	.05	55	.39	5.19	.78	38.6
11	.00	54	.30	5.69	.20	10.2
	.05	54	.31	5.65	.25	12.1
12	.00	54	.34	5.52	.19	10.8
	.05	54	.34	5.55	.20	12.5
13	.00	55	.35	5.56	.39	46.0
	.05	55	.34	5.58	.05	54.4
14	.00	60	.42	5.02	.87	37.9
	.10	60	.41	5.01	1.09	45.1

0188

TABLE 5. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Amc) CONTENTS OF NEUTRALIZED SOUR CREAM RIPENED WITH VARIOUS AMOUNTS OF ADDED CITRIC ACID TO ABOUT THE SAME ACIDITIES.

Laboratory trials						
	Cream					
Trial no.	% citric acid added	Ripening temp.	% acid	pH	Ac_2 p. p. m.	Amc p. p. m.
1	0.00	60° F.	0.37	5.14	1.80	44.2
	.05	60	.36	5.14	1.80	48.0
	.10	60	.38	5.09	1.80	37.2
	.15	60	.39	4.99	2.02	38.3
2	.00	60	.44	4.75	1.81	37.4
	.05	60	.45	4.73	2.11	36.2
	.10	60	.47	4.68	2.72	72.3
	.15	60	.44	4.71	2.72	78.0
3	.00	58	.40	4.79	1.73	24.3
	.05	58	.37	5.09	1.57	32.3
	.10	58	.39	5.05	2.09	41.4
	.15	58	.42	4.93	2.17	48.5
4	.00	58	.41	4.79	1.70	24.0
	.05	58	.45	4.79	2.02	44.9
	.10	58	.45	4.75	3.00	39.3
	.15	58	.45	4.74	3.21	44.9
5	.00	63	.43	4.73	1.59	33.2
	.10	63	.44	4.71	2.63	73.2
6	.00	63	.47	4.67	2.90	33.9
	.10	63	.49	4.63	3.76	37.4
7	.00	62	.42	4.92	1.41	31.1
	.05	62	.45	4.92	2.57	38.1
	.10	62	.45	4.90	3.37	36.8
	.15	62	.48	4.82	3.83	30.1
8	.00	62	.42	4.92	1.41	31.1
	.05	62	.37	5.10	1.83	39.8
	.10	62	.39	5.00	2.11	35.6
	.15	62	.42	4.95	2.57	37.3

TABLE 6. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Amc) CONTENTS OF SWEET (Swt.) OR NEUTRALIZED SOUR (Sr.) CREAM RIPENED WITH AND WITHOUT ADDED CITRIC ACID TO ABOUT THE SAME ACIDITIES.

Semi-commercial trials							
	Cream						
Trial no.	Type of cream	% citric acid added	Ripening temp.	% acid	pH	Ac_2 p. p. m.	Amc p. p. m.
1	Swt.	0.00	52° F.	0.48	4.76	[illegible]	76.1
	Swt.	.05	52	.51	4.70	4.44	108.7
2	Swt.	.00	50	.48	4.79	4.23	37.4
	Swt.	.05	50	.53	4.71	4.10	21.6
3	Swt.	.00	53	.42	5.29	1.50	46.2
	Swt.	.05	53	.44	5.24	2.28	59.0
4	Swt.	.00	51	.51	4.85	3.78	62.2
	Swt.	.05	51	.53	4.77	5.90	97.0
5	Sr.	.00	51	.31	5.35	1.55	62.0
	Sr.	.05	51	.34	5.20	1.67	62.0
6	Sr.	.00	54	.32	5.92	.67	34.0
	Sr.	.05	54	.35	5.55	.82	30.0
7	Sr.	.00	53	.37	5.34	1.40	26.3
	Sr.	.05	53	.37	5.31	1.60	31.2

TABLE 7. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Amc) CONTENTS OF SWEET (Swt.) OR NEUTRALIZED SOUR (Sr.) CREAM RIPENED WITH AND WITHOUT AGITATION.

Laboratory trials							
	Cream						
Trial no.	Type of cream	Agitation	Ripening temp.	% acid	pH	Ac_2 p. p. m.	Amc p. p. m.
1	Swt.	—	60° F.	0.25	6.31	0.53	42.7
	Swt.	+	60	.26	6.13	.54	106.6
2	Swt.	—	60	.52	4.81	2.35	72.7
	Swt.	+	60	.52	4.80	4.44	110.0
3	Swt.	—	60	.55	4.67	1.40	54.0
	Swt.	+	60	.56	4.69	3.35	108.0
4	Swt.	—	70	.26	6.01	.43	28.9
	Swt.	+	70	.26	6.01	1.22	59.9
5	Swt.	—	70	.35	5.65	.52	31.2
	Swt.	+	70	.35	5.68	1.35	78.3
6	Swt.	—	60	.27	5.32	.78	30.6
	Swt.	+	60	.26	5.46	1.08	42.6
7	Swt.	—	60	.47	5.02	.87	37.9
	Swt.	+	60	.45	5.20	1.61	56.3
8	Sr.	—	63	.43	4.73	1.59	33.2
	Sr.	+	63	.44	4.78	2.40	46.1
9	Sr.	—	63	.47	4.67	2.90	33.9
	Sr.	+	63	.46	4.70	3.26	51.8

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TABLE 8. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Amc) CONTENTS OF SWEET (Swt.) OR NEUTRALIZED SOUR (Sr.) CREAM RIPENED WITH AND WITHOUT AGITATION AFTER ADDITION OF 0.1 PERCENT CITRIC ACID.

Laboratory trials

Trial no.	Cream						
	Type of cream	Agitation	Ripening temp.	% acid	pH	Ac_2 p. p. m.	Amc p. p. m.
1	Swt. Swt.	— +	70° F. 70	0.38 .37	5.31 5.49	0.77 1.46	30.6 79.5
2	Swt. Swt.	— +	70 70	.45 .47	4.98 5.14	.91 1.90	42.7 96.9
3	Swt. Swt.	— +	60 60	.41 .40	5.01 5.02	1.09 1.57	45.1 61.0
4	Swt. Swt.	— +	60 60	.49 .46	4.74 4.92	1.73 2.75	65.3 101.2
5	Sr. Sr.	— +	63 63	.47 .46	4.71 4.71	2.06 3.50	73.2 83.9
6	Sr. Sr.	— +	63 63	.50 .50	4.62 4.65	3.27 5.60	77.1 108.7

TABLE 9. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Amc) CONTENTS OF SWEET (Swt.) OR NEUTRALIZED SOUR (Sr.) CREAM RIPENED WITH AND WITHOUT AGITATION.

Semi-commercial trials

Trial no.	Cream						
	Type of cream	Agitation	Ripening temp.	% acid	pH	Ac_2 p. p. m.	Amc p. p. m.
1	Swt. Swt.	— +	52° F. 52	0.48 .48	4.76 4.80	3.28 5.30	76.1 164.0
2	Swt. Swt.	— +	54 54	.35 .33	5.23 5.35	1.19 2.80	28.9 67.8
3	Swt. Swt.	— +	55 55	.38 .37	4.98 4.97	1.24 1.91	23.8 55.8
4	Sr. Sr.	— +	55 55	.37 .36	5.34 5.41	1.40 2.72	30.3 78.0
5	Sr. Sr.	— +	53 53	.49 .45	4.98 5.15	1.50 1.63	28.8 39.1

TABLE 10. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Amc) CONTENTS OF SWEET CREAM RIPENED AT DIFFERENT TEMPERATURES TO ABOUT THE SAME ACIDITIES.

Laboratory trials

Trial no.	Cream					Trial no.	Cream				
	Ripening temp.	% acid	pH	Ac_2 p. p. m.	Amc p. p. m.		Ripening temp.	% acid	pH	Ac_2 p.p.m.	Amc p.p.m.
1	50° F. 60 70	0.33 .32 .33	5.62 5.78 5.63	0.80 .66 .48	42.9 41.1 37.5	10	50° F. 60 70	0.33 .34 .35	5.68 5.61 5.60	0.55 .40 .28	25.8 23.7 24.8
2	50 60 70	.39 .39 .39	5.42 5.62 5.40	.89 .70 .49	45.0 45.0 38.5	11	50 60 70	.39 .38 .38	5.52 5.55 5.50	.62 .47 .34	27.9 25.9 25.9
3	50 60 70	.46 .44 .44	5.17 5.38 5.31	1.12 .72 .61	45.7 52.1 42.2	12	50 60 70	.43 .42 .42	5.43 5.43 5.45	.64 .39 .39	30.5 27.3 27.2
4	50 60 70	.33 .33 .34	5.66 5.78 5.71	.79 .58 .52	40.2 43.8 39.6	13	50 60 70	.40 .38 .37	4.96 5.12 5.13	1.32 1.02 .84	37.3 36.3 35.6
5	50 60 70	.40 .39 .40	5.40 5.45 5.45	.85 .58 .60	42.7 41.9 41.3	14	50 60 70	.44 .46 .44	4.81 4.89 4.89	1.63 1.52 1.49	46.6 41.4 43.0
6	50 60 70	.47 .45 .44	5.20 5.28 5.35	1.00 .60 .63	41.8 47.3 41.7	15	50 60 70	.51 .52 .51	4.72 4.70 4.67	2.03 1.77 2.28	53.7 52.5 52.4
7	50 60 70	.40 .37 .40	5.26 5.57 5.40	.60 .57 .31	27.1 26.8 20.0	16	50 60 70	.37 .36 .35	5.13 5.22 5.24	1.12 1.10 .75	35.6 31.2 34.8
8	50 60 70	.51 .49 .50	5.01 5.12 5.12	.82 .56 .37	32.2 29.8 28.0	17	50 60 70	.45 .44 .42	4.99 5.05 5.18	1.15 1.15 1.05	38.6 38.1 37.6
9	50 60 70	.58 .54 .55	4.90 4.98 4.95	1.01 .41 .44	36.2 29.0 29.0	18	50 60 70	.50 .40 .49	4.85 4.84 4.84	1.85 1.70 1.67	58.8 59.4 50.4

TABLE II. DIACETYL (Ac_2) AND ACETYLMETHYLCARBINOL (Amc) CONTENTS OF SWEET (Swt.) OR NEUTRALIZED SOUR (Sr.) CREAM RIPENED AT DIFFERENT TEMPERATURES TO ABOUT THE SAME ACIDITIES.

	Semi commercial trials					
	Cream					
Trial no.	Type of cream	Ripening temp.	% acid	pH	Ac_2 p. p. m.	Amc p. p. m.
1	Swt.	55° F.	0.55	4.95	2.57	36.8
	Swt.	62	.54	4.85	2.74	42.6
	Swt.	70	.57	4.72	2.99	45.8
2	Swt.	62	.55	4.65	1.95	42.9
	Swt.	70	.52	4.73	1.92	35.0
3	Swt.	61	.34	4.99	1.68	36.2
	Swt.	70	.36	4.95	.79	8.2
4	Sr.	62	.50	4.83	2.06	45.1
	Sr.	70	.51	4.75	2.13	16.2

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J. Dairy Sci. 48(7): 870-876, (June 1965)

CONSUMER PREFERENCES FOR FLAVOR IN CREAMED COTTAGE CHEESE[1]

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ABSTRACT

A triangular test and a paired comparison test were used to determine, respectively, the difference threshold and preference for diacetyl in creamed Cottage cheese samples by a consumer panel. Creamed Cottage cheese of 4% fat and 80% moisture was used in the tests. Levels of diacetyl compared were 0.2 vs. 1.4, 0.2 vs. 2.2, 0.2 vs. 3.5, and 0.2 vs. 5.0 ppm. The consumer panel could detect differences between samples when diacetyl content of the samples differed by 1.4 ppm or more. Preference was for the creamed Cottage cheese containing the higher level of diacetyl. Sex, age, and like or dislike of creamed Cottage cheese did not affect the ability to detect differences between diacetyl levels. Sex and age did not affect the preference choice in the preference test. Both sexes and all age groups expressed a greater preference for the high diacetyl creamed Cottage cheese. Preference of those individuals who liked Cottage cheese was not significantly different from those individuals who disliked creamed Cottage cheese. However, those who disliked the product showed a slight preference for the samples with low levels of diacetyl.

Creamed Cottage cheese is a product with considerable economic importance in the dairy industry. Per capita consumption increased from 0.9 lb in 1925 to 4.8 lb in 1960 (9). The per capita consumption forecast for 1963 was 4.4 lb, a drop of 0.4 lb per person over the last 3 yr. Leach (6) predicted that per capita consumption would not keep pace with the population increase unless the dairy industry took an interest in the desires of the consumer. A consumer might purchase a product once, but unless he found that the product had a desirable flavor he would not continue to purchase the item. Nelson and Trout (10) have emphasized that the people in the dairy industry should never forget that the increased sales of dairy products depend upon that pleasant, delicate flavor sensation which dairy products make on the palate of the consumer. Olson and Strozier (11) reported that one of the principal criticisms of creamed Cottage cheese was that it lacked a distinct flavor. Angevine et al. (1) suggested that creamed Cottage cheese should have a fresh, clean, slightly acid, salty taste, with the delicate flavor and aroma of a good lactic culture. Development of methods for standardizing the level of diacetyl in creamed Cottage cheese (3) has now made it possible to determine consumer preferences.

This research was designed to determine the difference threshold for diacetyl, and consumer preference for different diacetyl levels in creamed Cottage cheese. Analyses of data were made on the basis of difference threshold, age, sex, and like or dislike of creamed Cottage cheese, to determine the effect of these factors upon the detection of diacetyl and upon diacetyl levels preferred by consumers tested.

Received for publication March 12, 1965.

[1] Published with the approval of the Director of the Purdue Agricultural Experiment Station as Journal Series Paper no. 2518.

METHODS AND PROCEDURES

Preparation of creamed Cottage cheese samples. The large-curd Cottage cheese used in these experiments was manufactured by employees of the Purdue University Creamery. A long-set method similar to the one outlined by Hales (4) was used. The creaming mixture was made from a citrated *Streptococcus citrovorus* culture and pasteurized cream, as described by Mather and Babel (8). The citrated culture was added in various amounts to cream of selected fat percentages to give a creaming mixture of 12% fat and the desired diacetyl level in the creamed Cottage cheese. For the low level of diacetyl flavor, citrated skimmilk instead of the citrated *S. citrovorus* culture was used to prepare the creaming mixture. Creamed Cottage cheese containing 4% fat was prepared by adding one part of the creaming mixture to two parts of Cottage cheese. Previous work with an analytical panel (5) indicated that diacetyl was more correctly identified in the presence of 0.75% salt than at any other salt concentration. Therefore, salt was added at the rate of 0.75% of the final weight of the creamed Cottage cheese.

The pH of a macerated sample of the product was determined with a Beckman pH meter. The colorimetric method of Prill and Hammer

141) was used for the determination of diacetyl. The volatile acidity of the samples was determined by steam-distilling a 50-g sample of creamed Cottage cheese and titrating the volatile acids with 0.1 N NaOH.

Creamed Cottage cheese samples with four levels of diacetyl were used, with two replicates at each of three comparisons, 0.2 vs. 1.4, 0.2 vs. 2.2, 0.2 vs. 3.5, and one replicate at 0.2 vs. 5.0 ppm.

Consumer taste panel operation. Creamed Cottage cheese containing the two levels of diacetyl to be used was prepared 24 hr before the consumer taste panel was conducted and was stored at 4 C until used. The morning of the taste panel operation, the creamed Cottage cheese was placed in 1-oz Lily portion cups according to a random pattern. The cups were lettered A, B, and C for the triangle test and A and B for the preference test. There were six possible combinations for the odd sample in the triangular test, and two possible combinations for the two samples in the preference test. There were 12 orders, with 18 samples in each order. The samples were placed on trays, coded, and presented to panel members in random order.

Consumer taste panel evaluations were carried out on succeeding Fridays from April 19 to May 24, 1963, for the first six replications. The individuals consisted primarily of customers coming to the Departmental Dairy Retail Store. The seventh replicate was held on July 15 at the Purdue Dairy Center, on Purdue Dairy Day. These individuals were mostly dairy farmers. The same persons did not participate in every replicate.

Individuals who expressed a desire to participate in consumer research on cultured dairy products were directed into the tasting room, where they were seated at a table and given two answer forms to complete. The samples for the triangular test were presented and the individuals indicated which sample, A, B, or C, was the odd sample. They were then given a sample of each cheese and asked which sample they preferred or whether they had no preference. Age, sex, and like or dislike of creamed Cottage cheese was also indicated on the answer forms.

The answer forms were numbered from 1 to 1,512, so that the individual responses could be coded for analysis. The number of tasters ranged from 212 to 216 per replicate, depending upon the number of tasters fully completing the answer forms. Forms not completed were discarded. Statistical analyses were performed on the numerical data (12, 15, 16).

RESULTS AND DISCUSSION

Difference threshold in creamed Cottage cheese. The triangular test data for each comparison in Table 1 were tested separately (16), to determine whether the identification of the odd sample was made by change. The consumer panel could detect a difference between the 0.2 vs. 1.4 ppm comparison ($P < 0.05$). In Replicate Six, the pH difference between samples was larger than 0.1 unit. The *S. citrovorus* culture used in Replicate Six had produced only 7 ppm diacetyl, whereas the citrated cultures used for the other replicates had produced over 12 ppm. The increased amount of culture required was apparently large enough to cause a lowering in the pH of the higher-flavor-level creamed Cottage cheese. The ability of the panel to tell the difference between the diacetyl levels in Replicate Six was attributed to the pH difference between samples. When the difference in diacetyl level between samples became larger than 1.2 ppm, the consumer panel could easily distinguish between levels

TABLE 1

Results of triangular taste test and preference choice for the consumer panel

Diacetyl comparison	Rep.	No. answering	Triangular test: Correct identifications	Triangular test: Significance*	Preference choice: High	Preference choice: Low	Preference choice: No preference
(ppm)			*(%)*		*(%)*		
0.2 vs. 5.0	7	215	48.8	< 0.001	44.2	43.3	12.5
0.2 vs. 1.5	1	212	43.6	< 0.001	49.5	34.9	15.6
0.2 vs. 3.5	2	213	46.5	< 0.001	46.0	38.0	16.0
0.2 vs. 2.2	3	214	49.5	< 0.001	45.3	36.9	17.8
0.2 vs. 2.2	4	216	45.4	< 0.001	45.4	32.4	22.2
0.2 vs. 1.4	5	216	40.3	< 0.050	48.1	32.9	19.0
0.2 vs. 1.4	6	216	53.7	< 0.001	49.1	32.9	18.0
	Total	1,502		Average	46.9	35.9	17.2

* Determined by table of Roessler et al. (15).

of diacetyl ($P < 0.001$). An analytical panel (5) could differentiate between similar samples of creamed Cottage cheese with 0.7 ppm difference between them. If diacetyl is not present at least to 1.4 ppm, a consumer may not be able to detect it. Mather and Babel (7) analyzed 41 commercial samples of creamed Cottage cheese and found that 71.2% contained less than 1.0 ppm diacetyl.

It is questionable if a consumer could tell a difference in flavor between any two bland products. However, as the level approached 1.4 ppm, many consumers would be able to detect the diacetyl flavor. Preferences of those consumers who could differentiate between diacetyl levels (Table 2) were analyzed, using a 4×3 contingency table (12). Replicate Six was not included in the analysis, because of pH difference. Preference was dependent upon the level of diacetyl in the creamed Cottage cheese ($P < 0.025$). The area of preference most affected was the No Preference selection. At the 0.2 vs. 3.5 ppm comparison, the largest number of choices between high and low flavor was indicated. Of those who indicated a choice in this comparison, the high flavor was preferred, although not significantly, as tested using the method of Roessler et al. (15). Least-preference choice was indicated for the 0.2 vs. 2.2 ppm comparison. The data indicated that at the 0.2 vs. 3.5 ppm comparison consumers could easily differentiate between samples and, therefore, showed a preference for one or the other cheese. When the difference between comparisons was less than 3.5 ppm, differentiation became more difficult and the consumers had less preference for either sample. When diacetyl was considerably above 3.5 ppm, as in Replicate Seven, a harsh, unnatural flavor existed and preference between a high-harsh and a low-bland flavor became nearly equal. The trend was for the high-diacetyl cheese at all comparisons. Based on the combined seven replicates for those consumers who correctly identified the odd sample and expressed a preference, the creamed Cottage cheese containing a higher level of diacetyl was preferred to the sample containing the lower level of diacetyl ($P < 0.05$).

For those consumers who incorrectly identified the odd sample, Table 2, preference was independent of diacetyl content (5×3 contingency table). This was expected, because the triangular taste test indicated that they could not differentiate between diacetyl comparisons. For those consumers who incorrectly identified the odd sample and had a preference, the combined totals for the seven replicates indicated that the high-diacetyl-flavor levels were preferred to the low-flavor levels ($P < 0.001$). Preference for the high-diacetyl flavor was approximately the same for, and apparently independent of, consumers who could or could not identify the odd sample in the triangular test.

For the consumer panel as a whole (Table 1), preference was independent of diacetyl level (7×3 contingency table). Least choice for diacetyl was shown for the 0.2 vs. 2.2 ppm comparison. This was followed closely by 0.2 vs. 1.4 ppm. The highest choice for a comparison was shown at 0.1 vs. 5.0 ppm. Preference was not shown for either high or low diacetyl at this comparison. The consumer panelists in this comparison were mostly dairy farmers. The wide difference in flavor between samples and the tasting of a product that is of interest to the panelist (13) could account for the high number of choices for high- or low-diacetyl content. Preference over the combined seven replicates was for the high level of diacetyl ($P < 0.001$).

TABLE 2

Preference choice for diacetyl level by correct or incorrect triangular taste test

Diacetyl comparison	Rep.	Correct triangular taste test: No. answering	Preference choice: High	Low	No preference	Incorrect triangular taste test: No. answering	Preference choice: High	Low	No preference
(ppm)			(%)				(%)		
0.2 vs. 5.0	7	105	46.6	42.0	11.4	110	41.8	44.6	13.6
0.2 vs. 3.5	1 and 2	202	50.0	41.1	8.9	223	45.7	32.3	22.0
0.2 vs. 2.2	3 and 4	204	42.2	36.8	21.0	226	48.2	32.7	19.1
0.2 vs. 1.4	5	87	48.3	35.6	16.1	129	48.1	31.0	20.9
0.2 vs. 1.4	6	116	46.6	34.5	18.9	100	52.0	31.0	17.0
		714[a]	46.5[b]	38.2[b]	15.3[b]	788[a]	47.1[b]	33.8[b]	19.1[b]

[a] Total number answering questions.
[b] Average per cent for each preference level.

0794

Effect of sex. Leach (6) reported that more women than men eat creamed Cottage cheese. Because women are more familiar with the product, there may be a difference in detection between the sexes. Analysis of the data (2 × 2 contingency table) in Table 3 indicated that sexes were equally able to differentiate between diacetyl levels. Although more males than females correctly selected the odd sample, the difference in detection between the sexes was not greater than would be expected by chance. Therefore, the ability to detect diacetyl in creamed Cottage cheese samples did not appear to be dependent upon the sex of the individual.

Preference choice was independent of sex (2 × 3 contingency table) for the consumer panel (Table 4). Females showed less no preference choice for the high- or low-diacetyl-level cheese than males. At the 0.2 vs. 5.0 ppm level females showed more preference for low diacetyl than high. For those individuals who indicated a preference between the high- or low-diacetyl cheese, approximately the same percentage of males and females preferred the high level of diacetyl. The preference of the high level of diacetyl over the low level was significant for both sexes. Both men and women preferred diacetyl-flavored creamed Cottage cheese to a product with essentially no diacetyl present.

Preference of diacetyl level by sex for those individuals who correctly identified the odd sample in the triangular test was independent of diacetyl concentration. Females had less no preference choice between high or low diacetyl level than men; however, there was not much difference between the sexes. The preference between sexes for high or low diacetyl was approximately equal, with preference for both sexes shown for the high level of diacetyl.

No significant difference could be found in comparing the sexes for like or dislike of creamed Cottage cheese. Approximately 90% of the consumers participating in this study expressed a like for creamed Cottage cheese. This is not meant to imply that 90% of the population likes creamed Cottage cheese, for

TABLE 3

Effect of sex on identification of odd sample in triangular taste test

Diacetyl comparison	Rep.	Males		Females	
		No. answering	Correctly answering	No. answering	Correctly answering
(ppm)			*(%)*		*(%)*
0.2 vs. 5.0	7	169	49.4	46	46.8
0.2 vs. 3.5	1	154	48.7	58	48.3
0.2 vs. 3.5	2	134	44.8	79	49.4
0.2 vs. 2.2	3	144	49.3	70	50.0
0.2 vs. 2.2	4	143	48.2	73	39.7
0.2 vs. 1.4	5	119	42.0	97	38.1
0.2 vs. 1.4	6	128	53.1	88	54.5
		991[a]	48.0[b]	511[a]	46.6[b]

[a] Total number answering questions.
[b] Average per cent for each correctly answering.

TABLE 4

Effect of sex on preference of diacetyl level for consumer panel

Diacetyl comparison	Rep.	Males				Females			
		No. answering	High	Low	No preference	No. answering	High	Low	No preference
(ppm)			*(%)*				*(%)*		
0.2 vs. 5.0	7	169	46.2	40.8	13.0	46	37.0	52.1	10.9
0.2 vs. 3.5	1	154	48.7	35.1	16.2	58	51.7	34.5	13.8
0.2 vs. 3.5	2	134	48.5	37.3	14.2	79	41.8	39.2	19.0
0.2 vs. 2.2	3	144	39.6	36.8	23.6	70	57.2	37.1	5.7
0.2 vs. 2.2	4	143	42.7	35.6	21.7	73	50.7	26.0	23.3
0.2 vs. 1.4	5	119	50.4	26.9	22.7	97	45.4	40.2	14.4
0.2 vs. 1.4	6	128	53.1	28.1	18.8	88	43.2	39.8	17.0
		991[a]	46.8[b]	34.8[b]	18.4[b]	511[a]	46.8[b]	38.0[b]	15.2[b]

[a] Total number answering questions.
[b] Average per cent for each preference level.

0794

many persons declined to participate in our consumer preference test.

Effect of age. There is a difference in sensitivity to taste with age (2). In infants, taste buds can be found in all parts of the oral cavity. As a child grows, the most sensitive area shifts from the tip of the tongue to a position at the back of the tongue. In younger children, there is a greater abundance of taste receptors than at maturity. Age could affect the ability to detect diacetyl.

The data were analyzed by age groups and correct triangular test, to determine if one age group contained better tasters than another. Good consistency between age groups in their ability to select the odd sample over the seven replicates is shown in Table 5. A heterogeneity Chi-square test indicated that age did not significantly affect the ability to detect diacetyl in creamed Cottage cheese.

Preference choice by the consumer panel was not significantly influenced by age grouping (Table 5). One age group did not have a significantly different preference choice from another age group (5 × 3 contingency table). For those who indicated a preference between high or low diacetyl, there was a consistent and nearly equal preference for high- over low-diacetyl level in every age group. The 30–40 age group indicated a higher preference for the high-diacetyl level than the other age groups. Least preference for the high level was shown by the over-50 age group.

For those individuals who correctly identified the odd sample, preference choice was not dependent upon age group. The age groups were consistent in their preference of high- or low-diacetyl-flavored cheese.

Effect of like or dislike of creamed Cottage cheese. Individuals who liked creamed Cottage cheese are more familiar with the product than those who dislike creamed Cottage cheese. The data in Table 6 indicate that those who disliked creamed Cottage cheese were the better tasters.

Preference choice for those in the consumer panel who liked or disliked creamed Cottage cheese (Table 7) was not significantly different, but those who disliked the product showed fewer choices for high or low flavor. For the consumers who liked creamed Cottage cheese

TABLE 5

Effect of age on triangular test and preference choice for consumer panel

Age	No.	Triangular test: Correct identification	Preference choice: High	Preference choice: Low	Preference choice: No preference
		(%)	(%)	(%)	(%)
10-20	433	48.0	47.8	38.6	13.6
20-30	611	45.7	45.8	34.7	19.5
30-40	201	49.8	50.7	32.4	16.9
40-50	153	51.6	47.1	39.2	13.7
Over 50	104	46.1	40.4	33.7	25.9
	1,502		46.8[a]	35.9[a]	17.3[a]

[a] Average per cent for each preference level.

TABLE 6

Effect of like or dislike of creamed Cottage cheese on identification of odd sample in triangular taste test

Diacetyl comparison	Rep.	Like: No.	Like: Correctly answering	Dislike: No.	Dislike: Correctly answering
(ppm)			(%)		(%)
0.2 vs. 5.0	7	195	49.7	20	40.0
0.2 vs. 3.5	1	189	48.2	23	52.2
0.2 vs. 3.5	2	197	46.2	16	50.0
0.2 vs. 2.2	3	194	50.0	20	45.0
0.2 vs. 2.2	4	196	45.9	20	40.0
0.2 vs. 1.4	5	187	40.6	29	37.9
0.2 vs. 1.4	6	185	54.1	31	51.6
		1,343[a]	47.8[b]	159[a]	51.6[b]

[a] Total number answering questions.
[b] Average per cent for each correctly answering.

and showed a preference for the high or low level of flavor, the high-flavor level was consistently preferred ($P < 0.001$).

The over-all trend for the seven replicates for those who disliked creamed Cottage cheese was for the high level of diacetyl, although in Replicates Seven and One the low level was most preferred. Those who disliked creamed Cottage cheese seemed to have a greater objection to over 3.5 ppm diacetyl than those who liked creamed Cottage cheese. Those individuals who disliked the product were not the consumers who consistently purchase the product. Because they are less familiar with creamed Cottage cheese, a high level of diacetyl probably seems harsh and unnatural. Therefore, they prefer a lower level of diacetyl flavor than those who like creamed Cottage cheese.

Those consumers who liked creamed Cottage cheese and correctly identified the odd sample in the triangular test (Table 8) had a preference for the high-level cheese. The preference for the high level varied among replications; however, the total preference for the high level was significant ($P < 0.05$).

The total preference between samples for those who disliked creamed Cottage cheese and identified the odd sample was for the lower level of diacetyl flavor (Table 8). These persons did not prefer creamed Cottage cheese with over 2.2 ppm diacetyl present.

ACKNOWLEDGMENT

This research was supported in part by a grant from the American Dairy Association.

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TABLE 7

Preference by like or dislike of creamed Cottage cheese by consumer panel

Diacetyl comparison	Rep.	Like				Dislike			
		No.	High	Low	No preference	No.	High	Low	No preference
(ppm)			(%)				(%)		
0.2 vs. 5.0	7	195	45.6	42.6	11.8	20	30.0	50.0	20.0
0.2 vs. 3.5	1	189	51.3	33.9	14.8	23	34.8	43.5	21.7
0.2 vs. 3.5	2	197	45.2	37.5	17.3	16	56.2	43.8	0.0
0.2 vs. 2.2	3	194	45.4	37.1	17.5	20	45.0	35.0	20.0
0.2 vs. 2.2	4	196	46.5	32.1	21.4	20	35.0	35.0	30.0
0.2 vs. 1.4	5	187	46.0	35.8	18.2	29	62.1	13.8	24.1
0.2 vs. 1.4	6	185	50.3	34.0	15.7	31	41.9	25.8	32.3
		1,343[a]	47.1[b]	36.2[b]	16.7[b]	159[a]	44.0[b]	33.3[b]	22.7[b]

[a] Total number answering questions.
[b] Average per cent for each preference level.

TABLE 8

Effect of like or dislike of creamed Cottage cheese on preference choice of diacetyl level for those individuals correctly identifying odd sample in triangular taste test

Diacetyl comparison	Rep.	Like				Dislike			
		No. correct triangle	High	Low	No preference	No. correct triangle	High	Low	No preference
(ppm)			(%)				(%)		
0.2 vs. 5.0	7	97	49.5	40.2	10.3	8	12.5	62.5	25.0
0.2 vs. 3.5	1	91	58.2	33.0	8.8	12	33.3	58.1	8.3
0.2 vs. 3.5	2	91	45.1	45.1	9.8	8	37.5	62.5	0
0.2 vs. 2.2	3	97	39.2	40.2	20.6	9	22.2	44.5	33.3
0.2 vs. 2.2	4	90	45.6	34.4	20.0	8	62.5	12.5	25.0
0.2 vs. 1.4	5	76	47.4	36.8	15.8	11	54.5	27.3	18.2
0.2 vs. 1.4	6	100	48.0	35.0	17.0	16	37.6	31.2	31.2
		642[a]	47.5[b]	37.9[b]	14.6[b]	72[a]	37.5[b]	41.7[b]	20.8[b]

[a] Total number answering questions.
[b] Average per cent for each preference level.

0794

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The Diacetyl Test as an Aid for Quality Control of Citrus Products. I. Detection Of Bacterial Growth in Orange Juice During Concentration[a, b]

Food Technol. 11: 240-3 (1957) 0813

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(Manuscript received June 25, 1956)

THE DIACETYL TEST was developed to detect microbiological growth in orange juice during orange concentrate production in commercial evaporators. Diacetyl value is dependent upon the concentration of both acetylmethylcarbinol and diacetyl in the citrus product. These substances are metabolic products of the growth of acid-tolerant bacteria, such as *Lactobacillus* or *Leuconostoc*. Off-flavor, usually described as similar to buttermilk, occurs in a product if the diacetyl content becomes sufficiently large. Presence of acetylmethylcarbinol causes no off-flavor, but may be indicative of microbial activity.

The diacetyl test, a colorimetric method based upon the Voges-Proskauer reaction, was first suggested by Hill, Wenzel and Barreto *(5)* as a method for detecting bacterial growth in citrus juices or concentrate. Byer *(3)* modified this test and improved its sensitivity. Beisel *et al. (2)* used it to investigate the Voges-Proskauer reactants in California Valencia orange juice. Hays and Riester *(4)* and Murdock, Troy and Folinazzo *(6)* discussed the problem of the development in orange concentrates of off-flavors of bacterial origin.

Purpose of this report is to present additional data to demonstrate how the diacetyl test may be used to detect bacterial growth in orange juice during commercial concentration before off-flavor of the buttermilk-type has resulted. Spoilage and loss of the juice, which would occur if bacterial growth was allowed to continue, may thus be prevented by the application of the diacetyl test.

Results of the flavor evaluation of 435 commercial samples of frozen orange concentrate, collected during the 1953-54 and 1954-55 seasons, are reported to indicate that by the use of both good sanitary practices and quality control methods, including the diacetyl test and microscopic examinations, processors have been able to prevent the occurrence of buttermilk-type off-flavors in frozen orange concentrates. Diacetyl values for the 435 commercial concentrate samples are also reported.

[a] Cooperative research by the Florida Citrus Commission and Florida Citrus Experiment Station. Florida Agricultural Experiment Station Journal Series, No. 495.

[b] Presented at the Sixteenth Annual Meeting of the Institute of Food Technologists, St. Louis, Mo., June 13, 1956.

EXPERIMENTAL METHODS

The diacetyl test. The basis for this test is that diacetyl, in the presence of alkali, creatine and alpha-naphthol, forms a compound that results in a red solution, the intensity of which may be measured colorimetrically; also that acetylmethylcarbinol in the presence of atmospheric oxygen and strong alkali is oxidized to diacetyl.

Diacetyl value, as reported in this paper, is the diacetyl equivalent of diacetyl and/or acetylmethylcarbinol in 25 ml. of distillate from 300 ml. of reconstituted orange concentrate. The diacetyl value was determined by using the following reagents, equipment, and procedure.

Reagents. (a) Alpha-naphthol solution. Dissolve 5 g. of alpha-naphthol (Eastman No. 170) in 100 ml. of 95% ethyl alcohol.

(b) Concentrated alkali solution containing creatine. Dissolve 40 g. of KOH in sufficient distilled water to make 100 ml. of solution and then add 0.3 g. of creatine (Eastman No. 951).

The reagents should be prepared daily or may be kept in brown bottles for 2 weeks if refrigerated.

Equipment. The colorimeter used was a Fisher Electrophotometer. A 525 mμ filter and 3 ml. round glass absorption cells were used.

Procedure. (a) Reconstitute sample of citrus concentrate to be tested to 12° Brix ± 0.2°.

(b) Distill 25 ml. from 300 ml. of reconstituted juice using an efficient condenser. Collect the distillate in a 25 ml. graduated cylinder. The distillate should flow down the sides of the graduated cylinder to prevent dispersion of peel oil, which will cause cloudiness.

(c) Pipette 10 ml. of distillate from the graduated cylinder, without disturbing any floating peel oil, into a 25 mm. x 200 mm. test tube.

(d) Add 5 ml. alpha-naphthol solution and 2 ml. of KOH solution containing creatine, stopper the test tube, shake vigorously for 15 sec., and allow to stand for 10 min. Shake again vigorously for 15 sec. In the same manner and at the same time, prepare a reagent blank using distilled water in place of distillate from the juice.

(e) Fill colorimeter tubes with test sample and reagent blank. Null the colorimeter with a reagent blank and measure color intensity in test sample.

(f) Determine concentration in p.p.m. of diacetyl by using a calibration curve obtained by applying this procedure to distilled water to which known amounts of diacetyl (Eastman No. 1591) have been added.

The diacetyl test should be made using analytical grade reagents of known purity. Isopropyl alcohol, 99%, may be used in place of 95% ethyl alcohol; because of its accessibility without regulations, it is used in most plants as a solvent for alpha-naphthol. The color produced when ethyl alcohol was used had its peak absorption at 545 mμ; at 535 mμ when isophropyl alcohol was used. The reaction time allowed between the addition of

0813

the reagents and the time at which the colorimeter readings are made must be carefully controlled, because the color developed by the reaction changes and decreases steadily after reaching a maximum. The rate of change in color, after the addition of the reagents, is different when ethyl alcohol is used from that occurring with isopropyl alcohol.

Microbiological methods. Direct microscopic counts were made from slides prepared as described by Hill, Wenzel and Barreto *(5)*. The number of microorganisms in each of 25 fields was counted and the average count per field calculated. Total plate counts were made by using orange serum agar, pH 5.6, with incubation at 30° C. for 48 hr.

Pilot plant procedures. A 2-stage, high-vacuum, low-temperature pilot-plant evaporator, described by Atkins, Wenzel and Moore *(1)*, was used for the processing of 20° Brix orange concentrate for an extended period of 13 hr. so that bacterial growth would occur in the product. Sufficient pasteurized 20° Brix orange concentrate was divided into 2 batches. One of these was put into the first stage of the evaporator. The other batch was inoculated with a very actively growing culture of *Lactobacillus plantarum* and then pumped into the second stage of the evaporator. No mixing of the two batches of concentrate occurred initially or during subsequent processing, since the connecting pipe between the two stages was closed by means of a valve. Mixing of vapor from the separation chambers was possible during processing, but apparently did not occur to any significant extent.

The two batches of product were continuously processed under vacuum for 13 hr. during which time the concentration of the concentrate in each of the stages was maintained at 20° Brix by the addition of pasteurized deionized water. Temperature of 78° to 82° F. was maintained in the concentrate in the pan of the evaporator. At hourly intervals, samples of concentrate were withdrawn from both the first and second stages of the evaporator for examination using the diacetyl test and microbiological methods. Results are shown in Table 1 and Figure 1.

Commercial plant procedures. Data were collected during the 1953-54 season at a commercial concentrate plant. Diacetyl tests were made on orange juice going to the evaporator, products from the four stages of the evaporator, the pulpy cutback juice, and on the finished canned concentrate. It was thus determined that bacterial growth in juice during concentration could be detected most readily by testing juice from the second stage. Therefore, most of the data were obtained by determining diacetyl values and plate counts on samples of product withdrawn at regular intervals from the second stage of the evaporator. Some of the data are presented in Table 2 and Figure 2.

TABLE 1

Diacetyl values and microbiological counts in pasteurized 20° Brix orange concentrate, pH 4.0, after inoculation with Lactobacillus plantarum and processing in evaporator[1]

Processing time	Product inoculated—second stage of evaporator[2]			Product not inoculated—first stage of evaporator[2]	
	Diacetyl value	Direct microscopic count	Plate count	Diacetyl value	Plate count
hr.	p.p.m.	Average of 25 fields	Orange serum agar, pH 5.6	p.p.m.	Orange serum agar, pH 5.6
Initial	3.1	0.1	180,000	2.7	950
1	1.1	0.1	...	0.2	...
2	1.1	0.1	340,000	0.1	1,900
3	1.1	0.2	...	0.2	...
4	1.2	0.6	1,060,000	0.2	3,000
5	1.3	0.8	...	0.1	...
6	1.6	0.9	1,115,000	0.2	7,500
7	1.7	1.1	...	0.1	...
8	2.3	2.3	2,615,000	0.2	6,700
9	3.0	3.1	...	0.2	...
10	3.8	3.6	4,850,000	0.2	39,000
11	4.1	5.0	...	0.1	...
12	5.7	5.3	7,500,000	0.1	57,000
13	7.3	5.9	6,100,000	0.3	196,000

[1] The concentration of the product in both stages of the evaporator was held constant at 20° Brix during processing by the addition of pasteurized deionized water. The temperature of the juice in the pan of the evaporator was maintained at 78° to 82° F.

[2] There was no circulation of product between the first and second stages of the evaporator.

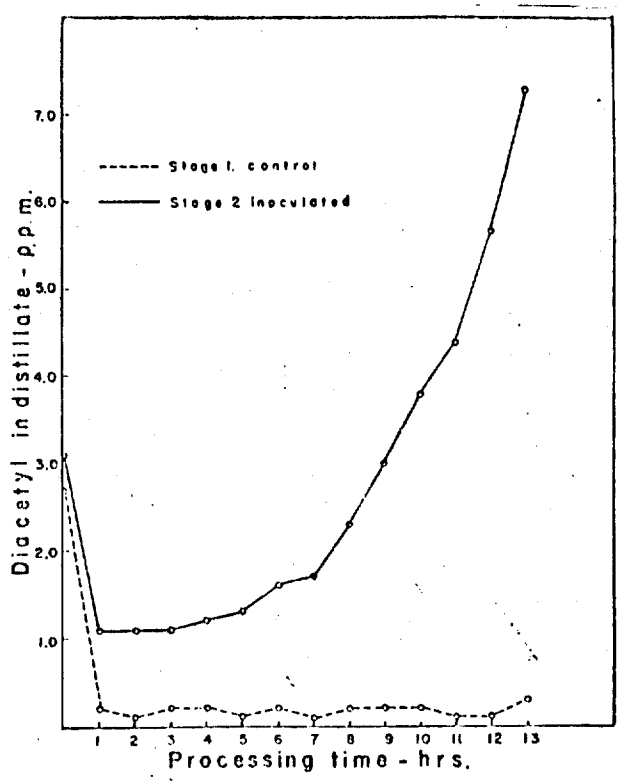

Figure 1. Diacetyl values in pasteurized 20° Brix orange concentrate, pH 4.0, after inoculation with Lactobacillus plantarum and processing in pilot plant evaporator. Addition data are reported in Table 1.

Examination of samples of commercial frozen orange concentrate. During the 1953-54 and 1954-55 seasons, samples of commercial frozen orange concentrate were collected twice each month from 24 Florida plants. The diacetyl value in 435 samples was determined; a frequency graph of these values is shown in Figure 3. The flavor of these commercial products was also evaluated by a taste panel which averaged 7 persons, varying from a minimum of 5 to a maximum of 9. The reconstituted orange concentrates were tasted at room temperature and flavor defects noted when juices were graded as poor in quality.

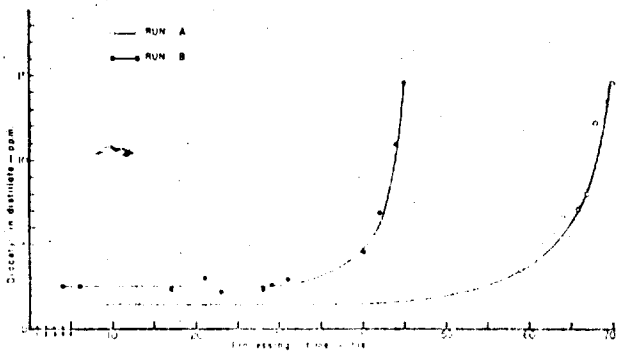

Figure 2. Diacetyl values in orange concentrate in second stage of commercial evaporator during continuous operation.

RESULTS AND DISCUSSION

Pilot plant investigation. Data reported in Table 1 and Figure 1 for diacetyl values, microscopic and plate

0813

counts show that the growth of *Lactobacillus plantarum* increased gradually in the 20° Brix orange concentrate in the second stage of the evaporator, with the rate of growth becoming markedly faster after 7 hr. of processing. The microscopic counts increased during the 13 hr. processing period from 0.4 to 5.9 organisms per field; the plate counts from 480,000 to 7,300,000 colonies per ml. and the diacetyl values from 3.1 to 7.3 p.p.m. The decrease during the first hour in the diacetyl value from 3.1 to 1.1 p.p.m. resulted because of the volatility of both diacetyl and acetylmethylcarbinol; this also indicated that some of these substances are removed from products being processed under vacuum. Off-flavor of significant intensity was detectable in the concentrate from the second stage after 9 hr. of processing. There was some microbial growth in the uninoculated concentrate in stage 1 of the evaporator as shown by the plate counts in Table 1; however, the diacetyl values in this product were very small, did not increase, and off-flavor of the buttermilk-type did not develop. It should be pointed out that this pilot plant run was different from continuous commercial processing in that there was no addition of raw juice or withdrawal of concentrate, since the concentration of the product in the two stages of the evaporator was held at 20° Brix by the continuous addition of water.

TABLE 2

Diacetyl values and microbiological counts in orange concentrate in second stage of commercial evaporator during continuous operation

Processing time	Diacetyl value	Plate count	Concentration of juice from evaporator
hr.	p.p.m.	Orange serum agar, pH 5.6	° Brix
9	1.3	...	22.0
19	1.3	2,000	18.5
29	1.7	20,500	21.0
39	1.6	13,500	19.5
49	2.4	30,500	20.0
51	2.2	61,000	21.0
53	2.3	63,500	20.5
55	2.6	75,500	22.0
57	3.4	200,000	21.5
59	3.5	155,000	20.5
60	4.0	...	18.0
61	4.2	560,000	18.8
62	4.0	...	21.5
63	5.6	585,000	16.5
64	6.6	560,000	17.5
65	5.6	600,000	21.8
66	7.1	1,360,000	20.0
67	8.0	985,000	20.0
68	12.2	2,300,000	14.5
69	13.5	3,350,000	19.5
69.5	14.6	2,700,000	20.5

Commercial plant investigation. Data were accumulated chiefly from the examination of orange juice from the second stage of the commercial evaporator. The juice concentration, determined with a hand refractometer, in this stage of the evaporator was usually in the range of 18° to 22° Brix as is shown in Table 2. Using the diacetyl test, preliminary data were obtained to find out existing conditions in juice being concentrated in this plant. Before the evaporator was to be shut down for a general cleaning of the entire plant, a diacetyl value of 8.8 p.p.m. was found in juice from the second stage; this increased very rapidly to 19.7 p.p.m. at which time processing was discontinued. The evaporator and plant were then thoroughly cleaned. During the following day, the diacetyl value ranged between 1.2 and 2.2 p.p.m. and during the second day of operation the range was 1.8 to 2.8 p.p.m. After 64 hr. of processing, a diacetyl value of 12.8 p.p.m. was obtained and followed

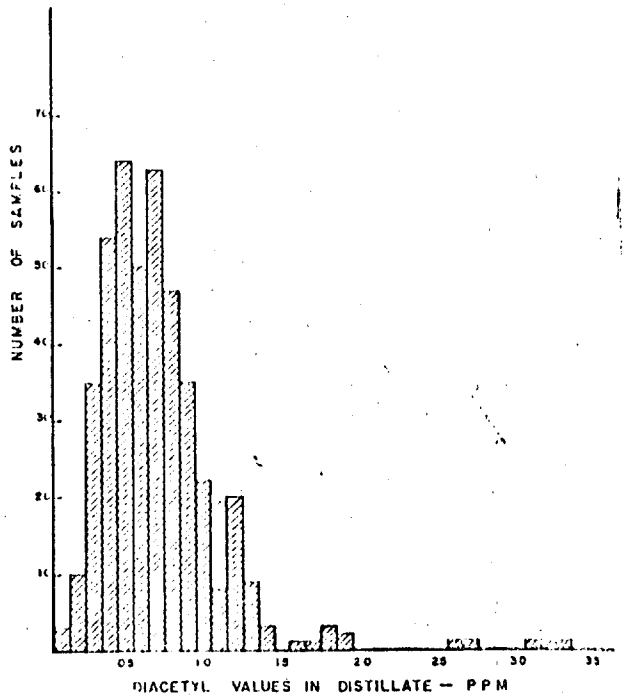

Figure 3. Frequency graph of diacetyl values in 435 commercial samples of frozen concentrated orange juices collected from 24 Florida plants during the 1953-54 and 1954-55 seasons.

by a very rapid increase to 28.7 p.p.m. At this time evaporation of juice was again stopped. It should be pointed out that during continuous operation frequent cleaning of extractors, finishers and other equipment was done, but in such a manner that the evaporators did not have to be closed down.

Having the previously mentioned data, it was decided to obtain diacetyl values and plate counts from juice in the evaporator every 1 or 2 hr., starting shortly after the plant was cleaned thoroughly and continuing until the evaporator was again stopped. The diacetyl values obtained during a period of continuous operation of almost 70 hr. are shown graphically in Figure 2, Run A; some of the plate counts, together with the corresponding diacetyl values, are reported in Table 2. There was a gradual rise in the diacetyl values after 45 hr. of operation, which increased above 5 p.p.m in 63 hr., and then the diacetyl content in the distillate increased rapidly to 14.6 p.p.m. during the next 6.5 hr. Good correlation was found between the diacetyl values and the plate counts.

Diacetyl values found in juice being evaporated during another run (Run B) are also shown in Figure 2. In this instance, the 5 p.p.m. level was reached after the relatively short time of only 38 hr. of operation; the rapid increase in the diacetyl and/or acetylmethylcarbinol in the product after the 5 p.p.m. level was reached was again evident.

On the basis of the information obtained at this plant, it was recommended that concentration of juice should be discontinued when a diacetyl value of 5 p.p.m. was reached in juice in the second stage of the evaporator. All data indicated that bacterial growth would increase very rapidly, if processing was continued after the 5 p.p.m. level was reached, and that consequently off-flavor would eventually become apparent in the finished 42° Brix orange concentrate. Buttermilk-type off-flavor was definitely detectable in concentrate from the last stage of the evaporator after 69 hr. of operation and this sample had a diacetyl value of 4.9 p.p.m. Comparison of this value with 12.2 p.p.m. in juice from the second stage after 68 hr. indicated that a large amount of the diacetyl and/or acetylmethylcarbinol produced by bacterial growth in juice in the low-Brix stages was removed during the concentration of the product in the high-Brix stages of this commercial evaporator. Hill, Wenzel and Barreto *(5)* previously reported a decrease of 70% in diacetyl value when spoiled 20° Brix orange juice was concentrated from 20° to 60° Brix in a pilot plant evaporator.

It should be mentioned that the maximum limit for the diacetyl value to be used as an indication for imminent bacterial growth and subsequent spoilage of product will not be the same for all concentrate plants, because it may be affected by differences in evaporators and plant procedures. Therefore, the maximum diacetyl value permissible in evaporator juice has to be determined by quality control personnel in each plant. At the present time, both the diacetyl and direct microscopic count methods are being used in control laboratories in Florida plants for the detection of microbial growth in citrus juices during concentration.

Finally, attention should be called to the fact that thermal stabilization of orange juice for concentration was being used in the commercial plant where data in this report were obtained. Nevertheless, bacterial growth occurred in juice in the evaporator. Therefore, thermal treatment of juice prior to concentration does not eliminate completely the possibility of bacterial spoilage, even though the stabilization procedures used, which protect the product against enzymic changes, do reduce the bacterial content of the evaporator-feed juice to a very low level.

Diacetyl values in commercial frozen orange concentrates. Although the primary purpose of the diacetyl test is to aid in the detection of microbiological activity in citrus juices in evaporators, some interest has developed and questions have been asked about the diacetyl values found in citrus juices or concentrates. Therefore, diacetyl values were determined and are reported for some commercial samples of frozen orange concentrates. Because of various factors involved, the relationship of diacetyl values to the quality of citrus juices or concentrates remains to be determined after sufficient data on this problem have been obtained.

A frequency graph, Figure 3, shows results obtained when 435 samples of commercial frozen concentrates were reconstituted and diacetyl values determined. These samples were collected twice monthly from 24 Florida plants during the 1953-54 and 1954-55 seasons. The diacetyl values found in the reconstituted juices ranged from 0.1 to 3.3 p.p.m. and 88% of the juices had diacetyl values of 1.0 p.p.m. or less. Flavor evaluation of the 435 samples by the taste panel resulted in only 5 or 1.1% being rated as poor in quality because of buttermilk-type off-flavor. The diacetyl values found in these 5 orange concentrates were 1.2, 2.6, 3.1, 3.2 and 3.3 p.p.m.

Results from the flavor evaluation of the commercial concentrates indicate that, even though bacterial spoilage can occur in juice during concentration in commercial evaporators, processors are preventing such spoilage by using good sanitary plant practices together with quality control tests, such as the diacetyl test and microscopic counts, so that today the consumer seldom finds buttermilk-type off-flavor in commercial frozen orange concentrates.

SUMMARY

Orange concentrate, pH 4.0 and 20° Brix, was inoculated with *Lactobacillus plantarum* and recirculated in a pilot plant evaporator until spoilage of the product occurred. Diacetyl values, direct microscopic counts, and plate counts, determined on samples of concentrate withdrawn from the evaporator every hour, are reported and were indicative of bacterial growth.

Data obtained during commercial plant operation are presented and illustrate the use of the diacetyl test for the detection of bacterial growth in orange juice in commercial evaporators before spoilage occurs, thereby preventing loss of product.

Diacetyl values were determined for 435 samples of commercial frozen orange concentrate, which were collected during two years from 24 Florida plants.

Acknowledgments

The help of personnel of the Agricultural Marketing Service of the U. S. Department of Agriculture, Winter Haven, Florida, in collecting commercial frozen orange concentrate samples was appreciated.

The assistance of Miss Betty Jo Andrews and Mrs. Maxine Melvin in performing many of the tests during this investigation is also acknowledged.

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0833

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J. Food Sci. 30(4): 604-9, (1965) (Eng.)

The Effects of Storage Conditions Upon Acetylmethylcarbinol, Diacetyl, and Ethyl Alcohol in Apple Juice

(0833)

SUMMARY

A total of 576 samples of apple juice stored at 0, 37, 72, and 91°F were analyzed during a period of 242 days. Of these, 283 were analyzed for acetylmethylcarbinol (AMC), 195 for diacetyl, and 98 for ethyl alcohol. There was no significant decrease in quantity of AMC or ethyl alcohol in apple juice in tin cans and glass bottles during the storage periods. The level of diacetyl, however, declined at a significant rate when diacetyl had been added to apple juice in bottles and stored at 91 and 72°F for 219 days. Only AMC was tested for its stability during repeated thawing and refreezing periods. No significant changes occurred in the quantities of AMC present in these frozen samples.

INTRODUCTION

Acetylmethylcarbinol (AMC) and diacetyl, metabolic by-products of yeasts, molds, and bacteria, have been used as indicators of the microbial quality of apple juice (Fields, 1962). Fields also proposed standards for AMC content of commercially processed apple juice. Based on data in his study, apple juices with 1.0 ppm AMC or below should be acceptable and are generally indicative of sound raw materials and good processing conditions. Values of 1.1–1.6 ppm AMC would be questionable, and values above 1.6 ppm AMC would indicate poor raw materials and/or poor sanitary conditions.

Although diacetyl is found in very small quantities in fermenting liquids (Brewer *et al.*, 1938), as compared to AMC, both chemicals can be used as indicators of quality. Fields (1964) stated that the main producers of diacetyl in apple juice are yeasts, namely *Saccharomyces* spp. He therefore postulated that diacetyl in apple juice may be considered presumptive evidence of poor sanitary practice in the processing plant. Murdock (1964) stated that diacetyl is destroyed or converted to a non-Voges-Proskauer reactant by yeasts in orange juice, thus possibly limiting the use of diacetyl.

The chemical reactions which bring about changes in canned foods during storage are extremely complicated. It was generally assumed by Murray (1953) that the rate at which chemical reactions occur in canned foods doubles with each 18°F rise in temperature.

Prevailing temperatures reported in a survey of 79 canned-food warehouses throughout the U. S. showed that, even in the hottest areas of the South, the average yearly warehouse temperature did not exceed 80°F (Monroe *et al.*, 1949). In the areas in which the greatest annual volume of canned food is warehoused, the average yearly temperature was about 65°F.

Irvine (1964) stated that the expected shelf life of canned apple juice, based on an average warehouse temperature of 70°F, is approximately 2–2½ years. McConnell (1964) estimated that the shelf life of canned apple juice ranged from 15 months to over 24 months. In any case, a technologist might expect to find apple juice that is over one year old in warehouses. No information could be found on the shelf life of bottled apple juice.

Fields (1964) reported a 23.4% loss of AMC in apple juice after 5 months of storage at 72–74°F. No data have been published on the effects of thawing and refreezing upon the AMC or diacetyl content of apple juice. Such data are important in determining the quality of apple juice.

The ethyl alcohol content has also been suggested as a means of detecting poor raw materials and poor processing conditions in apple juice manufacturing (Hill, 1965). Specific standards have been suggested for ethyl alcohol by Hill. In a 300-ml sample of juice ethyl alcohol values of 0.90 mg per ml in 80 ml of distillate or less are to be considered satisfactory. Values greater than 0.90 mg ethyl alcohol per ml in 80 ml of dis-

0833

tillate are to be considered unsatisfactory. However, no work has been done showing the effects of time and temperature of storage upon ethyl alcohol. If this compound is to be used as an indicator of quality of apple juice, its stability must be determined when juices containing ethyl alcohol are subjected to different storage conditions.

This research was done to determine whether storage conditions influence the quantities of AMC, diacetyl, and ethyl alcohol present in apple juice. Such data are important in establishing a chemical index of quality. If the storage temperature and/or the type of container (glass bottles or tin cans) influences the level of the chemical index, the food technologist should consider these factors in making a final judgment as to whether the product complies with the chemical standard of quality.

EXPERIMENTAL METHODS

Juice with added AMC and diacetyl. Approximately 100 gal. of juice were prepared from Jonathan variety apples with commercial equipment and methods. Sound apples were washed, ground, pressed, and treated with pectinase regular (Takamine) at the rate of 16 oz per 20 gal. of juice. This was 4 times the amount of enzyme recommended by Tressler and Pederson (1938). The increased amount compensated for the added filter in the enzyme preparation.

After the juice was allowed to stand 2 days with the enzyme at refrigerated temperatures, the juice was heated to 150°F to inactivate the enzyme. The filtering process was aided with diatomaceous earth. The juice was pumped through a standard juice filter until clear. The juice was again heated, this time to 160°F, to allow a filling temperature of at least 140°F for both cans and glass bottles.

One-quart bottles with screw-top lids were filled with juice. AMC was added to bottles to yield a concentration of 10 ppm of AMC in 30 ml of distillate. Diacetyl was added to other bottles to yield a concentration of 30 ppm in 30 ml of distillate. The bottles were capped and laid flat to enable sterilization of the caps.

Apple juice was also processed in tin cans in the same manner as the juice in bottles. After the juice was heated the second time it was put into 46-oz cans. Diacetyl was added to specified cans to yield a concentration of 20 ppm in 30 ml of distillate, while AMC was added to other cans of juice to yield a concentration of 10 ppm in 30 ml distillate. The cans were sealed, inverted, and held to sterilize the can.

Juice from apples with added rot. Juice with rots of 0, 1, 3, and 5% were prepared from Golden Delicious apples by weighing exact amounts of rots cut from rot-containing apples and by adding to sound apples at the rate of 0, 1, 3, and 5 lb per hundredweight. These mixtures were ground, pressed, and treated with pectinase regular (Takamine) at the rate of 16 oz per 20 gal. of juice.

The method of processing was as previously described except that the juice was heated to a higher prefilling temperature of 180°F to ensure sterilization because of the inclusion of rot. One-quart bottles were the only containers used in this study.

Juice with added alcohol. Frozen apple juice prepared in 1957 under conditions similar to those described was used for this study. A mixture of 4 parts York, 2 parts Winesap, 4 parts Red Delicious, 1 part Rome Beauty, 1 part Golden Delicious, and 1 part York Imperial was used. To ensure a filling temperature of 140°F, the juice was heated to 160°F. Each 1-quart bottle contained 900 ml of juice to which ethyl alcohol stock solution was added to yield 5.2 mg/ml in 30 ml of distillate using a 300-ml sample of juice.

Influence of thawing and refreezing on AMC. Apple juice used in studying the effects of thawing and refreezing was prepared in 1961 by the procedure given for preparation of apple juice. The juice was stored in 1-gal. glass jugs. The juice was divided into the same experimental categories, Experiment I and Experiment II, as used by Fields (1962), the only difference between the experiments being the varieties of the apples used: in Experiment I, 50% Jonathan, 25% Red Delicious, and 25% Golden Delicious; and in Experiment II, equal amounts of Jonathan and Golden Delicious. At the time of testing, the juice was allowed to thaw at room temperature and was mixed by vigorous shaking. Three hundred ml of juice were removed for each analysis. Immediate refreezing of the apple juice remaining after each testing helped minimize time and temperature effects.

Storage conditions. A walk-in cooler provided storage space for apple juice samples held at 37°F. Samples held at 72°F were stored in an air-conditioned room. A 91°F storage area was achieved in an incubator. These latter samples were likely to show accelerated chemical reactions if any were to occur. Samples were removed at various intervals during storage periods for analyses for AMC, diacetyl, and ethyl alcohol.

Samples of juice used in the thawing and refreezing study were held at 0°F in a cold-storage room locker until sampling time, and were analyzed for AMC.

Determination of AMC and diacetyl. The method used was that of Hill and Wenzel (1957). Three hundred ml of apple juice were distilled in a 1-L Erlenmeyer flask attached to a Liebig condenser with a jacket length of 500 mm. Thirty ml of distillate were collected except when distilling the frozen apple juice, in which case 25 ml were collected.

A standard curve was prepared using Eastman No. P3788 acetylmethylcarbinol and Eastman No. 1591 diacetyl. Ethyl alcohol (95%) was used in preparation of the alpha-naphthol solution (Eastman No. T170); Eastman's No. 951 creatine was used in preparing the creatine-sodium-hydroxide solution. Ten minutes after the reagents and the distillate solution were mixed, colorimetric readings were made on a Bausch and Lomb Spectronic 20 colorimeter at 545 mμ.

Determination of ethyl alcohol. Three hundred ml of apple juice which had been adjusted to pH 8 were placed in a 1-L Erlenmeyer flask attached to a Liebig condenser with a jacket length of 500 mm. Eighty ml of the distillate were collected. To 2 ml of the distillate was added 10 ml of potassium dichromate in sulfuric acid solution as recommended by Shupe and Dubowski (1952) and modified by Niss (1953).

Twenty-five ml of 95% ethyl alcohol were added to a volumetric flask and the volume of liquid increased to 1 L with distilled water. Twenty-five ml of ethyl alcohol (95%) at 25°C are equal to 19.99 or 20 mg alcohol ml in 1 L of water (corrected for temperature and specific gravity, Hodgman, 1932). Using the stock solution, dilutions were prepared and colorimetric readings were made at 615 mμ. A standard curve was prepared to determine the ethyl alcohol content in stored apple juice.

RESULTS AND DISCUSSION

AMC added. There were 53 samples in glass bottles and 56 samples in tin cans held in storage for periods of 228 days and 242 days, respectively. The samples were analyzed at 5 different times. The levels of AMC were adjusted to give approximately 10 ppm in bottles and tin cans so that the rate of any loss could be followed. The amounts added were well within the limits found in commercial apple juice (Fields, 1964).

There was no significant change in AMC content during storage. The variations found in the amount of AMC present are within the limits of the accuracy of the methods used (Holck, 1964). One can assume that the AMC found in apple juice in these containers is the amount present at processing time. This is contrary to findings by Fields (1964), who stated that an average loss of 24.3% of AMC in apple juice occurred in tin cans during 5 months at 72–74°F. However, in Fields' study the storage data were limited in scope. Other possible reasons for the loss, as reported by Fields, are reactions in the juice and/or reactions with the tin plate due to faulty enamel in the tin cans.

Diacetyl added. Sixty-three samples of apple juice in glass bottles with added diacetyl in the amount of 30 ppm were analyzed at 5 sampling times in a 219-day storage period. As with added AMC, the levels of diacetyl were high enough that the rate of any loss could be followed. It was thought that greater losses might occur with diacetyl than with AMC because of the "extra" ketone group in diacetyl. Of all the samples stored at the 3 different temperatures, only those stored at 91 and 72°F showed a decline in the quantity of diacetyl (Table 1). At present there is no explanation as to why this loss of diacetyl occurred in bottles. Further research needs to be done concerning the effects of storage conditions on diacetyl.

The samples stored at 37°F showed no substantial changes in diacetyl levels; rather, the variations seemed to be due to the greater volatility of diacetyl than of AMC, whether in its purified form or in naturally occurring rots. Before any definite conclusions can be drawn, further study is required with more samples. However, since

Table 1. Storage temperatures and time and their effects upon diacetyl (ppm)[a] added to apple juice in glass bottles.

Storage time (days)	Storage temperature		
	91°F[b]	72°F[c]	37°F
00	30	30	30
22	35	34	28
66	30	30	32
179	23	24	29
219	21	25	27

[a] Mean of four replicate samples.

[b] Any difference between any two means which is greater than the D value of 2.9 is significant at the 5% level.

[c] Any difference between any two means which is greater than the D value of 4.0 is significant at the 5% level.

0833

the average yearly temperature did not exceed 80°F in the study of Monroe *et al.* (1949), one may conclude that diacetyl would be retained in sufficient quantity during normal warehouse temperatures to be of value as a presumptive indicator of quality.

During a 288-day period, 59 samples of apple juice in tin cans were analyzed for diacetyl at six sampling times. Losses of diacetyl could occur more readily if there were any flaws in the enamel of the tin cans. According to Table 2, however, no substantial changes occurred in any samples, regardless of storage temperature. Variations could, again, be accounted for by the limits of the accuracy of the methods used.

Table 2. Storage temperature and time and their effects upon diacetyl (ppm)[a] added to apple juice in tin cans

Storage time (days)	No. of samples	Storage temperature 91°F	72°F	37°F
0	02	17	17	17
11	04	21		
19	12	22	21	21
61	12	22	24	22
174	12	19	20	21
228	17	19	19	21
Mean for storage period		20	20	20

[a] Figures listed as mean.

AMC content with added rot. As shown in Table 3, apple juice samples were tested with 0, 1, 3, and 5% rot. Two samples of each level of rot were analyzed from each of the 3 storage temperatures at each of the testing dates. Zero-time readings were calculated using only 2 samples per rot level.

Since no samples were lost from spoilage, it was possible to report data on all 128 samples prepared. After 219 days of storage, there appeared to be no substantial change in quantities of AMC present in apple juice samples with added rot. This indicated that AMC, as contained in natural rotting apple tissue, was present after storage in concentrations similar to those found during the production of the apple juice. This study emphasized the validity of AMC as an indicator of quality.

Ethyl alcohol with added rot. The same distillates were used for analyses of AMC

Table 3. Influence of storage temperatures and time upon AMC (ppm)[a] in apple juice made from apples with added rot.

No. of samples	0 91°F	0 72°F	0 37°F	1 91°F	1 72°F	1 37°F	3 91°F	3 72°F	3 37°F	5 91°F	5 72°F	5 37°F
00	0.1	0.1	0.1	0.9	0.9	0.9	1.5	1.5	1.5	2.2	2.2	2.2
22	0.1	0.1	0.1	1.5	1.6	1.3	2.1	2.5	2.5	3.0	3.0	2.7
96	0.2	0.2	0.3	0.9	1.0	0.9	1.6	1.8	1.7	2.7	2.7	2.7
187	0.1	0.2	0.1	1.1	1.2	1.3	2.0	2.1	2.1	2.0	2.1	3.0
219	0.1	0.1	0.1	0.9	0.9	1.0	1.5	1.7	2.1	2.1	2.2	2.6
Mean for storage period	0.1	0.1	0.1	1.1	1.1	1.1	1.7	1.9	2.0	2.4	2.4	2.6

[a] Figures listed as mean.

[b] Two samples tested except at 0 days, when only two samples group were tested. The total amount of samples tested

and ethyl alcohol. The total analyses of 128 samples of apple juice for ethyl alcohol are shown in Table 4. Here again, the variations in readings recorded appear to be due to the methods used and not to reactions within the bottled juice during the 219 days of storage at different temperatures.

Ethyl alcohol added. Forty-three samples were analyzed over a 164-day period during which 5 sampling times were selected. Three storage temperatures, as listed previously, were used to show any effects of storage temperature and time upon ethyl alcohol in apple juice. There were no appreciable changes in the ethyl alcohol content of the samples regardless of the temperature at which it was stored. Ethyl alcohol remained stable in apple juice for this period, so it appears that it could be used as a chemical indicator of quality.

Thawing-refreezing. The study was designed to show the influence, if any, of thawing and refreezing upon AMC content in juices made from apples with added rot. As shown in Table 5, there were 4 separate testing times in Experiment I and 3 separate testing times in Experiment II. During these times the samples were thawed and distilled, and readings were recorded using the Voges-Proskauer test. Because of a shortage of the pilot-plant-produced juice, only 1 sample was run per level of rot in each experiment at each sampling time. Forty samples were analyzed in both experiments.

According to results found in these experiments, there appeared to be no loss of AMC contained in the apple juice after 4 successive thawing and refreezing times. This would indicate that the amounts of AMC found in frozen samples would be similar to the amounts found at the time of processing, thus implying that freezing would be an efficient means of handling samples until laboratory work could be carried out.

General conclusions. Both AMC and ethyl alcohol fulfill the criteria for a useful chemical index for determining the microbial quality of apple juice as proposed by Fields (1964). The fact that there was no decline in the quantities of AMC and ethyl alcohol in this study strengthens the use of these compounds as indicators since they reflect the conditions at the time of manufacturing.

Table 4. The influence of storage temperatures and time upon ethyl alcohol (mg/ml)[a] in apple juice made from apples with added rot.

No. of days	% rot																	
	1						3				5							
	32°F	72°F	87°F	32°F	72°F	87°F	32°F	72°F	87°F	32°F	72°F	87°F	32°F	72°F	87°F	32°F	72°F	87°F
00[b]	0.4	0.4	0.4	0.4	0.4	0.4	0.8	0.8	0.8	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
22	0.5	0.0	0.5	0.6	0.1	0.4	0.9	0.4	0.7	1.7	1.7	1.7	1.7	1.7	1.0	1.0	1.0	1.0
69	1.0	0.6	0.4	0.7	0.6	0.7	1.2	0.9	0.0	1.4	1.0	1.0	1.4	1.0	1.0	1.0	1.0	1.0
187	0.4	0.6	0.4	0.5	0.7	0.5	0.8	0.8	1.0	1.0	1.3	1.0	1.0	1.3	1.0	1.0	1.0	1.0
219	0.5	0.5	0.4	0.6	0.8	0.7	0.9	1.1	0.9	1.2	1.4	1.2	1.4	1.4	1.2	1.2	1.2	1.2
Mean for storage period	0.6	0.4	0.5	0.6	0.5	0.5	0.9	0.8	0.8	1.2	1.1	1.0	1.1	1.1	1.0	1.0	1.0	1.0

[a] Figures listed as mean.
[b] At 0 day only two samples rot level were tested. The remaining figures are representative of two samples temperature rot level.

0833

Table 5. AMC (ppm)[a] observed by Voges-Proskauer test in frozen pilot-plant-produced apple juice.

Experiment[b]	% ret	Sampling time (days) 90	94	151	185	Mean for storage period
I	0	0.2	0.6	0.5	0.6	0.47
	2	1.2	1.5	1.4	1.0	1.27
	4	2.8	4.0	3.5	3.6	3.47
	6	1.6	2.3	2.2	2.3	2.10
	8	5.3	5.2	5.1	4.3	4.97
	10	3.3	3.2	3.7	3.7	3.47
	12	7.5	6.5	6.0	6.7	6.67
II	0	0.5		0.9	0.4	0.60
	4	0.9		1.5	0.9	1.10
	8	1.1		1.9	1.6	1.53
	12	1.8		2.3	2.5	2.20

[a] Figures listed are representative of one sample per testing date.
[b] In Experiment I, a mixture of 50% Jonathan, 25% Red Delicious, and 25% Golden Delicious was used. Juice in Experiment II was 50% Jonathan and 50% Golden Delicious.

Diacetyl, although declining in quantity during storage in this study, would still be effective as an indicator of quality if any were found in a product. Fields (1964) proposed that the presence of diacetyl be used as presumptive evidence of poor sanitary practice in the processing plant because it is produced by yeasts and usually is not found in tissue decomposed by filamentous fungi.

The analyses for AMC and diacetyl and for ethyl alcohol, as described herein, are relatively inexpensive and may be performed rapidly. The validity of the methods used for these indicators are sufficiently accurate for commercial purposes.

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Ms. rec'd 10/16/64.

Journal Series Paper No. 2833, approved by the Director of the Missouri Agricultural Experiment Station.

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Nature 201(4925), 1223, (1964)

Carbonyl Compounds in Toasted Oat Flakes

THE nut-like flavour of properly toasted oat flakes is associated with volatile and non-volatile compounds developing mostly during the toasting process. The volatile compounds are represented primarily by carbonyl compounds. Their amount, and in some cases even the content, are much higher after processing. In the original oat flakes, only slightly steamed, we established the presence of the following compounds: furfural, formaldehyde, propanal, acetone and two unidentified carbonyl compounds. In toasted oats a certain decrease of formaldehyde was observed, but a number of other aldehydes and ketones appeared. In laboratory tests we found acetaldehyde, butanal, 2-methylpropanal, 2-methylbutanal (α-methylbutanal), 3-methylbutanal (β-methylbutanal), hexanal, octanal and 2-butanal.

Of the ketones present, 2-pentanone (methylpropylketone), 2-hexanone (methylbutylketone), 2-octanone (methylhexylketone) and diacetyl (2,3-butadione) were established with certainty. The carbonyl compounds were separated by paper chromatography and their identification carried out by transferring them to 2,4-dinitrophenylhydrazone.

Furfural being present in considerable quantities, but not exceeding other compounds in amount, cannot be assumed to be the main cause of the specific flavour of toasted oat flakes. It seems to contribute to the aroma in the same way as hydroxymethylfurfural. The two methylbutanals as well as butanal, hexanal and octanal and propanal show a characteristic flavour. We suppose that even the presence of methylketones, which themselves possess a clear-cut aroma, support the characteristic flavour of the oat-meal. The presence of a comparatively large amount of 2-hexanone confirms our assumption.

A thorough knowledge, not only of the number of components developing during the toasting process, but also their amount and their proportions may help us to foretell the flavour of toasted oat flakes.

The carbonyl compounds cannot be regarded as the sole source of flavour, since there is a number of other volatile amines, the presence of which has been recorded elsewhere.

Considering the problem of flavour we must also mention the developed non-volatile compounds, primarily the melanoidins and similar substances, which are doubtless also responsible for the unique flavour of oatmeal, according to earlier findings.

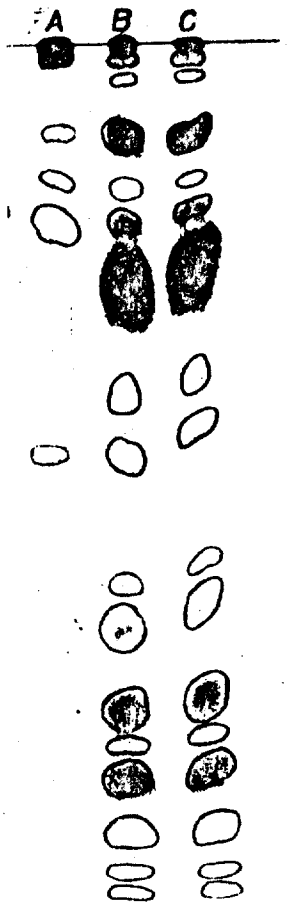

Fig. 1. Carbonyl compounds in toasted oat flakes. For the separation of mixtures of 2,4-dinitrophenylhydrazones, descending paper chromatography was used. Whatman paper No. 1 impregnated with 30 per cent *N,N'*-dimethylformamide in ether. Developing system hexane saturated with *N,N'*-dimethylformamide. After the detection by potassium hydroxide solution. *A*, Untoasted oat flakes. Spot from the start: 1, unidentified; 2, furfural; 3, formaldehyde; 4, unidentified; 5, acetone + propanal. *B*, Toasted oat flakes at a higher temperature and for a longer time. *C*, Toasted oat flakes at a higher temperature and for a shorter time. Spot from the start: 1, Unidentified; 2, hydroxymethylfurfural; 3, furfural; 4, formaldehyde; 5, diacetyl; 6, acetaldehyde; 7, 2-butanal; 8, acetone + propanal; 9, *n*-butanal; 10, 2-methylpropanal; 11, 3-methylbutanal; 12, 2-pentanone; 13, *n*-hexanal + 2-methylbutanal; 14, 2-hexanone; 15, *n*-octanal; 16, 2-octanone

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THE BUTTER INDUSTRY

PREPARED FOR

FACTORY, SCHOOL AND LABORATORY

THIRD EDITION

REWRITTEN AND ENLARGED

BY

OTTO FREDERICK HUNZIKER

B.S.A., M.S., D.Sc.

PUBLISHED BY THE AUTHOR
LA GRANGE, ILLINOIS
(1940)

his country H. W. Conn,[5] the eminent biologist of Wesleyan University and of the Connecticut Agricultural Experiment Station, made extensive studies and independent discoveries regarding the possibilities and the use of butter cultures. As early as 1889 he began the publication of a series of bulletins on cream ripening and pure culture starters, and his labors played an important role in the initial introduction and use of commercial starter cultures in America during the years 1890 to 1900.[6]

Progress with pure starter cultures in American creameries was slow at first. Among the principal reasons for the industry's reluctance to use pure culture starters were the facts that the majority of buttermakers were not in condition to pasteurize their cream, and that the American consumer demanded a higher flavored butter than resulted from the use of the then available commercial cultures of lactic acid bacteria, used in connection with pasteurized cream.

As the merits of pasteurization of cream for buttermaking became more fully recognized, and as the possibilities of the proper use of pure culture starters became more generally known and appreciated, the initial objections gradually yielded to the more general adoption of the use of commercially prepared starter cultures. While even today the economic merits of making butter from cream ripened with pure culture starters are still a controversial issue, and while the use of pure culture starters is by no means universal in American creameries, it is now fully recognized that the proper ripening of efficiently pasteurized cream, with starter cultures of selected lactic acid-producing and citric acid-fermenting bacteria, accentuates the desired butter aroma and flavor, and makes for greater uniformity of flavor and aroma in butter.

Bacteriology of Pure Culture Starters.—It was early recognized by the great pioneers of starter cultures prepared from selected bacteria, that the problem of organisms involved and products formed is an exceedingly complex one, and that the typical result of a strictly pure culture of S. lactis is far from the desired flavor and aroma; that it is in fact a flat, acid, sour milk flavor.

Thus, Storch's trials in 1888 in which he supplied the cultures for use in the making of butter from pasteurized cream,

showed that the pure lactic acid cultures did not produce fine flavored a butter as when using buttermilk from a churning of good butter for inoculation of the cream. Conn, as early as 1889, concluded that "the ripening of cream is a complex matter." In 1896 he pointed out that, while the acid is developed from the sugar of milk, the flavor probably comes from other sources. He held that acid and flavor should be considered separately and that aroma should be distinguished from flavor. Weigmann,[7] and McDonnell[8] found a variation of the products formed by acid-producing bacteria, they were inclined to attribute the production of aroma to several species of lactic acid bacteria, and believed that more than one species is necessary to produce a good starter. Later, in 1908, after much further study of and experience with starters, Weigmann[9] expressed his belief that a good starter should contain ferments of the type of Streptococcus lacticus and of the aroma type, and that the specified aroma is produced by several bacteria.

Starter Cultures Must Contain Lactic Acid Bacteria and Flavor Bacteria.—These early impressions and observations were clarified and definitely confirmed in 1917 to 1919 by the researches of Storch,[10] Hammer and Bailey,[11] Boekhout and Ott de Vries[12] and Orla-Jensen.[13] These investigators, independently and almost simultaneously, found and demonstrated that a good butter culture is not a pure culture of one single species of organisms.

Thus, Hammer,[14,15,16,17] found in commercial butter cultures not only the lactic acid producing S. lactis, but in addition, and associated with S. lactis, two associated organisms. He likewise discovered that citric acid is the primary source of the volatile acids formed in starter cultures by the organisms associated with S. lactis and he named the two closely related citric acid-fermenting organisms, S. citrovorus and S. paracitrovorus.

Flavor and Aroma Depend on Presence of Diacetyl.—Another important step forward in our knowledge of butter cultures and butter aroma was brought about by the discovery in 1928-29, on the part of the German chemists Schmalfuss,[18] and Schmalfuss and Barthmeyer,[19] and by the Dutch scientists Van Niel, Kluyver and Derx,[20] that diacetyl and its mother substance acetylmethylcarbinol (acetoin) are largely responsible for the aroma of fine butter made from ripened cream. Subsequent

studies by Michaelian, Farmer, and Hammer,[21] and other investigators, showed that the citric acid-fermenting S. citrovorus and S. paracitrovorus of Hammer, or Betacoccus creamoris (Orla-Jensen), when associated with the lactic acid producing S. lactis or S. cremoris, yield in addition to lactic acid, volatile acids, such as acetic and propionic, CO_2, and diacetyl (i.e., principally its mother substance, acetylmethylcarbinol from which diacetyl is believed to result through oxidation).

Michaelian and Hammer[22] reported that "their results indicate that the formation of diacetyl from acetoin in a butter culture is due to the activity of organisms rather than to a simple chemical oxidation," and they attribute this oxidation to the activity of the aroma organisms. Van Beynum,[23] as the result of independent research, also held that it is the aroma bacteria that are responsible for the production of diacetyl, but not as the result of oxidation of acetoin, but by oxidation of an intermediate product, probably acetaldehyde.

The work of Hammer, Stahley, Werkman and Michaelian[24] further indicates that the flavor-producing organisms may, under certain conditions, reduce acetylmethylcarbinol and diacetyl to 2, 3-butylene glycol. Thus, an abnormal development of the flavor type of bacteria in the starter culture may cause the dissipation of the initial products which they produced, causing a lack of the diacetyl character of flavor and giving the impression of an "over-ripe" condition.

Still more recently a new flavor-producing lactic streptococcus was isolated from spontaneously soured potato mash by Matuszewski et al.[25] These authors reported that this organism which they have named S. diacetilactis, unites the essential features of S. lactis and of S. citrovorus and S. paracitrovorus. It produces large amounts of both lactic acid, and of volatile and aroma products such as acetylmethylcarbinol (in the form of diacetyl), acetic acid and CO_2. However, Ritter and Nussbaumer[26] who experimented with cultures of S. diacetilactis reported that the flavor and aroma produced by these cultures was not quite equal to that of mixed cultures of acid organisms and aroma organisms, and that continued propagations showed a gradual decline of the desired, characteristic butter aroma and flavor.

Effect of Citric Acid Content on Intensity of Starter Flavor.

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—Michaelian and Hammer[22] discovered that an increase of citric acid by addition of citric acid to the milk tended to increase volatile acidity and flavor products. They obtained similar results by the addition of other acids such as acetic acid, phosphoric acid and sulphuric acid, which suggests that those other acids assisted in the liberation of the citric acid from its salts, thus accelerating its breaking down. This assumption was further supported by their findings that when these other acids were added to milk from which the citric acid had previously been removed, there occurred no increase in the volatile acidity.

Fabricius and Hammer[27] found that generally, the addition of 0.15 per cent citric acid to the milk intended for culture, resulted in higher scoring butter than when no citric acid was used. Similar results of the addition of citric acid to starter or to cream (adding .2% citric acid) are reported by Templeton & Sommer[28,29] and by Templeton.[30] Vas and Csiszar[31] likewise emphasized the advantages of added citric acid or sodium citrate. They found that the addition of small amounts of citric acid (.06%) stimulated aroma production as much as larger amounts (.12 to .25%). While the addition of citric acid has proven effective in increasing the aroma products in most cases, there have occurred exceptions also, where this practice failed to yield satisfactory aroma and flavor, and where it was necessary to start with a new culture. Cordes[32] also reported somewhat negative results in that the addition of citric acid to milk with otherwise satisfactory solids content, failed to increase the intensity of the desired diacetyl type of flavor, and he suggested that the addition of citric acid to milk to be made into starter, should not be considered a cure-all for starter troubles, or as an insurance for satisfactory results.

ESSENTIAL REQUIREMENTS OF GOOD STARTER CULTURES

The essentials in the production of a good starter are ably discussed by Cordes.[32] They embrace the presence and proper balance of the right organisms; milk of good quality and of suitable chemical composition; dependable control of incubation as to rate of inoculation, temperature, time, and air supply; suitable utensils and equipment; and sanitation in handling.

Selected Bacteria.—The foregoing discussion showed that the neutral flavor substance diacetyl is the primary product

Davies[39] also reported that full-flavored butter (meaning butter made from ripened cream) did not keep well, developing fishy and later tallowy flavor. He attributed the poor keeping quality not so much to the aroma constituents, as to the high serum acidity and its effect on the liberation of organic acids from the butter fat (oleic acid). He further reported superior keeping quality of butter made from low-acid cream and from pasteurized, neutralized cream.

Ritter[40, 41] stated that in his experiments fishy butter occurred only in sour-cream butter and never in butter from cream churned sweet. He further emphasized that the presence of traces of copper or iron greatly hastens the oxidative changes leading to fishiness and similar flavor defects of chemical nature. Mohr and Ahrens[42], on the other hand reported that their experimental salted butter made from 20% cream ripened to 28° S.H. (.63% cream acidity or .787% cream serum acidity) kept as well as butter made from sweet cream. After storage for 8⅔ months at 21 to 42°F. the butter was of good quality. In their trials they used perfectly fresh cream, pasteurized at 95°C. (203°F.) and all operations were conducted under faultlessly aseptic conditions of equipment and handling, eliminating the possibility of bacterial and metallic contamination, and of air during storage. The butter was stored in sealed glass containers. Results of later work convinced Mohr and Ritterhoff[43] that sweet cream butter, if it is not over-neutralized, is not as sensitive regarding maintenance of its quality when it comes out of storage, as ripened cream butter.

Effect of Diacetyl on Keeping Quality of Ripened Cream Butter.—The aroma substance, diacetyl, is present in butter, even when made from fully ripened cream, in exceedingly small amounts. The diacetyl content of such butter usually averages approximately 2.5 p.p.m., and it rarely exceeds 4 p.p.m. While recognized as a potent oxidizing catalyst, capable of intense oxidation of such constituents as butter fat, it is present in such small amounts as to render its effect apparently negligible.

King[44] studied the effect of diacetyl on butter fat oxidation and found that in the presence of air, the butter fat is attacked by the diacetyl, causing tallowiness and bleaching. In the presence of light, oxidation was hastened. However, he worked with much higher concentrations of diacetyl than apply to butter. The lowest concentration of diacetyl used in his experiments was 50 p.p.m, or from 15 to 20 times the amount normally contained in butter. Even a concentration of 50 p.p.m. had practically no effect on, and did not change the aroma of the butter fat held for 97 days at 22-24°C. (71.6-75.2°F.) in the dark, while higher concentrations caused tallowy flavor and bleaching.

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That it is the high churning acidity and not the diacetyl content of butter made from ripened cream was further demonstrated by the work of Hunziker and Cordes, involving several thousand churnings made from unripened cream to the butter of which was added diacetyl to the extent of approximately 3 to 4 p.p.m. This butter was held for from 2 to 9 months. In no case was there any indication of bleaching, tallowy flavor, or other flavor deterioration, such as might be attributable to oxidation due to diacetyl.

Other Factors that Influence the Effect of Cream Ripening on the Keeping Quality of Butter.—The above citations of experimental results show, practically without exception, better keeping quality of sweet-cream salted butter, than of salted butter made from cream ripened to an acidity sufficiently high to insure a full aroma and flavor. There are certain conditions of manufacture, however, that permit of controlled ripening without jeopardizing keeping quality and there are still other conditions of manufacture and distribution, under which ripening to a full aroma and flavor is definitely beneficial to keeping quality.

The extent to which cream may be ripened and still have the butter reach the consumer without objectionable flavor deterioration due to chemical changes, is influenced by such factors as: condition and quality of the cream before manufacture, temperature at which butter is held between churn and consumer, whether intended for fresh consumption or for cold storage, whether the cream is used for salted or for unsalted butter, and salt concentration in the salted butter.

Quality of Cream.—The fresher and sweeter the cream, and the more complete its freedom from such metallic salts as the salts of copper and iron, the less is the danger of impairing the keeping quality of butter by chemical changes, when made from ripened cream. Cream that has never been allowed to sour or ferment at any time prior to pasteurization, such as fresh fac-

tory-separated cream, or cream that arrives at the factory in perfectly fresh and sweet condition, will stand ripening to a higher acidity without jeopardizing the chemical keeping quality of butter, than cream that arrives at the factory in sour, fermented condition.

Thus, in the case of cream of inferior quality, the unstable lecithin may have yielded to hydrolysis in the cream itself, inviting early oxidation of the choline and the formation of trimethylamine and fishiness in the resulting butter. Fermentation products and metallic salts may have dissipated the resistance of the fat and exhausted its induction period to a point where active autoxidation commences in the freshly churned butter. Proteolysis may have progressed in the cream, and protein cleavage products may be present by the time of its arrival at the factory, causing the early appearance of objectionable cold storage flavors, such as often develop in butter made from low grade cream.

That cream of inferior quality contains such decomposition products as liberated organic acids (particularly oleic), and nitrogen decomposition products such as soluble nitrogen compounds, amino nitrogen and ammonia substances, in larger quantities than fresh sweet cream, was shown by the work of Ferris[45, 46], and Ferris, Redfield and North[47]. These investigators further found that the above products of decomposition were present to a greater extent in fresh butter and increased more rapidly during storage, in the case of butter made from sour cream than from sweet cream.

Effect of Cream Ripening on Fresh-Consumption Butter. In the United States butter intended for fresh-consumption usually is from about 2 to 6 weeks old before it reaches the consumer. The temperature at which it is held between churn and pantry of consumer varies obviously with many conditions. It is, however, seldom at or below the freezing point (32°F.). The coolers of the creamery and of commission merchants and wholesalers usually carry it at a temperature of approximately 35 to 45°F. In transit, although cars and trucks are iced during the hot weather season, the butter may be exposed to somewhat higher temperatures. The temperature of the ice box or refrigerator in the retail store may vary between about 40 and 60°F.

These very approximate and widely varying temperature conditions suggest that, in general, the temperature to which

fresh-consumption butter is exposed and the period of exposure, are such as to permit of deterioration due to bacterial causes. Bacterial deterioration of fresh-consumption butter is, in fact, a constant menace. It was shown in earlier paragraphs that cream ripening, by reason of the retarding effect of lactic acid bacteria, lactic acid and its salts on the activity of putrefactive and other forms of quality-damaging germ life, assists in delaying flavor deterioration due to bacterial causes. On the other hand, the damaging chemical changes that are promoted by the higher acidity of ripened cream butter, progress more slowly and their jeopardy to the keeping quality of fresh-consumption butter is largely negligible.

For these several reasons it has been found advantageous, in the case of fresh-consumption butter, to neutralize the sour cream to as low an acidity as practicable without giving the butter an objectionable neutralizer flavor, and then ripen it back to the maximum churning acidity (0.25 to 0.30%) that is still low enough to prevent the development of fishy flavor. Such butter averages a more pleasing flavor and is less subject to bacterial deterioration than butter made from cream of similar quality without ripening, and yet it has sufficient resistance to chemical deterioration to keep satisfactorily for fresh-consumption purposes.

Effect of Cream Ripening on Butter Held in Commercial Cold Storage.—In the case of butter intended for commercial cold storage at the usual temperature of -10 to -20°F., the ripening of cream is of doubtful merit, especially when the butter is made from sour, neutralized cream. Under American conditions, storage butter may be held in cold storage from 3 to 12 months, or even longer. From the standpoint of flavor and aroma, the ripening of cream intended for cold storage butter is of little value. As shown in earlier paragraphs, prolonged cold storage of the butter dissipates the diacetyl and its accompanying flavor and aroma. Again, bacteriological deterioration is a negligible factor with butter held in commercial cold storage. The retarding effect of cream ripening on bacterial deterioration of butter, therefore, is unimportant. On the other hand, oxidation processes that are enhanced by the high acidity of butter made from ripened cream, continue their work of flavor deterioration, and the prolonged period of cold storage intensifies the damage done.

For these various reasons the quality of cold storage butter is not benefitted by cream ripening. On the contrary, it suffers greater depreciation than that of butter made from unripened, low-acid cream. Cream churned sweet, and sour cream (.45% acid or less), neutralized to a churning acidity of .15% or lower, gives the resulting butter held in cold storage excellent keeping quality. In the case of high-acid cream the menace of neutralizer flavor usually does not permit of neutralizing to such low acidities. For such cream, a churning acidity of not to exceed .21% has been found preferable. See also Chapter XX under "Keeping Quality of Butter in Commercial Cold Storage."

The Effect of Cream Ripening on Unsalted Butter.—The foregoing discussion of the relation of cream ripening to keeping quality referred to salted butter. In the case of unsalted butter the situation is somewhat different. Bacteriologically, unsalted butter is the most perishable type of butter made. It contains no added preservative, such as salt, that would retard or inhibit bacterial activity. To be sure, efficient pasteurization and a faultless standard of cleanliness and sanitation in manufacture and packing provide maximum freedom from damaging germ life in the butter and assist in retarding bacterial deterioration. However, unless sterilized after manufacture, and packed in hermetically sealed containers, or held at temperatures low enough to permanently inhibit all bacterial growth, unsalted butter is inevitably destined to suffer bacterial flavor deterioration with age.

If made from cream that is churned at a low acidity, such as sweet cream, or sour, neutralized cream, without ripening, early flavor deterioration due to bacterial activity is practically unavoidable, when held at temperatures above the freezing point. If, made from cream ripened to a full aroma and flavor, bacterial deterioration is very noticeably retarded by the great predominance of lactic acid bacteria and the relatively high acidity. These agencies assist in holding in check, the type of organisms which, if present, are prone to cause bacterial flavor defects in unsalted butter.

OPERATION OF CREAM RIPENING IN THE FACTORY

Equipment.—The equipment should be as simple as possible, consistent with sanitary construction, freedom from damaging metal surfaces, efficient temperature control, and economy of operation.

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The ripening of cream is usually most conveniently done in vertical or horizontal coil vats, in vats with oscillating coil, or in square or circular tanks with coil, blade or impeller agitator. Tanks with removable coil have also been found suitable. In the creamery that uses vat pasteurization, the vat pasteurizer provides the needed equipment for cream ripening. Plain vats, with or without jacket, and with or without cover, are also used

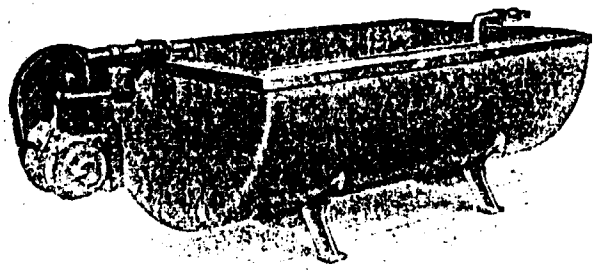

Fig. 58. Cream ripener, jacketed, with swinging coil, stainless steel
Courtesy of Silkeborg Maskinfabrik

for this purpose, especially in some of the European creameries. Their operation usually necessitates the addition of crushed ice for cooling the ripened cream. Insulated coil vats have been found to provide the most practical and labor-saving means of temperature adjustment and control.

Amount of Starter to Use.—The starter is an item of expense in the operating cost of the creamery. For economic reasons, therefore, the amount of starter added to the cream should be limited to that necessary to accomplish the full benefits of cream ripening. The amount of starter actually needed for this purpose depends on such factors as condition and richness of cream, ripening temperature, and ripening period that best fits into the operating routine of the factory.

Fresh, sweet cream requires less starter than sour, neutralized cream. A good quality of sweet cream provides a favorable medium for the activity of the starter organisms. Even when using a small amount of starter in such cream, their ascendency is assured. Old, stale, sour neutralized cream, even after efficient pasteurization, still contains a considerable num-

ber of bacteria, especially species foreign to starter organisms, and usually more or less antagonistic to their activity. Such cream may also contain fermentation products that are a hindrance to the desired starter action. A larger amount of starter is necessary, therefore, to overcome these objectionable agencies. In fact, at best, the flavor and aroma results of starter action in cream of poor quality are not as dependable nor as pronounced as in cream of good quality.

Rich cream requires more starter than cream low in fat. The lower the ripening temperature, and the shorter the ripening period, the larger the amount of starter required to attain the desired, pronounced flavor and aroma that is expected of ripened cream butter.

The amount of starter used by different creameries ranges from about one to ten per cent of the volume of cream to be ripened. The Danish buttermaker who has the advantage of fresh, factory-separated sweet cream, containing about 20 to 25% fat, which he ripens at a relatively high temperature, adds approximately 3% starter to his cream. In this country it has been found helpful to use from 5 to 7% or more starter in sour, neutralized cream of about 90 score quality and containing well over 30% fat. In the northern tier of the dairy belt where the cream averages a lower fat content (usually below 30% fat), and where much of it is of 92 score quality, the addition of 3 to 4% starter is usually sufficient.

Per Cent Acid to Which the Cream Should Be Ripened.—On the European continent where a pronounced flavor and high aroma of butter is preferred, but little attention is paid to the churning acidity of the cream, the ripening process is carried to the point where the cream has a granular, glistening appearance and a pronounced flavor and aroma. Acid tests are not made regularly. Under these conditions the ripened cream usually has a titratable acidity of about 0.55 to 0.65%. This is the general practice in Danish creameries.

In the United States the churning acidity is looked upon as an important factor in determining the keeping quality of the butter, and the churning acidity is controlled by the acid test of the cream. Likewise, in order to hold the churning acidity of cream intended for salted butter down to a safe point, especially with regard to prevention of fishy flavor, the ripening is usually

done at a low temperature (about 48 to 53° F.). In some case the starter is added to the cream at a higher temperature during the cooling of the pasteurized cream, such as at 70° F., and cooling to churning temperature is continued.

For fresh consumption salted butter, cream of moderate richness (testing about 30% fat) may safely be ripened to about 0.25 to 0.30% acid. In the case of salted butter intended for commercial cold storage it has been found preferable not to ripen the cream, and to churn it at an acidity of about 0.21% acid or lower.

In order to avoid excessive acid development due to cream ripening, some buttermakers follow the practice of adding the starter just before churning. In this case the use of 5 to 7% starter usually raises the acidity of the cream approximately 0.03 to 0.04%. The flavor and aroma of the resulting butter here depend solely on the degree to which they are present in the starter. At best their effect on the butter is slight, and it is pertinently questionable whether this practice is economically justifiable.

In the case of unsalted butter, the cream may be ripened to any acidity without jeopardizing keeping quality. In fact, it is desirable to ripen the cream for this purpose to a full aroma and flavor. Excellent results are obtained by low-temperature ripening, such as at about 50 to 55° F. over night. By this method the cream usually shows a pronounced flavor and aroma the following morning and an acid range of about 0.35 to 0.45% titratable acidity. When ripening at the higher temperatures (60 to 70° F), the aroma character tends to be somewhat more pronounced and the acid somewhat higher (0.5 to 0.65% titratable acidity).

Addition of Citric Acid to Cream for Ripening.—As explained in Chapter XIII on "Starters and Starter Making," the major substances responsible for the characteristic butter aroma and flavor, such as diacetyl, acetoin, volatile acidity and CO_2, are products resulting from the fermentation of citric acid by the starter bacteria. It was further shown that the addition to the starter milk of a small amount (about 0.2%) of citric acid, or its equivalent as sodium citrate, stimulates the production of these products and increases the desired aroma and flavor in the starter.

0878

These facts suggest the possibility of augmenting the desired aroma and flavor in the cream and butter by adding citric acid or sodium citrate to the cream during ripening. According to the findings of Sherwood and Hammer[39] the citric acid content of cream direct from the separator varied from 0.09 to 0.19%, averaging 0.15%; in mixed farm cream it varied from 0.10 to 0.20%, averaging 0.15%.

Data showing the effect of adding citric acid to the cream at the time of ripening, on the resulting butter are limited. Ritter and Stüssi[48] made experimental churnings on a commercial scale, ripening the cream with and without the addition of 0.2% citric acid. The scores of the resulting butter, judged by seven groups of three judges per group, favored the control butter. The butter made from the citric acid cream had a less desirable flavor and aroma than that of the control churnings. This was true of the fresh butter as well as after one month of storage.

In the absence of more favorable experimental data, the addition of citric acid to cream appears to be of doubtful merit, suggesting the wisdom of avoiding this added expense, until more convincing knowledge of its merits is available.

Adding the Flavor Substance Direct to the Butter.—It has been shown in the foregoing paragraphs that cream ripening increases the cost of manufacture, may jeopardize the keeping quality of butter from the standpoint of chemical stability, and that the major portion of the aroma and flavor substances passes into the buttermilk and water used for washing the butter, and is lost to the butter. These facts have suggested, and have led to the practice in some instances, of dispensing with cream ripening, and of working the desired aroma substance direct into the butter, thus eliminating much of the added operating expense, avoiding the quality-jeopardizing effect of high churning acidity, and preventing the waste of flavor principle. This may be done by mixing starter, starter distillate, or synthetic flavoring compounds with the butter during the working process.

Working Starter Into the Butter.—This is a common practice in many creameries. Instead of using water for wetting the salt and for bringing the moisture up to the desired point, several gallons of starter are added to the butter with the salt. The starter so used does assist in freshening the flavor of the butter

0878

made from unripened cream somewhat. It does not increase the acidity of such butter sufficiently to augment the danger of deterioration due to chemical reactions. The keeping quality of butter so treated is similar to that of butter made from unripened cream without the addition of any starter. The resulting improvement in flavor, however, is slight. The butter still lacks the full aroma and flavor of ripened cream butter.

Working Distillate of Starter Into the Butter.—The process of distilling the butter flavor essences from the starter and adding them to the butter in the form of standardized starter distillate was developed by Ruehe,[49] whose method of preparation of the starter distillate is as follows:

Incubate the pasteurized starter milk that has been inoculated with the usual butter culture containing lactic acid bacteria and flavor organisms, at 70° F. for 24 hours. Then add 0.25 to 0.3% citric acid and incubate at 70° F. for another 24 hours.

Place the starter in a still which has steam and condenser connections. Add a small amount of ferric chloride ($FeCl_3$ oxidizes the acetoin to the aroma-producing diacetyl, greatly increasing the amount of diacetyl). Distill at the rate of 50 cc. distillate from 200 cc. starter. Standardize the resulting distillate to a definite diacetyl content and acidity. This distillate keeps at ordinary cooler temperature (40° F.) without appreciable loss of diacetyl.

For use in butter, add the distillate to the butter with the salt. Use the distillate at the rate of approximately 2 to 2.5 lbs. of distillate per 1,000 lbs. of butter.

Ruehe reports that butter flavored with starter distillate in the above, and even in larger amounts, "stood up" satisfactorily during storage for 14 months. There was no indication of impairment of keeping quality due to the distillate. He suggests, however, the necessity of further study before definite conclusions are justifiable.

Working Preparations of Synthetic Diacetyl into Butter.—The discovery by Van Niel, Kluyver and Derx[50] in 1929, of diacetyl as the principle source and cause of the characteristic aroma and flavor of butter, has been followed by the appearance on the market of diverse so-called butter cultures in the form of commercial preparations containing synthetically prepared diacetyl. Some of these preparations are clear concentrates of

diacetyl in milk, concentrated milk or other vehicles, still others contain, in addition to diacetyl, volatile acids, such as acetic, or propionic, or both.

Their use in amounts that confine the diacetyl content of butter to 4 p.p.m. or less, has not been found damaging to the chemical stability of butter, but when used in excessive proportions they may easily cause damaging oxidation of the butter fat, leading to bleaching and tallowy flavor, as shown by King.[44]

The addition of synthetic compounds of diacetyl obviously does give the butter some of the diacetyl type of aroma. In the absence of other aroma products that are normally associated with citric acid fermentation, such as volatile acids, CO_2, and probably acetaldehyde and other as yet undetermined volatile essences, which blend with the diacetyl aroma to give a composite effect of the characteristic, pleasing aroma of good butter, these commercial preparations containing synthetically prepared diacetyl only, give the butter a somewhat unnatural, harsh aroma character. The butter lacks the pleasing, mellow, blended aroma of ripened cream butter.

Furthermore, the mere working into the butter of these flavor compounds fails to fuse them with the butter constituents as completely as when they are produced and absorbed in the cream and churned into the butter. They do not become a truly integral part of the butter, and the aroma of such butter lacks permanency. After comparatively short storage the butter is relatively devoid of the aroma that it had at the churn.

In addition, these butter flavor compounds have no merit from the standpoint of retarding bacterial deterioration of butter. Their use, therefore, has no quality-stabilizing effect on unsalted butter, the keeping quality of which, being chiefly a bacteriological problem, is materially and definitely enhanced by cream ripening. This objection applies likewise to the use of starter distillate.

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0878

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range of —10 to —20° F., as standard for butter storage in American commercial cold storage houses.

Cause of Flavor Deterioration of Butter Held in Commercial Cold Storage.—In the brief discussion of the relation of pasteurization of cream to keeping quality of butter (Chapter XI), it was shown that the bacterial count of butter, both salted and unsalted, almost invariably decreases during the cold storage of butter, indicating that keeping quality of butter held at the low temperatures of cold storage (—10 to —20° F.) appears not to be a biological problem.

Our present state of knowledge of the mechanism of butter deterioration during cold storage, while incomplete, reveals strong evidence that these storage flavor defects are the result of progressive chemical changes due to oxidation processes, causing cleavage of one or more of the constituents of butter. However, neither hydrolysis of the fat due to lipase action, yielding free fatty acids of low molecular weight, i.e., butyric, capronic and caprylic acid, that are associated with rancid flavor; nor autoxidation of unsaturated fatty acids, such as oleic and linoleic, that lead to tallowy flavor, are characteristic flavor defects of cold storage butter. While fat splitting reactions and the production of rancid and tallowy flavor are prone to occur in aging butter at ordinary temperatures, they rarely, if ever, occur in butter stored at —15° F. These observations are supported by the work of Dyer[5] who found that "the development of undesirable flavor in butter held in cold storage at a temperature of 0° F. is not dependent upon oxidation of the fat itself," and "that the production of off flavors so commonly found in cold storage butter is attributable to a chemical change expressed through a slow oxidation progressing in some one or more of the non-fatty substances occurring in the buttermilk." Since such butter is usually high in soluble nitrogen compounds, the results suggest that the protein constituents of butter are involved.

The problem of preventing flavor deterioration in cold storage butter has been the subject of extensive study by dairy experiment station staffs, as well as in the laboratories within the industry. It is now definitely known that among the dominating factors that either incite or accelerate the flavor-damaging chemical reactions are: poor quality of cream, presence of metallic salts, high churning acidity, high salt content and air content of butter.

0878

Keeping Quality of Butter After Cold Storage.—The final test of keeping quality of cold storage butter lies in its ability to withstand rapid flavor deterioration, when it is removed from commercial cold storage on its journey to the trade and the consumer. Upon removal from cold storage, butter may be and usually is exposed for a considerable period (probably from one to three weeks) to temperature conditions that tend to hasten deterioration.

In general, butter that has been held for six to nine months in commercial cold storage, develops off-flavors more readily than fresh butter under the same marketing conditions. This is as might be expected. The progressive chemical changes that occur in cold storage, while slow at the low temperature of commercial cold storage, have the effect of lowering the resistance of the butter constituents involved. When the butter is removed from the protecting influence of low temperature, these changes, already begun, are accelerated and flavor deterioration goes on more rapidly.

Here again the quality of the cream and the churning acidity exert a controlling influence. Butter made from fresh, sweet cream of high quality, having suffered practically no chemical changes during cold storage, has a resistance similar to that of fresh butter of the same grade. It usually is capable of holding its score when taken out of cold storage, and it gives every promise to reach the consumer in satisfactory condition. On the other hand, butter that, because of a poor quality of cream, or of high churning acidity, or both, has suffered chemical changes to the extent of showing noticeable flavor deterioration during the cold storage period, has dissipated its resistance and will tend to depreciate rapidly in flavor, when removed from cold storage. Border-line lots that suggest "weakness" at the end of cold storage, are rarely safe to put out among the fresh butter trade. They almost invariably cause serious quality complaints from the trade and the consumer.

Effect of Cold Storage on Diacetyl Content.—The general tendency is for the Diacetyl and Acetoin content of butter to diminish during the cold storage of butter. However, the diacetyl flavor developed in the cream generally stays with the butter fairly permanently throughout the storage period. On the other hand, the diacetyl flavor of fresh butter, resulting from the addi-

tion to and working into the butter of diacetyl compounds, is relatively unstable and disappears almost completely from butter held in cold storage.

Predicting the Keeping Quality of Butter Intended for Cold Storage.—Details of methods and merits of keeping quality tests are given in Chapter XXV on "Factory Tests." By reason of the low temperature, long period, and relatively slow progressive changes that take place in butter held in commercial cold storage, the results of short-duration incubation tests made at room temperature or higher, cannot be expected to be comparable with the probable behavior of the butter in cold storage. In addition, the merits of incubation tests are largely confined to the revelation of the relative resistance of butter to flavor deterioration due to bacterial causes, while deterioration of butter held in cold storage is not a microbiological problem.

As shown in earlier paragraphs, the churning acidity is a controlling factor of the keeping quality of butter in cold storage. There exists a fairly close relation between the churning acidity and the acidity of butter. It follows, therefore, that the acid test of butter should give some indication of the fitness of butter for cold storage.

A titratable acidity of butter of 0.05% is a dependable indication that the churning acidity of the respective cream was sufficiently low to insure satisfactory keeping quality, relative to absence of flavor-damaging chemical reactions that are incited or accelerated by acidity. Unfortunately, the end point in the acidity titration of butter is somewhat evasive and not as easily detected by the unskilled operator as it should be. Because of this difficulty, the results are occasionally open to question.

The pH determination of butter provides an index of the acidity that, if wisely interpreted, may serve as a fairly dependable indication of the probable keeping quality of the butter. In the case of butter made from sour, neutralized cream, a pH range of from 6.6 to 7.3 has been found a reasonably safe limit. In the case of sweet cream and sweet cream ripened to a controlled limit of safe churning acidity, the pH usually ranges from about 5.8 to 6.5. The colorimetric method of determining the pH of butter serum, given in Chapter XXV, provides a very simple test, the performance of which requires no technical skill.

0878

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equipment and methods of manufacture, the defect that involves the entire mass of butter, causing an intense tallowy flavor and bleaching, has been practically completely eliminated. Today, off-flavors in butter suggestive of tallowy flavor, are confined principally to surface deterioration of butter with age, the butter developing a tallowy or related flavor on the surface, in the absence of complete protection from air and light.

Similarly as rancid flavor, tallowy flavor is due to fat decomposition. However, while rancidity has to do with fat hydrolysis initiated by the action of lipolytic bacteria and enzymes, tallowiness is caused by oxidation of the fat, involving the unsaturated fatty acids in butter, such as the oleic acid. It was demonstrated by the early study of tallowiness in butter by Hunziker and Hosman[34], however, that butter does not show a tallowy flavor when oleic acid which has been exposed to the air until there has been a reduction in the iodine number, is added to it, but that butter containing free oleic acid becomes tallowy rapidly when exposed. These investigators concluded that tallowiness is the result of fat oxidation, but that the product which it is necessary to link up with the fatty acids, in order to make butter tallowy, is derived from free glycerol produced by a slight hydrolysis of the fat. They found this product to be glycollic acid ester of oleic acid. Only very small amounts (.25%) of this reagent added to butter fat produced a distinct tallowy flavor and odor. They point out further that glycollic acid is naturally found in the fat of sheep's wool, suggesting the probable close relation between glycollic acid and tallowy odor. See also Chapter XXI under "Decomposition of Butter Fat."

Causes and Prevention of Tallowy Flavor in Butter.—AIR, LIGHT AND HEAT.—Exposure to air is the most common cause of oxidation producing tallowiness in butter. Prolonged storage of butter without protection against the atmosphere causes surface oxidation associated with tallowy flavor on the surface of the butter. Such oxidation is greatly accelerated in the presence of heat and light. The development of tallowiness proceeds more rapidly in unprotected butter held at room temperature and higher, than at ice box temperature. At the temperature of commercial cold storage, butter does not become tallowy. Daylight, and especially direct sunlight greatly hasten fat oxidation and the development of tallowy flavor, accompanied by bleaching.

0878

The use of air- and light-proof liners and wrappers, such as "parchfoil," or transparent wrappers, stained green or red, greatly retards surface oxidation and assists in minimizing, if not preventing surface tallowiness. Treatment of the wrapper with a harmless anti-oxidant, such as oat flour solution (Avenex) has also been found helpful in retarding surface oxidation of butter.

METALS. The presence in butter of certain metals, their salts or oxides, greatly hastens reactions that lead to tallowy flavor. Of the metals commonly used in dairy equipment and for cream containers, copper and iron are the most damaging. Other metals used in creamery equipment, such as tin, aluminum, stainless steel, chrome-nickel, and nickel, while not completely inert, have been found incapable of producing tallowy flavor in butter. In order, therefore, to eliminate metallic contact or contamination as a source of tallowy flavor in butter, iron surfaces, such as in cream cans, should be kept properly tinned and rusty iron cans should be eliminated. Copper surfaces in plant equipment should be kept properly tinned. The growing use of stainless steel equipment in the creamery within recent years has done much to eliminate occurrences of tallowy flavor in butter.

NEUTRALIZATION. The presence of an unnatural alkaline condition in cream or butter accelerates oxidation by promoting fat hydrolysis and resulting in accelerated oxidation, by making the resulting compounds (free fatty acids and glycerol) more susceptible to oxidation. Over-neutralization of cream aggravates the tendency of tallowy flavor in the resulting butter.

DIACETYL. It was shown by the work of King[35] that diacetyl is capable of causing tallowy flavor and bleaching of butter fat in the presence of air. In this experiment the amount of diacetyl added to the fat was 50 p.p.m. or more. This emphasizes the danger of fortifying the flavor of butter with excessive amounts of diacetyl compounds. When producing butter flavor naturally, i.e., by ripening cream with a starter containing lactic and aroma streptococci, the diacetyl content of the resulting butter usually falls within the range of 2.5 to 4.0 p.p.m. Experience in commercial butter manufacture has conclusively demonstrated that a diacetyl content of 4 p.p.m. does not produce tallowiness in storage butter, regardless of whether the diacetyl is produced by the starter in the cream, or added to the butter in the form of a synthetic diacetyl compound.

(0888)

Amer. Soc. Brew Chem Proc., (1968)

Mechanism of Diacetyl Formation in Beer

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It is generally thought that diacetyl (DA) in beer is formed from acetoin (3,5,15,16), but sufficient experimental evidence for this has not been accumulated.

During an investigation on the amounts of DA and related compounds present during beer fermentation, the authors found a substance (material X) in fermented liquor, which was easily converted into DA when the liquor was exposed to air. In further studies it was found that the substance was identical with α-acetolactic acid (α-AL).

The data presented in this paper support the thesis that α-AL is formed in yeast cells as an intermediate of valine biosynthesis, passes into the fermenting medium, and is spontaneously converted into DA if the redox potential of the fermenting medium is sufficiently high.

DA is not formed from acetoin during brewery fermentation.

Experimental

Diacetyl (DA). DA content was determined by a modification of the micro method of Owades and Jakovac (14). The following modifications were made. Traps containing hydroxylamine were cooled with ice-cold water while sweeping with carbon dioxide. Condensation of DA with hydroxylamine was carried out at 80°C. for 1 hr. When the presence of material X was suspected in samples such as the fermented liquor, the following treatment was made prior to the determination. The sample solution was adjusted to pH 2 with sulfuric acid, held at 60°C. for 10 min. and after cooling to room temperature, the pH was readjusted to 4.2 with sodium hydroxide. This treatment is called "acid-treatment" hereafter. As mentioned later, material X was quantitatively converted into acetylmethylcarbinol (acetoin) (AMC) by this treatment.

Material X. This material is defined as the material present in fermenting liquor which is converted into DA when the liquor is exposed to air. It was determined quantitatively as follows: Sample solution was adjusted to pH 4.2, shaken well to expose it to air, and heated at 60°C. for 20 min. in an Erlenmeyer flask fitted with a plug. This treatment is called "autoxidation" hereafter. The amount of DA in the treated solution was measured by the modified micro method. The amount of material X was calculated by subtracting the amount of DA in the acid-treated solution from that in the autoxidized solution.

α-AL. This acid was synthesized by the method described by Krampitz (9) and determined quantitatively by the aniline-citrate method of Edson (4).

AMC. This substance was estimated by subtracting the amount of DA plus material X from the amount of DA measured by the modified micro method after oxidation with ferric chloride (22).

Sampling and Preparation of the Fermented Liquor. Fermentation was carried out in a pilot brewery or in a laboratory. Samples of fermenting liquor from the pilot brewery were transferred to a container previously filled with carbon dioxide gas to avoid exposure of the sample to air. Yeast was removed from the liquid by centrifugation at 3,000 rpm. for 10 min. at 0°C. giving a clear liquid. In the laboratory, bottom-fermenting brewery yeast was

cultured in stationary cultures at 20°C. in wort or in a synthetic medium in Erlenmeyer flasks filled to the neck (17). Clear liquid was prepared by centrifugation, as in the case with fermented liquor from the pilot brewery.

Results

1. *Presence of Material X in Fermenting Liquor.* It was found during an investigation of the changes in the amounts of DA and related compounds in beer fermentation that the DA content in the fermented liquor increased when the liquor was exposed to air.

Fermented liquor, taken after 5 days of fermentation in the pilot brewery, was incubated at 60°C. by varying the pH and the incubation period as indicated in Fig. 1. After cooling to room temperature the liquor was readjusted to pH 4.2, and was subjected to DA determination by the modified micro method. The DA content became smaller on lowering the pH and the minimum DA content was observed after incubating the liquor at pH 1.8 to 2.3 for 10 min. at 60°C. (Fig. 1). Since the acid-treatment has no effect on DA, the DA content measured after this treatment indicates the content of DA originally present in the liquor.

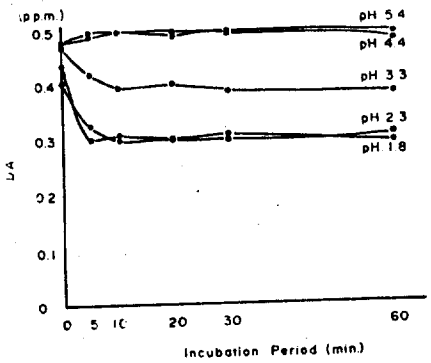


Fig. 1. Change in DA content in fermented liquor in relation to pH value.

The fermented liquor was incubated at 1°, 20°, and 60°C. for various periods at its original pH, and the DA content was measured after acid-treatment. The DA content increased when the temperature was raised, and the maximum content was attained by treatment at 60°C. for 20 min. (Fig. 2).

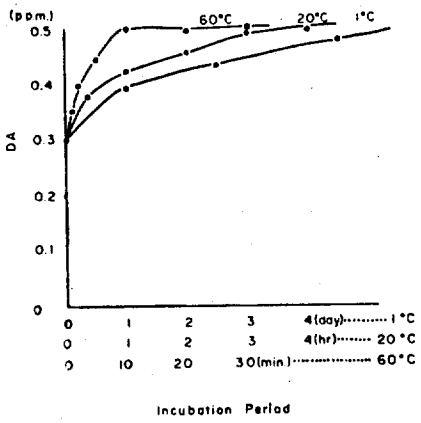


Fig. 2. Increase in DA content in fermented liquor on incubation.

Thus, a material (material X) seems to be present in the fermenting liquor, and to be converted into DA on exposure to air and to a substance other than DA on treatment with acid. Changes in the amounts of this material, DA, and AMC during fermentation in the pilot brewery are shown in Fig. 3.

As the characteristics of material X observed above were similar to those of α-AL (2,8,18,19), the material was thought to be α-AL.

2. *Identification of Material X with*

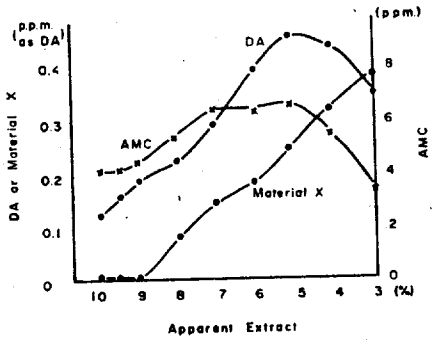


Fig. 3. Changes in amounts of DA, material X, and AMC during fermentation in the pilot brewery.

α-AL. To identify material X with α-AL, the following experiments were carried out using synthetic α-AL as a standard.

A. α-AL is known to be converted into DA by oxidative decarboxylation (2). A known amount of α-AL was added to the fermented liquor or to buffered solution and the amount of DA formed after autoxidation was measured. As shown in Table I, added α-AL was quantitatively recovered as DA. (In experiments 1–4, 9.3 micromoles of α-AL was added, and 29 micromoles in other cases. Fermented liquor was taken from the fermenting tank in the pilot brewery on the 5th day of fermentation. The amounts of pre-existing DA and material X were subtracted in calculation of the DA/α-AL ratio.)

TABLE I

Formation of DA by Autoxidation of α-AL

Expt. No.	Medium	Condition	DA/α-AL Ratio[a]
1	Fermented liquor	autoxidation	1.02
2	Fermented liquor	autoxidation	0.96
3	KH_2PO_4 (pH 4.5), 1.0*M*	autoxidation	0.89
4	KH_2PO_4 (pH 4.5), 1.0*M*	autoxidation	0.89
5	Acetate buffer, 0.01*M* (pH 4.0)	60°C., 5 min.	0.97
6	Acetate buffer, 0.01*M* (pH 4.0)	25°C., 3 hr.	0.96

[a] DA/α-AL ratio = $\frac{\text{DA formed}}{\text{α-AL added}}$

B. It has been reported that α-AL does not dissociate below pH 3.2 (13) and is easily decomposed to AMC by decarboxylation (9). Material X also seemed to be converted into a substance other than DA at low pH (Fig. 1).

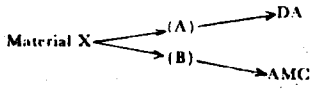
A sample of fermented liquor was subjected to acid-treatment and another sample to autoxidation. The contents of DA and of AMC in the two liquors were measured. As shown in Table II, material X (1.06 ppm.) was quantitatively recovered as an increase in the amount of AMC (1.0 ppm.) by acid-treatment.

TABLE II

Change in Amounts of DA and AMC after Autoxidation or Acid-Treatment of Fermented Liquor[a]

	After Autoxidation (A)	After Acid-Treatment (B)	(B)−(A)
	ppm.	*ppm.*	*ppm.*
DA	1.68	0.62	−1.06
AMC (expressed as DA)	8.4	9.4	+1.0

[a] The fermented liquor was prepared by laboratory fermentation of wort at 20°C.



C. α-AL is known to be an intermediate in valine biosynthesis (10, 20). In brewery yeast, as well as in *Escherichia coli* (20), the pathway of its biosynthesis is known to be inhibited by valine (15). Increasing amounts of valine were added to glucose-ammonium medium and, after a certain period of incubation with yeast, the amount of material X formed was measured and expressed as a percentage of that obtained when no valine was added. Valine inhibited the formation of the material (Table III). The medium used contained (per liter): glucose, 50 g.; ammonium sulfate, 4 g.; salts; vitamins; 0.1*M* citrate buffer solution at pH 5.5, 25 ml.; valine, as indicated in table. Yeast was inoculated to give a concentration of 1.2×10^8 cells per ml. and incubated at 25°C. for 2 hr.

D. 2,4-Dinitrophenylhydrazine reagent was added to fermented liquor and the hydrazones of carbonyl compounds were prepared by a procedure similar to that used by Bassett and Harper (1). These hydrazones were analyzed by paper chromatography (Whatman 3MM, 30% acetone) using authentic samples of DA, AMC, and α-AL for comparison. A spot with the

TABLE III

Effect of Valine on Formation of Material X

Amount of Valine Added	Material X Formed
ppm.	%
0	100
1	100
2	96
5	84
20	76
50	55
100	27
200	8

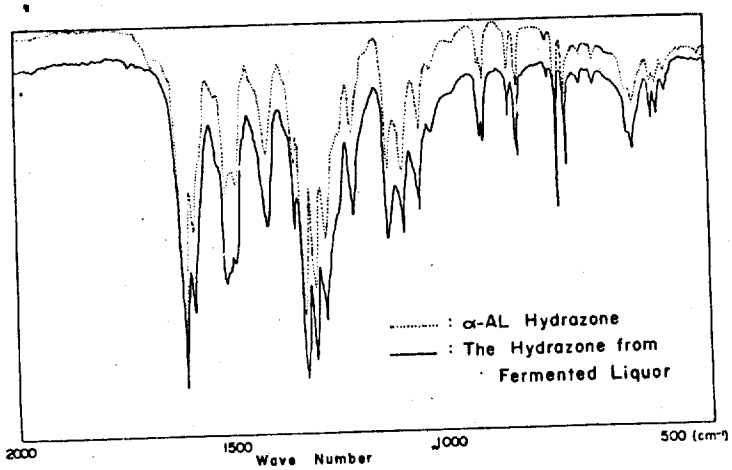


Fig. 4. Infrared spectrometric patterns of α-AL hydrazone and the hydrazone from fermented liquor.

same R_f value (0.65) as that of the hydrazone of α-AL could be detected on the paper. Hence, the hydrazones originated from 60 liters of fermented liquor of the pilot brewery were subjected to paper chromatography, and the area of R_f 0.65 was eluted with ethyl acetate. After removal of the solvent by evaporation under reduced pressure, the material was recrystallized several times from acetone. Five milligrams of yellow needle-like crystals was obtained. These crystals and those prepared from standard α-AL were decomposed at 92°C. with evolution of gas.

Mass-spectrometric analysis of both the isolated crystalline material and authentic α-AL hydrazone showed the peaks at 266 (DA 2,4-dinitrophenylhydrazone) and at 45 (carboxyl group), while no peak at 312 (α-AL 2,4-dinitrophenylhydrazone) was present. Heat generated during the analysis (about 100°C.) might decompose the hydrazone to the DA derivative.

The infrared spectrometric patterns of α-AL and the isolated hydrazone were identical (Fig. 4).

Further, amino acids were prepared from these 2,4-dinitrophenylhydrazones by hydrogenation with platinic oxide as a catalyst (12) and purified by passage through a column of ion-exchange resin (Amberlite IR 120). The purified amino acids were subjected to paper-chromatographic analysis on Whatman 3MM filter paper by the ascending method with butanol, acetic acid, water (4:1:2) as the solvent. A purple spot of R_f 0.65 was formed by the amino acid prepared from the α-AL and the isolated hydrazone (Table IV). The coexisting reddish-pink spot (R_f 0.28) was supposed to be that of the amine formed from DA and AMC hydrazone owing to spontaneous decomposition of α-AL and the isolated hydrazone during preparation of the amino acids.

E. The amount of α-AL in the fermented liquor was determined by the method of Juni and Heym (7), in combination with the method of Whiting and Coggins (23) as illustrated in Fig. 5. The fermented liquor from laboratory fermentation with and without

TABLE IV

Paper-Chromatographic Analysis of the Amino Acid Derived from the 2,4-Dinitrophenylhydrazone with R_f 0.65 from the Fermented Liquor

Compound	R_f Value	Color Developed on Spraying with Ninhydrin Reagent
DA 2,4-DNP hydrazone	0.28	reddish-pink
AMC 2,4-DNP hydrazone	0.28	reddish-pink
α-AL 2,4-DNP hydrazone	0.28	reddish-pink
	0.65	purple
2,4-Dinitroaniline	0.55	black
2,4-DNP hydrazone with R_f 0.65	0.28	reddish-pink
	0.65	purple

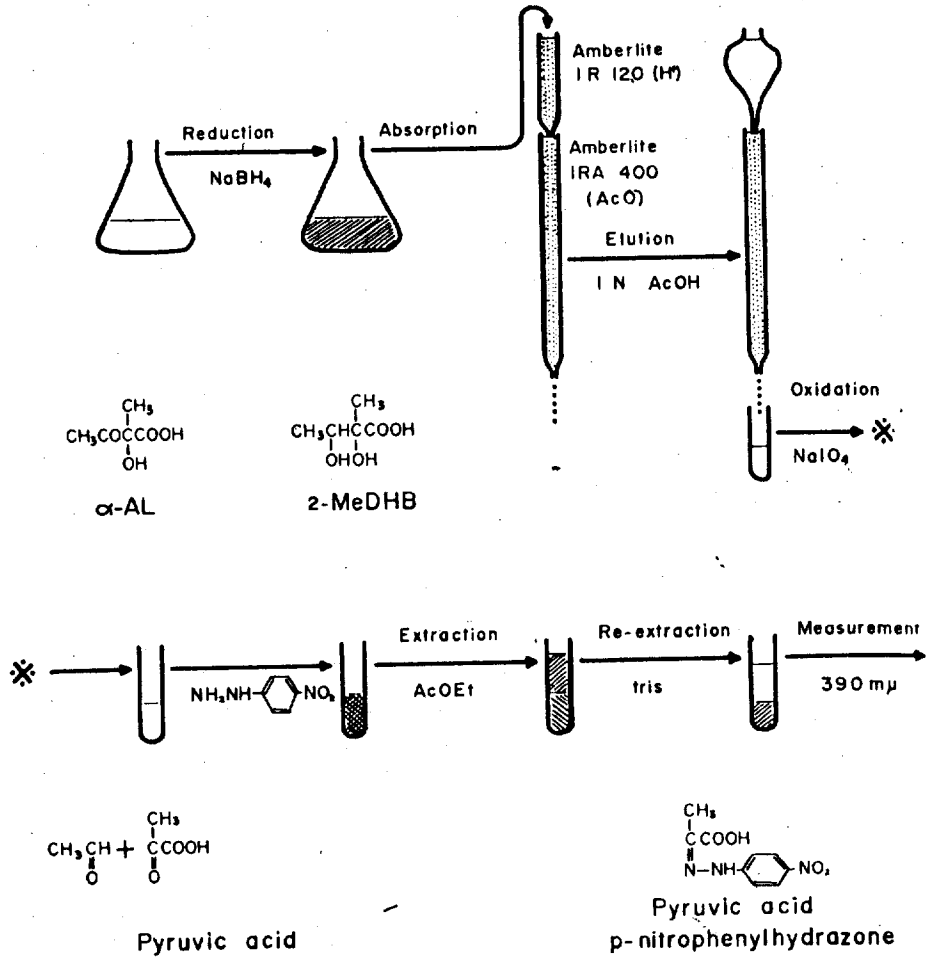


Fig. 5. Procedure for quantitative determination of α-AL in the fermented liquor.

autoxidation was adjusted to pH 7.0 and treated with sodium borohydride to convert α-AL to a stable acid, 2-methyl-2,3-dihydroxybutyric (2MeDHB). The amount of 2MeDHB was estimated by the method of Juni and Heym (7). The difference between the amounts of 2MeDHB in the fermented liquor with and without autoxidation represents the amount of α-AL. This difference coincided with the amount of material X (Table V). (Fermentation was carried out for 1 day at 25°C. in medium consisting of (per liter): glucose, 50 g.; amino acids except valine and isoleucine, 4 g.; salts; vitamins; 0.1*M* citrate buffer solution at pH 5.5, 25 ml.)

TABLE V
Determination of Material X and α-AL in the Fermented Liquor

	Amount of Material X	Amount of α-AL
	ppm. (as DA)	*ppm. (as DA)*
Fermented liquor 1	0.62	0.72
Fermented liquor 2	0.96	1.01

The results of the experiments described here show that material X was identical with α-AL.

The variation in the amount of DA estimated by the analytical methods used (6,11) might be owing to the coexistence of this unstable substance in

samples.

3. *Mechanism of DA Formation during Fermentation.* It was first necessary to ascertain whether yeast cells take part in the final reaction of DA formation in beer fermentation.

If DA were formed in the cells, the DA concentration in the cells during DA formation would be higher than that in the medium. Yeast cells were harvested from pilot brewery fermentation medium during DA formation and disrupted in ethanol with a cell homogenizer ("Cell Homogenizer MSK," B. Braun Melsungen Apparatebau) for 2 min. at 0°C., by the method described by Wagner *et al.* (21). The amounts of material X and DA in the cell-free extract, obtained by centrifuging the homogenate at 10,000 rpm. for 30 min., were measured. Ethanol in the supernatant was removed by evaporation after the condensation process in the DA determination.

The results (Table VI) show clearly that DA itself, unlike α-AL, was not formed in the yeast cells but in the medium.

Since α-AL could be oxidized to DA as described previously, α-AL might be a precursor of DA in beer fermentation if the oxidative state of the medium was high enough to decarboxylate α-AL oxidatively.

Accordingly, the effect of the redox potential of the fermenting medium on the formation of DA was investigated. The potential was altered by addition of ferric chloride or sodium hydrosulfite. Fermentation was carried out in stationary cultures at 20°C. and formation of DA and material X was measured at intervals. To prevent erroneous results caused by the effects of the added substances on the determination of DA, excess ethylenediamine tetraacetic acid was added to the fermented liquor containing ferric chloride before the quantitative analyses and the medium was shaken for 5 min. when it contained sodium hydrosulfite. Results are shown in Fig. 6. In the fermentation liquid which was kept at a high redox potential with ferric chloride (a), no material X accumulated but DA did. In the liquid of low redox potential obtained with sodium hydrosulfite (b), however, material X accumulated but DA did not.

Fermentation without addition of any substance was carried out in stationary cultures in wort at 20°C. The amounts of DA and material X were measured periodically in relation to the change in redox potential. The redox potential of the fermenting medium was measured with a platinum electrode against a saturated calomel electrode. The result in Fig. 7 indicates that while the potential was still high, only DA accumulated while material X did not.

TABLE VI

Intra- and Extracellular Concentration of DA and Material X

	Intracellular Concentration (I)	Extracellular Concentration (E)	(I)/(E)
	$\left(\frac{mcg.\ as\ DA}{g.\ wet\ wt.}\right)$	$\left(\frac{mcg.\ as\ DA}{ml.}\right)$	
DA	0.08	0.14	0.6
Material X	0.50	0.14	3.6

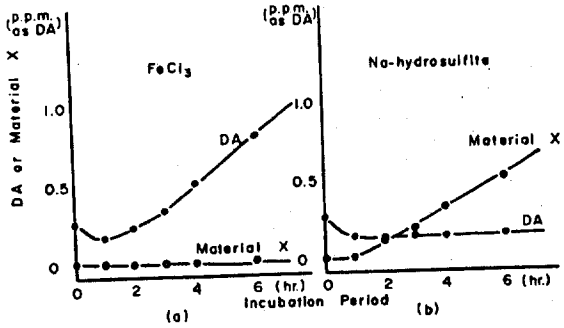


Fig. 6. Changes in amounts of DA and material X during fermentation of wort with $FeCl_3$ (100 ppm.) or Na-hydrosulfite (200 ppm.).

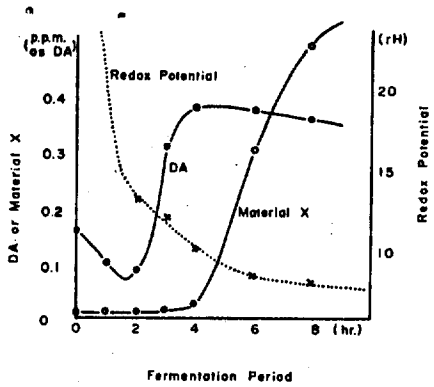


Fig. 7. Changes in amounts of DA and material X and in redox potential during fermentation in wort at 20°C.

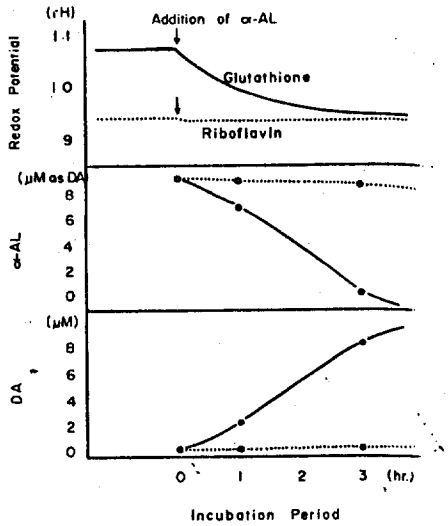


Fig. 8. Oxidative decarboxylation of α-AL with the redox substances.

As the fermentation proceeded, the rH value decreased. When the rH value reached about 10, formation of DA terminated and accumulation of material X began.

In another experiment, the critical redox potential below which α-AL is not oxidized to DA was estimated by use of redox substances. Ten micromoles of α-AL was added to 50 ml. of 1*M* acetate buffer solution containing 20 micromoles of the redox substance (pH 4.5, 20°C., in atmosphere of N_2) after equilibration at the desired redox potential. When the potential was higher than the critical potential, decrease in the potential of the solution, consumption of α-AL, and formation of DA were observed. Below the critical potential, addition of α-AL induced neither change in the potential nor DA formation (Fig. 8).

The critical potential value measured by this method was above 9.4, but not above 10.4, which was similar to that observed in the fermentation of wort.

These results show that material X was a precursor of DA in beer fermentation and was oxidatively decarboxylated to DA immediately after its formation at above rH 10, but at lower rH values, no more DA was formed and material X accumulated.

From the data presented in this work, the authors propose the mechanism of DA formation in beer illustrated in Fig. 9. α-AL is formed by yeast cells through carbohydrate metabolism as an intermediate of valine biosynthesis and a part is passed into the fermenting medium. While the redox potential of the medium is still higher than rH 10, α-AL is converted into DA by oxidative decarboxylation, and later when the potential falls below rH 10 as the fermentation proceeds, α-AL begins to accumulate and formation of DA terminates.

It has generally been thought that DA is formed from AMC. Even if the precursor of DA is AMC, DA must be

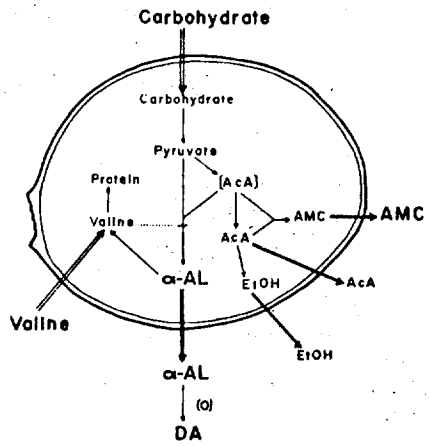


Fig. 9. Proposed mechanism of DA formation in beer.

formed in the medium, not in the yeast cells, as shown previously (see Table VI). But AMC was not oxidized to DA even during autoxidation. Therefore, AMC will not be a precursor of DA in beer fermentation.

Summary

1. Material X, which was easily converted into DA when the liquor was exposed to air, was found in fermented liquor.

2. Material X was decomposed to AMC by acid-treatment, and to DA by autoxidation.

3. Formation of material X was inhibited by addition of valine.

4. The behavior of the 2,4-dinitrophenylhydrazine derivative of material X was identical with that of α-AL on paper chromatography, mass-spectrometry, and infrared spectrometry.

5. An amino acid which was derived from the 2,4-dinitrophenylhydrazone of material X by hydrogenation behaved identically on paper chromatography with that derived from α-AL hydrazone.

6. The amount of material X in the fermented liquor coincided with that of α-AL measured by the method of Juni and Heym (7).

7. The conversion of material X to DA was regulated by the redox potential of the fermenting medium, and occurred only above about rH 10. This potential coincided with the critical potential at which α-AL was oxidatively decarboxylated.

8. The concentration of DA in yeast cells harvested during DA formation was lower than that outside the cells, but the concentration of material X was much higher in the cells.

9. DA was not formed from AMC during brewery fermentation.

10. The authors propose a mechanism of DA formation in beer in which α-AL is formed in yeast cells, passes into the fermenting wort, and is spontaneously converted into DA by oxidative decarboxylation if the redox potential of the fermenting wort is sufficiently high.

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CYCLIC PATHWAY FOR THE BACTERIAL DISSIMILATION OF 2,3-BUTANEDIOL, ACETYLMETHYLCARBINOL AND DIACETYL

II. The Synthesis of Diacetylmethylcarbinol from Diacetyl, a New Diphosphothiamin Catalyzed Reaction[1]

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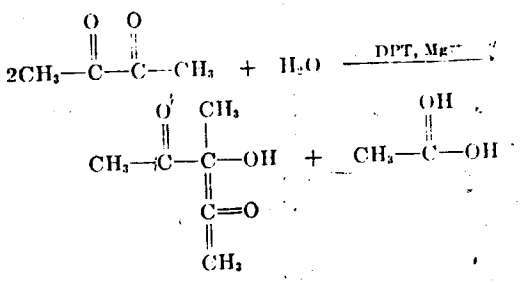
Received for publication May 7, 1956

It has been demonstrated (Juni and Heym, 1956*a*) that microorganisms capable of using either 2,3-butanediol or acetylmethylcarbinol (AMC) as the sole source of carbon and energy for growth develop a cyclic pathway that results in the conversion of these compounds to acetic acid. Evidence has been presented for the occurrence in these organisms of enzymes that oxidize 2,3-butanediol to acetylmethylcarbinol followed by the oxidation of the latter compound to diacetyl. Diacetyl then undergoes a non-oxidative diphosphothiamin (DPT) dependent acyloin condensation to form diacetylmethylcarbinol (DAMC) and acetic acid as indicated:



Once formed diacetylmethylcarbinol is further metabolized to acetylbutanediol (ABD) which, in turn, is hydrolyzed to 2,3-butanediol and acetic acid. These last two reactions are now being investigated and will be considered in detail in future reports.

The present paper is concerned with the purification and properties of the enzyme system that catalyzes the synthesis of diacetylmethylcarbinol and acetic acid from diacetyl.

[1] Aided by National Science Foundation Grant G-1269.

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MATERIALS AND METHODS

Preparation of crude enzyme extract. Cells of an organism tentatively identified as *Micrococcus ureae* were grown and harvested as described previously (Juni and Heym, 1956*a*). Cell-free extracts were prepared by suspending the wet packed cells, obtained by centrifugation in a Sharples supercentrifuge, in five times their volume of distilled water and subjecting 60-ml aliquots to sonic vibration for 30 min with a Raytheon 10-kc oscillator. The resulting suspension was then centrifuged at 25,000 × G for 30 min at 5 C and the precipitate discarded.

Chemicals and chemical assays. The assays used for acetic acid, diacetyl, and diacetylmethylcarbinol have already been described (Juni and Heym, 1956*a*). In addition to these methods a new colorimetric procedure for determining small quantities of diacetylmethylcarbinol quantitatively has been devised (Juni and Heym, 1957) and is now used as the method of choice. During enzyme purification protein was determined either by the colorimetric method of Lowry *et al.* (1951) or by the optical method of Warburg and Christian (1941). The determination of protein by measuring the turbidity produced in the presence of trichloracetic acid (Stadtman *et al.*, 1951) was found to be unsatisfactory since purification of the enzyme system resulted in protein solutions that no longer react with this reagent. Alumina C γ was prepared according to Willstätter and Kraut (1923). "Amphojel," a commercial preparation of aluminum hydroxide gel, was obtained from Wyeth Inc. Diphosphothiamin and protamine sulfate were obtained from Nutritional Biochemicals Corp. The dimerization of diacetyl was followed by determining the residual unpolymerized diacetyl according to Westerfeld (1945). Diacetylmethylcarbinol was synthesized as previously described (Juni and Heym, 1956*b*). Enzymatically formed diacetylmethylcarbinol may be prepared in large quantities using the enzyme fractions described below (Juni and Heym, 1957). Since phosphate interferes with the colorimetric method for the determination of diacetylmethylcarbinol this compound must first be removed quantitatively from reaction mixtures containing phosphate buffers (Juni and Heym, 1957).

Enzyme assay methods. Acetic acid formation may be followed manometrically by measuring the initial rate of CO_2 evolution from bicarbonate buffer in the conventional Warburg apparatus at 30 C, with 100 per cent CO_2 as the gas phase. Suitable precautions concerning the volatility of diacetyl during gassing should be observed (Juni and Heym, 1956*a*).

A simple assay procedure has been devised (Juni and Heym, 1957) making use of the fact that diacetylmethylcarbinol reduces molybdate anion to the so-called molybdenum blue. The following assay mixture should be freshly prepared each day and kept in an ice bath to avoid loss of diacetyl due to volatilization: 2.0 ml of 0.5 M sodium maleate buffer, pH 6.4, 0.6 ml of a diphosphothiamin solution containing 0.5 mg of DPT per ml, 0.6 ml of 0.5 per cent $MgSO_4$, 0.1 ml of 1.0 M diacetyl, and 4.7 ml of distilled water. To a colorimeter tube is added 0.4 ml of the above assay mixture and the solution temperature equilibrated in a 30 C water bath for several min. The reaction is initiated by adding 0.1 ml of a suitably diluted enzyme fraction (usually 1:10 for the crude enzyme), mixing thoroughly, and incubating at 30 C for 10 min. To stop the reaction 1.0 ml of acid-molybdate reagent (Juni and Heym, 1957) is added, followed by 4.5 ml of distilled water. The color is permitted to develop for the required length of time for this test and read in a Klett colorimeter at 660 mμ (Juni and Heym, 1957). The amount of phosphate present in the diluted enzyme fraction is not sufficient to interfere in the color-forming reaction. A colorimeter blank is prepared by addition of the acid-molybdate reagent to 0.4 ml of assay mixture followed by 0.1 ml of the diluted enzyme solution. Activity is defined as μmoles of diacetylmethylcarbinol formed in 10 min per ml of enzyme. Specific activity is defined as μmoles of diacetylmethylcarbinol formed in 10 min by 1.0 mg protein using the above assay procedure.

RESULTS

Stoichiometry of the reaction. Crude sonic extracts generally contain all the enzymes of the 2,3-butanediol cycle (Juni and Heym, 1956*a*). Addition of any of the intermediates of the cycle, except 2,3-butanediol, results in the conversion of the compound to 2,3-butanediol and acetic acid (Juni and Heym, 1956*a*). It has been observed, however, that high concentrations of diacetyl irreversibly inhibit several of the other reactions of the system and permit a relatively quantitative conversion to diacetylmethylcarbinol and acetic acid (table 1). Purification of the enzyme resulted in a fraction that was no longer capable of further metabolizing diacetylmethylcarbinol even when diacetyl was present in low concentration. The results obtained with such a fraction are shown in table 1. Studies with purified enzymes gave stoichiometric balances for the cases where the reaction proceeded either partially or to completion.

Aubert and Millet (1953) have reported the presence of a diacetyl mutase enzyme system (Green *et al.*, 1947) in extracts of *Neisseria winogradskyi* grown with 2,3-butanediol as the source of carbon and energy. Our studies (Juni and Heym, 1956*a*), however, have shown that several different genera of bacteria all carry out the same reactions of the 2,3-butanediol cycle and convert diacetyl to diacetylmethylcarbinol and acetic acid, there being no evidence for a diacetyl mutase reaction. Using diacetylmethylcarbinol as the substrate for partially fractionated extracts

TABLE 1

Balances for the anaerobic breakdown of diacetyl by cell-free extracts

Substrate and Products	Crude Enzyme		Purified Enzyme	
	Theoretical	Found	Theoretical	Found
	μmoles	*μmoles*	*μmoles*	*μmoles*
Diacetyl	141	—	25.4	—
DAMC	70.5	70.5	12.7	12.1
Acetic acid	70.5	66.0	12.7	12.1

$NaHCO_3$, 100 μmoles; diphosphothiamin, 50 μmoles; $MgSO_4$, 0.1 ml of a 0.5 per cent solution; enzyme, 0.5 ml; diacetyl, as indicated; gas phase, 100 per cent CO_2; final vol, 1.6 ml; incubation at 30 C.

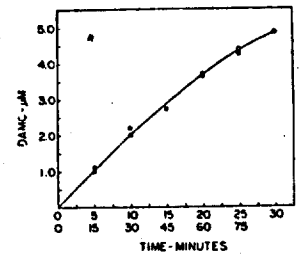

Figure 1. Diacetylmethylcarbinol formation as a function of time and enzyme concentration. Each tube contained the following: potassium phosphate buffer, 100 μmoles; pH 6.4; diphosphothiamin, 50 μg; $MgSO_4$, 0.1 ml of a 0.5 per cent solution; diacetyl, 50 μmoles; enzyme, 0.06 or 0.02 ml; final vol, 1.6 ml; gas phase air. The incubation temperature was 30 C. The reaction was stopped by acidifying samples at 5 min intervals when 0.06 ml of enzyme was used (⊙), and at 15-min intervals with 0.02 ml of enzyme (●).

it has been shown in our laboratory that there is sufficient oxidizable endogenous material in the extracts to reduce diacetylmethylcarbinol to acetylbutanediol in the presence of diacetylmethylcarbinol reductase, the latter enzyme always being present in extracts from cells grown on 2,3-butanediol (Juni and Heym, 1956*a*). The methods used by Aubert and Millet (1953) would have failed to differentiate between acetylbutanediol and acetylmethylcarbinol and could account for the results they reported.

General properties of the system. The production of diacetylmethylcarbinol is linear with time until the DAMC concentration approaches 1.5×10^{-3} M, at which time the rate of its formation begins to fall off (figure 1). To rule out the possibility that diacetyl inactivates the enzyme slowly with time the points of the curve of figure 1 were also obtained by diluting the enzyme 1:3 and incubating each sample three times longer. The values obtained are virtually identical to those for shorter contact with diacetyl, thus proving that prolonging the exposure time to diacetyl with a diluted enzyme does not result in inactivation. These data also illustrate that the rate of the reaction is a linear function of the amount of enzyme employed. This last fact has also been verified in independent experiments where the enzyme concentration was varied over a five-fold range.

A typical curve for acetic acid production as a function of time may be seen in figure 2. The rapid departure from linearity is due to end product inhibition and is very similar to the result obtained for the decarboxylation of pyruvic acid by wheat germ carboxylase (Singer and Pensky, 1952). When diacetylmethylcarbinol was added initially, together with diacetyl, the reaction was considerably inhibited but proceeded linearly with time (figure 2) since the small amount of diacetylmethylcarbinol formed during the course of the experiment did not essentially alter the concentration of this end product in the reaction vessel. The influence of diacetylmethylcarbinol concentration on the initial rate of the reaction is illustrated in figure 3.

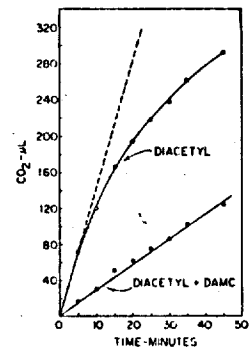

Figure 2. Acetic acid formation from diacetyl as a function of time in the presence and absence of diacetylmethylcarbinol. Standard conditions for the manometric assay were used. Diacetyl, 50 μmoles; diacetylmethylcarbinol, 34 μmoles; final vol 1.6 ml.

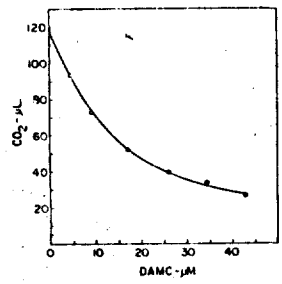

Figure 3. Inhibition of the initial rate of diacetyl dissimilation as a function of the concentration of diacetylmethylcarbinol. Standard conditions for the manometric assay were used. Diacetyl, 50 μmoles; final vol, 1.6 ml. The values for CO_2 evolved from bicarbonate buffer were obtained after incubation for 20 min in the presence of the indicated amounts of diacetylmethylcarbinol.

TABLE 2

Summary of purification procedure for diacetylmethylcarbinol-forming enzyme

Fraction	Volume	Protein	Activity	Specific Activity
	ml	*mg per ml*		
Crude	222	20.2	11.3	0.56
Amphojel supernatant	235	14.5	13.2	0.91
Protamine supernatant	250	13.3	11.7	0.88
C γ eluate	175	5.8	9.6	1.6
1st $(NH_4)_2SO_4$ precipitate	5.3	6.8	130	19.1

A diacetylmethylcarbinol concentration of 8.8×10^{-5} M inhibited the rate of the reaction by 50 per cent.

Partial purification of the enzyme system. During all the steps of the fractionation enzyme solutions were kept at 0–5 C. To 222 ml of crude sonic extract were added 55 ml of Amphojel (81.3 mg dry weight per ml) and the suspension stirred for 10 min. The gel was removed by centrifugation for 10 min at 25,000 × G and discarded. A fairly extensive study of this enzyme system showed it to be refractory to all the classical protein purification procedures until it was first treated with Amphojel or some other gel such as alumina C γ. The results in table 2 show that this treatment also resulted in a slight increase in activity due presumably to the removal of interfering substances.

Nucleic acid was removed by the dropwise addition, with continuous stirring, of 19.6 ml of a 2.0 per cent protamine sulfate solution, which had been adjusted to pH 6.4, to 235 ml of the supernatant obtained after the Amphojel treatment. The turbid suspension was stirred for 20 min and clarified by centrifugation for 20 min. The active protein was then adsorbed by first adjusting the supernatant after protamine treatment (250 ml) to pH 6.0 with 2.0 M sodium acetate buffer, pH 4.7, and then adding 175 ml of alumina C γ (27.8 mg dry weight per ml) slowly with stirring. After mixing for 10 min the gel suspension was centrifuged for 10 min and the supernatant fluid discarded.

The C γ gel was eluted with 175 ml of 30 per cent saturated ammonium sulfate solution (diluted from the following saturated solution: 70 g of $(NH_4)_2SO_4$ dissolved in 100 ml of water and adjusted to pH 7.3 with NH_4OH—the pH was measured with a sample diluted 1:50), the gel being thoroughly suspended with the aid of a cold mortar and pestle. The gel was removed by centrifugation for 10 min and the precipitated material discarded.

The ammonium sulfate eluate was found to remain stable when stored at −18 C for several weeks. To 175 ml of eluate were added 35 ml of saturated ammonium sulfate solution (pH 7.3) with continuous stirring. The slightly turbid suspension was centrifuged and the precipitate dissolved with distilled water to a final volume of 5.3 ml. This last fraction contained 27.8 per cent of the activity of the crude extract with an over-all purification of 34-fold. Storage of the purest fraction at −18 C overnight resulted in the loss of approximately 50 per cent of the activity. No attempt was made to stabilize the enzyme, since it is so readily derived from the stable ammonium sulfate eluate when required. It does seem likely, however, that reducing agents such as glutathione afford adequate protection since dialysis overnight against 0.025 M veronal buffer, pH 7.6, containing 0.01 per cent glutathione, resulted in the recovery of nearly all of the original activity in the fraction used.

Cofactor requirements. The activities of purified enzyme fractions can be stimulated several-fold by diphosphothiamin and magnesium ions. To obtain a better resolution a sample of purified enzyme was dialyzed against 0.6 per cent disodium dihydrogen ethylenediaminetetraacetate dihydrate, adjusted to pH 7.5 with sodium hydroxide for 12 hr at 5 C. The cofactor requirements for this dialyzed fraction are shown in

TABLE 3

Cofactor requirements for diacetylmethylcarbinol formation

Additions	Diacetylmethylcarbinol
	μmoles
Complete*	0.148
− DPT	0.014
− Mg^{++}	0.051
− DPT, − Mg^{++}	0.011

* Complete system: diacetyl, .5 μmoles; diphosphothiamin, 0.03 μmoles; $MgSO_4$, 1.2 μmoles; sodium maleate buffer, pH 6.4, 50 μmoles; enzyme, 0.01 ml; final vol, 0.5 ml; gas phase, air. Temperature, 30 C. Time of incubation, 10 min.

TABLE 4

Effect of divalent cations on the activity of diacetylmethylcarbinol-forming enzyme

Cation	Per Cent of Maximum Activity
Mg^{++}	100
Co^{++}	96
Mn^{++}	86
Ni^{++}	82
Ca^{++}	64
Cd^{++}	50
none	38

Assay system: diacetyl, 5 μmoles, diphosphothiamin, 0.03 μmoles; divalent cation, 8 μmoles; sodium maleate buffer, pH 6.4, 50 μmoles; enzyme 0.01 ml; final vol, 0.5 ml; gas phase, air; temperature, 30 C; time of incubation, 10 min.

table 3. Other divalent cations also activate the dialyzed preparation (table 4). Of all the cations tested some stimulation was observed in every case except for ferrous ions where an inhibition of 30 per cent of the basal activity (no cations added) was observed. Singer and Pensky (1952) report that ferric ions inhibit the basal activity of wheat germ carboxylase, and it is possible that the observed inhibition of the diacetylmethylcarbinol synthesizing enzyme may be due to the presence of ferric ions in the ferrous sulfate solution employed.

The Michaelis-Menten constant for diphosphothiamin was found to be 1.1×10^{-6} M. The comparable K_m for diphosphothiamin for the diacetyl enzyme from *N. winogradskyi* was reported to be 1×10^{-6} M (Aubert and Millet, 1953), while the K_m for the α-acetolactic acid forming system from *Streptococcus faecalis* was found to be 1×10^{-6} M (Dolin and Gunsalus, 1951).

Effects of pH. Unlike most diphosphothiamin enzymes the diacetylmethylcarbinol synthesizing system has a broad pH optimum from pH 6.0 to 8.0. Caution must be observed, however, since diacetyl dimerizes in alkaline solutions (Diels *et al.*, 1914). The curves in figure 4 show the disappearance of unpolymerized diacetyl as a function of the pH of incubation. It is apparent that some diacetyl dimerizes even at pH values less than 7.0; above pH 6.5 dimerization is very rapid. It is important to be aware of this behavior of diacetyl especially when attempting to do balance studies with this substrate. The experiments reported in this paper were all performed at pH 6.0 to 6.4 and 30 C.

Inhibitors. The various inhibitors of this reaction tested are listed in table 5. The enzyme does appear to contain sulfhydryl groups, since it is strongly inhibited by heavy metals and by *p*-chloromercuribenzoic acid and iodoacetic acid. The *p*-chloromercuribenzoic acid reagent is not as potent an inhibitor in this system as it is for the pyruvic acid carboxylases from *Acetobacter suboxydans* (King and Cheldelin, 1954) and from wheat germ (Singer and Pensky, 1952). Diacetylmethylcarbinol, the end product of the reaction, is also a fairly strong inhibitor of the system as shown in figure 3. The structural similarity of diacetyl and pyruvamide probably accounts for the inhibitory action of the latter compound.

Reversibility of the reaction. When purified enzyme preparations react with diacetyl very nearly all of the substrate is converted to diacetylmethylcarbinol and acetic acid. This indicates that the equilibrium of the reaction is in the direction of acyloin synthesis. To test for the possible reversibility of this reaction diacetylmethylcarbinol (25 μmoles) and sodium acetate (25 μmoles) were incubated with 0.5 ml of an active enzyme, together with the same amounts of the cofactors used to assay for the forward reaction, for 1 hour (reaction volume, 1.6 ml). No net synthesis of diacetyl could be detected using the sensitive method of Prill and Hammer (1938). It would thus appear that this reaction

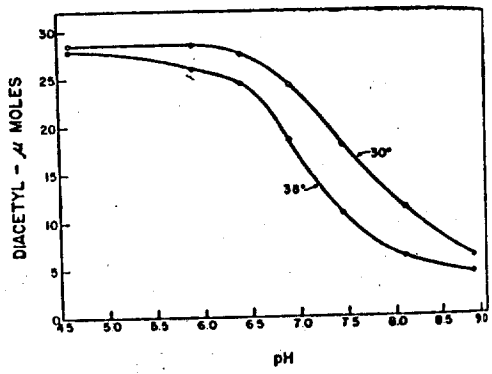

Figure 4. Dimerization of diacetyl as a function of pH. Each vessel contained 140 μmoles of potassium phosphate buffer at the pH indicated, 28.5 μmoles of diacetyl for the 30 C curve and 27.9 μmoles of diacetyl for the 38 C curve. Total vol, 1.6 ml. Incubation in 100 per cent nitrogen for 110 min.

0971

is also similar to the analogous α-acetolactic acid forming system in that reversibility cannot be demonstrated in either case. To date, the only diphosphothiamin catalyzed reaction that has been shown to be freely reversible is transketolase (Horecker *et al.*, 1953).

Substrate specificity. Since the formation of diacetylmethylcarbinol from diacetyl is completely analogous to the synthesis of α-acetolactic acid from pyruvic acid (Juni and Heym, 1956*a*, *b*) the possibility that the same enzyme might catalyze both reactions was investigated. Pyruvic acid was found to be inert in the diacetyl system, and diacetyl is not a substrate for the α-acetolactic acid forming enzymes. Analogues of diacetyl such as acetyl isobutyryl and acetyl benzoyl were found to substitute for diacetyl in both the manometric and colorimetric assays. The nature of the products obtained with these analogues is now being investigated and will be the subject of a future report. The Michaelis-Menten constant for diacetyl is 6.3×10^{-4} M.

Evidence against the possible direct oxidation of diacetyl. The cyclic mechanism for the oxidation of 2,3-butanediol (Juni and Heym, 1956*a*) involves the acyloin condensation of diacetyl to diacetylmethylcarbinol as outlined above. Since the cyclic pathway was elucidated primarily from anaerobic studies there is the possibility that diacetyl may also be directly oxidized under aerobic conditions as well as undergo an acyloin condensation. The concept that both of these reactions may be catalyzed by a single enzyme is not an unlikely one, since it has been shown (Schweet *et al.*, 1951; Juni and Heym, 1956*b*) that pyruvic acid oxidase can oxidize its substrate aerobically or give rise to acyloin condensation products when oxygen or a suitable electron acceptor is absent. For the case of the diacetylmethylcarbinol system, however, it was shown that extracts active anaerobically on diacetyl will not oxidize diacetyl either in air or anaerobically in the presence of ferricyanide. Furthermore, a particulate cytochrome containing fraction[3] obtained from *Proteus vulgaris*, which can couple the oxidation of pyruvic acid to molecular oxygen (Moyed and O'Kane, 1956), did not serve to activate the diacetylmethylcarbinol forming system for oxygen uptake. The accumulation of acetylbutanediol in the medium when cells are grown aerobically on acetylmethylcarbinol (Juni and Heym, 1956*a*) may be taken as further evidence that a considerable portion, if not all, of the diacetyl metabolized condenses to form diacetylmethylcarbinol and acetic acid.

[3] Kindly supplied by W. E. Razzell.

TABLE 5

Inhibitors of diacetylmethylcarbinol-forming enzyme

Inhibitor	Concentration	Per Cent Inhibition
	molarity	
$HgCl_2$	2×10^{-5}	16
$HgCl_2$	2×10^{-4}	100
$AgNO_3$	8×10^{-6}	41
$AgNO_3$	4×10^{-5}	100
$CuSO_4$	4×10^{-4}	51
$CuSO_4$	4×10^{-3}	100
p-Chloromercuribenzoic acid	4×10^{-3}	88
Iodoacetic acid	7×10^{-3}	67
Pyruvamide	1×10^{-1}	86
Pyruvamide	2×10^{-2}	62
Acetaldehyde	2×10^{-1}	95
Acetaldehyde	4×10^{-2}	69
Diacetyl monoxime	4×10^{-2}	65
Acetylmethylcarbinol	1×10^{-2}	52
Methyl ethyl ketone	2×10^{-1}	55
Sodium arsenite	2×10^{-2}	51
Sodium pyruvate	2×10^{-1}	35
Sodium acetate	2×10^{-1}	15
Sodium fluoride	5×10^{-2}	13
Acetamide	2×10^{-1}	17

Artifacts encountered using diacetyl as a substrate. Diacetyl is a fairly reactive compound and can give rise to many artifacts when used as a substrate in biological systems. The volatility of diacetyl makes it necessary to employ suitable gassing controls when using an atmosphere other than air in the conventional Warburg vessel. Solutions of diacetyl slowly decrease in strength even when stored in the refrigerator and should be checked periodically or, preferably, made up before each experiment from freshly distilled diacetyl. Under even slightly alkaline conditions diacetyl can dimerize fairly extensively (figure 4) and experiments of long duration should be conducted at pH 6.0, or as low as possible. During enzyme fractionation it was noted that fairly strong solutions of ammonium sulfate react with diacetyl to give an acid in the manometric assay using bicarbonate buffer. The products formed in this non-enzymatic reaction have not yet been identified. Ammonium sulfate frac-

tions assayed during the purification procedure described above were not dialyzed before being tested because the high dilution used in the colorimetric assay did not introduce sufficient ammonium sulfate to cause any interference. When used in the large scale preparation of diacetylmethylcarbinol (Juni and Heym, 1957), however, ammonium sulfate fractions should be dialyzed before coming into contact with diacetyl. Tris buffer (tris-(hydroxymethyl)-aminomethane) also reacts rapidly with diacetyl and cannot be used in any system where diacetyl is the substrate.

DISCUSSION

For many years the only naturally occurring acyloin condensation product known was acetylmethylcarbinol. Recent studies (Juni, 1952*a*, *b*; Dolin and Gunsalus, 1951) have shown that there is only one enzyme system that appears to be uniquely concerned with acetylmethylcarbinol synthesis, namely the α-acetolactic acid forming and decarboxylating enzymes from bacteria that carry on a typical 2,3-butanediol fermentation of sugars. All other enzymes that form acetylmethylcarbinol do so as side reactions. In these cases the rates of acetylmethylcarbinol production are usually very small compared with the rates of the chief reactions of the enzymes (Juni, 1952b; Juni and Heym, 1956*b*). The discovery of transketolase (Horecker and Smyrniotis, 1953; Racker *et al.*, 1953), the enzyme concerned with the synthesis of 5 and 7 carbon sugars, revealed a second diphosphothiamin enzyme whose main function is acyloin synthesis. The diacetylmethylcarbinol forming enzyme, described in this paper, represents still another diphosphothiamin enzyme whose primary function is to catalyze the synthesis of a carbon to carbon bond containing adjacent carbonyl and carbinol groups (ketols). Pyruvic oxidase preparations also convert diacetyl, in part, to diacetylmethylcarbinol (Juni and Heym, 1956*b*) but this has been shown to occur as the result of a lack of substrate specificity of these systems and is probably of no physiological significance.

All acyloin condensations may be pictured as two stage reactions. The first step yielding an activated aldehyde which is condensed with an aldehyde or ketone in the second step. No evidence for the participation of two separate enzymes has been obtained during the fractionation of the diacetylmethylcarbinol forming system. Transketolase has been crystallized from baker's yeast (de la Haba and Racker, 1955) and only one enzyme seems to be concerned here with both the formation of the activated aldehyde ("active glycolaldehyde") and with its subsequent condensation to a suitable acceptor aldehyde. Strecker and Ochoa (1954), however, have presented evidence that two distinct enzyme fractions may be involved in the synthesis of α-acetolactic acid from pyruvic acid by *Aerobacter aerogenes*. Combination of these fractions resulted in a 2-fold increase over the sum of the activities of the fractions assayed separately. Unpublished studies from our laboratory on the purification of the α-acetolactic acid forming system from the same organism did not disclose any evidence for the participation of two enzymes in spite of the fact that over a 100-fold purification was achieved. The detailed mechanisms whereby diphosphothiamin serves to generate an aldehyde activated for acyloin condensation have yet to be elucidated.

One of the chief reasons for the great interest in the enzymatic synthesis of acetylmethylcarbinol was the fact that the reaction represents the formation of a carbon to carbon bond. This was thought by some to be a model for the synthesis of complex organic molecules from simple ones by living cells. It is only in recent years that the importance of acyloin condensations for the biosynthesis of sugars has served to focus attention once again on this reaction. The studies reported in this paper show that acyloin condensations play a role in the aerobic dissimilation of certain compounds by bacteria. Ketol condensation products have been postulated as possible intermediates in the synthesis of branched chain amino acids (Strassman *et al.*, 1955). It seems likely that as more acyloin condensations are discovered this reaction may assume a role of considerable more importance in the area of biosynthesis than was hitherto suspected.

SUMMARY

Cell-free extracts from bacteria grown in a medium containing 2,3-butanediol or acetylmethylcarbinol as the sole source of carbon and energy contain an enzyme that irreversibly con-

0971

verts diacetyl to diacetylmethylcarbinol and acetic acid. The enzyme has been purified and the stoichiometry of the reaction studied. Diacetylmethylcarbinol is an acyloin condensation product, and diphosphothiamin and a divalent cation, such as magnesium, are the cofactors for the enzyme involved in its synthesis. Several inhibitors of the system have been studied. Diacetyl is not oxidized either aerobically or anaerobically in the presence of ferricyanide. The enzyme is specific for diacetyl and related alpha-diketones and will not degrade pyruvic acid. Diacetylmethylcarbinol is a fairly strong inhibitor for its own synthesis. Artifacts encountered when using diacetyl as a substrate have been discussed.

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1000

Diacetyl Production and Utilization by *Lactobacillus* Species[1,2]

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J. Dairy Sci. 51(2) 188-91, (1968)

Abstract

Single-strain cultures of *Lactobacillus casei* and *Lactobacillus plantarum* accumulated detectable amounts of diacetyl when grown in milk culture at both 8 and 30 C, but strains of *Lactobacillus lactis* and *Lactobacillus brevis* did not. *Lactobacillus casei* strains produced relatively large amounts of diacetyl under these incubation conditions, whereas *L. plantarum* accumulated this compound in levels of less than 1 ppm. Significant differences in the production of diacetyl were noted between strains of a species.

Diacetyl reductase activity was demonstrated in single-strain cultures of *L. casei, L. lactis,* and *L. brevis.* Diacetyl reductase could be induced in *L. plantarum* by growth in the presence of citrate. Growth in a milk medium supplemented with citrate resulted in a stimulation of diacetyl reductase activity with *L. casei.*

Although the presence of lactobacilli in Cheddar cheese is well documented (15), there have been few reports on the volatile compounds produced and utilized by these organisms. Nakae and Elliot (11, 12) and Kristoffersen and Nelson (10) have demonstrated the ability of some lactobacilli to produce volatile fatty acids from amino acids and casein hydrolysates. In a previous paper (8), the quantities of acetaldehyde and ethanol produced by single-strain cultures of *Lactobacillus brevis, L. casei, L. lactis,* and *L. plantarum* were determined after incubation at both their optimum growth temperature and 8 C. All organisms studied were capable of reducing added aldehydes to alcohols. In addition, *L. brevis* strains reduced added butanone to 2-butanol.

There have been few reports on the ability of lactobacilli to produce diacetyl. Bassette, Bawdon, and Claydon (1) reported diacetyl production by *L. casei* when grown in milk culture. Christensen and Pederson (5) have shown that extensive growth of *L. brevis* and *L. plantarum* during the concentration of fruit juice cause spoilage by production of diacetyl from the citrate present in the juice. Since diacetyl is produced in Cheddar cheese and reportedly contributes to the typical Cheddar flavor in low levels (3, 4), the research reported herein was designed to determine the qualitative and quantitative aspects of diacetyl production and utilization by those *Lactobacillus* species common to Cheddar cheese.

Received for publication August 5, 1967.

[1] Technical Paper no. 2334, Oregon Agricultural Experiment Station.

[2] Supported by a National Aeronautics and Space Administration Fellowship to the senior author, by PHS Grant EF-269, Division of Environmental Engineering and Food Protection and by the Nutrition Foundation, Inc.

Experimental Procedure

Cultures and culturing conditions. Cultures obtained from the American Type Culture Collection, and culturing conditions were identical to those described previously (9). All plate counts were made by plating dilutions of cultures in lactic agar (Difco).

Production of diacetyl. Reconstituted nonfat milk cultures of each of the organisms were analyzed for diacetyl content after 48 hour incubation at their optimum growth temperature (2). Duplicate determinations of diacetyl content were made on all cultures by the method described by Pack et al. (13), except that aliquots of culture were weighed rather than measured volumetrically. Based on the results obtained in this initial study, sterile milk medium was inoculated with *L. casei* and then split into two equal portions. A small amount of a sterile sodium citrate solution of sufficient concentration to yield 0.2% citrate in the resultant medium was added to one portion of each culture. Incubation was carried out for 60 hours at 30 C and diacetyl content, pH, and microbial numbers were determined at 12-hour intervals. The same type study was made with incubation at 8 C, except that citrate was not added to cultures. Analyses were made at weekly intervals for four weeks.

Utilization of diacetyl. To determine if these organisms are capable of utilizing diacetyl, an amount of a sterile diacetyl solution was added to sterile milk and the resultant medium was divided into several equal portions. One portion was held as a control and the others were inoculated with the respective single-strain culture. *L. brevis* and *L. plantarum* cultures were

bated along with a control at 30 C for hours and then analyzed for diacetyl con-. *L. lactis* strains were incubated at 40 C 18 hours. The 40 C control was acidified pH 4.5 with sterile 20% phosphoric acid be- incubation. This study was repeated with bation at 8 C for two weeks.

Diacetyl reductase activity. All cultures were assayed for diacetyl reductase activity by a ification of the method used by Seitz et al. (4). Cells were grown for 48–50 hours at their imum growth temperature in one-liter vol- of lactic broth (Difco) and in lactic broth taining 0.2% added citrate. The enzyme ree was prepared as follows: Cells were re- vered by centrifugation, resuspended in 40 ml pH 7.2 phosphate buffer (6), and disrupted a Raytheon 10 KC sonic oscillator for 15 utes. Cell debris was removed by centrifu- tion at 16,000 × *g* for 20 minutes. The su- natant obtained served as the enzyme source was frozen and held at −20 C until used. rotein determinations were made by the Biuret thod (7).

Diacetyl reductase activity was measured by ing the decrease in absorbancy at 340 mμ used by the oxidation of NADH with a Beck- Model DK-1 recording spectrophotometer. assay system contained 2 μM NADH, μM diacetyl, and 1 ml of the crude enzyme ration made up to a total volume of 3.0 ml.

Results and Discussion

of the two strains of *L. brevis* or studied accumulated detectable amounts yl on incubation for 48 hours at their growth temperature. Both strains of *brevis* accumulated diacetyl, but in levels below 1 ppm under similar incubation conditions. *L. casei* strains produced relatively high amounts of diacetyl and were studied more fully.

Data presented in Table 1 show that *L. casei* 393 accumulated 7 ppm diacetyl in 60 hours at 30 C and *L. casei* 334 accumulated 2.23 ppm in a similar period. Diacetyl content of the cultures increased with increasing cell population. The data of Table 1 show that when these organisms were grown in milk medium supplemented with citrate, diacetyl utilization was stimulated. With *L. casei* 393, 7 ppm diacetyl accumulated in the milk culture without added citrate in 60 hours and there was no evidence of diacetyl utilization. This is in contrast to the decrease in diacetyl content from an apparent high of 3.74 ppm after 36 hours to 1.08 ppm after 60 hours in the citrated culture. Although not as pronounced, this citrate effect was also evident with *L. casei* 334. These data were confirmed by analyses of new inoculums of cultures. Plate counts on cultures grown in citrated milk were generally higher during later stages of incubation, demonstrating that this effect was not due to a lower microbial population.

Table 2 presents data showing that both *L. casei* strains produce diacetyl at 8 C, a temperature near that used to age Cheddar cheese. These results indicate that *L. casei* probably contributes to the production of the diacetyl normally found in Cheddar cheese and partially explains the increase in diacetyl content sometimes noted during manufacture of Cheddar cheese (2, 3). The pH and plate count values indicate the metabolism and reproduction of these organisms is slow under these conditions.

Results presented in Table 3 show that both

TABLE 1

Diacetyl production by *L. casei* at 30 C in milk and milk fortified with 0.2% citrate

Organism	Incubation time *(hr)*	pH		Diacetyl *(ppm)*		Plate counts	
		Control	Citrate[a]	Control	Citrate[a]	Control	Citrate[a]
L. casei 393							
	0	6.30	6.30	0.11	0.11	67×10^6	67×10^6
	12	6.10	6.20	1.00	0.96	44×10^6	.....
	24	5.80	5.88	2.32	2.04	16×10^7	26×10^7
	36	5.38	5.38	3.92	3.74	38×10^7	69×10^7
	48	5.01	4.92	6.00	3.64	13×10^8	17×10^8
	60	4.70	4.58	7.00	1.08	13×10^8	14×10^8
L. casei 334							
	0	6.30	6.30	0.06	0.06	14×10^6	14×10^6
	12	6.11	6.20	0.14	0.14	12×10^7	.....
	24	5.80	5.91	0.41	0.50	37×10^7	34×10^7
	36	5.42	5.48	0.41	0.83	70×10^7	72×10^7
	48	5.13	5.13	2.19	1.96	79×10^7	87×10^7
	60	4.82	4.80	2.23	1.76	81×10^7	12×10^8

[a] Two-tenths per cent citrate added to milk medium.

TABLE 2
Diacetyl production by *L. casei* at 8 C

Organism	Incubation time *(weeks)*	pH	Diacetyl *(ppm)*	Plate count ($\times 10^6$)
L. casei 393				
	0	6.31	0.14	22
	1	6.21	1.50	33
	2	6.21	3.01	38
	3	6.05	4.05	32
	4	6.01	4.03	34
L casei 334				
	0	6.30	0.07	15
	1	6.21	0.71	20
	2	6.20	0.97	32
	3	6.19	1.18	36
	4	6.15	1.27	68

strains of *L. brevis* and *L. lactis* utilize added diacetyl when incubated at their optimum growth temperatures. Both strains of *L. plantarum* increased the diacetyl content of the medium somewhat, with *L. plantarum* 10776 producing more diacetyl than *L. plantarum* 4008. At 8 C *L. brevis* 14434 and *L. lactis* 11061 utilized small amounts of added diacetyl in two weeks, but there was no evidence for diacetyl utilization by *L. brevis* 4006 and *L. lactis* 12315 under these conditions. Both strains of *L. plantarum* increased the diacetyl content of the medium at 8 C, suggesting that these organisms can also contribute to the diacetyl content of Cheddar cheese.

To determine the mechanism of diacetyl utilization, these lactobacilli were assayed for diacetyl reductase activity. Results (Table 4) show that, with the exception of *L. plantarum* 10776, there was a measurable amount of diacetyl reductase in all organisms. Seitz et al.

TABLE 3
Diacetyl utilization by *Lactobacillus* species

Organism	Diacetyl *(ppm)* Incubation at optimum temp[a]	Diacetyl *(ppm)* Incubation at 8 C[b]
Control	1.42[c]	2.50
L. brevis 4006	0	2.50
L. brevis 14434	0.55	1.87
L. plantarum 4008	1.55	3.18
L. plantarum 10776	2.00	2.84
L. lactis 11061	0	2.23
L. lactis 12315	0	2.50

[a] Forty-eight-hour incubation at optimum growth temperature; 40 C for *L. lactis*, 30 C for *L. brevis* and *L. plantarum*.
[b] Incubated for two weeks.
[c] Values for 30 and 40 C uninoculated controls were identical.

TABLE 4
Diacetyl reductase activity of *Lactobacillus* sp.

Organism	Specific activity[a] Control	Citrate
L. casei 393	16	[illegible]
L. casei 334	16	[illegible]
L brevis 4006	3	[illegible]
L. brevis 14434	8	[illegible]
L. plantarum 4008	<1	[illegible]
L. plantarum 10776	<0	[illegible]
L. lactis 11061	5	[illegible]
L. lactis 12315	5	[illegible]

[a] Expressed as units per milligrams of protein where one unit was defined as a 0.001 change in absorbance at 340 mμ per mg protein during the second 30-second time interval after addition of NADH.
[b] Organisms grown in lactic broth fortified with 0.2% citrate.

(14) have shown that diacetyl reductase is widely distributed among bacteria. They found that the reaction required NADH and was reversible. The specific activities found in these lactobacilli compare favorably with the values Seitz et al. obtained for lactic streptococci. These data also help to explain the citrate effect discussed previously. Cultures of *L. casei* 393, *L. plantarum* 4008, and *L. plantarum* 10776 showed a significantly greater specific activity when grown in citrated lactic broth compared to cultures grown in the plain broth. This effect was greatest with strains of *L. plantarum*. To confirm this, both *L. plantarum* strains were grown for 48 hours at 30 C in sterile milk and in milk medium with 0.2% added citrate. Diacetyl was detected in the controls but not in the citrated cultures at the end of this incubation period. To determine if citrate stimulated enzymatic activity, 1 μx of citric acid was added to the assay mixture and decrease in absorbance of NADH was measured as described. The results provided no evidence for stimulation of diacetyl reductase. Thus it can be concluded that with *L. casei* 393 and both *L. plantarum* strains, growth in the presence of citrate stimulates the formation of diacetyl reductase. In view of the work of Harper (8), who found that some lactobacilli metabolize citrate, it cannot be concluded that citrate itself is responsible for this increase in diacetyl reductase activity.

The results presented herein indicate that *L. casei* and *L. plantarum* strains can contribute to the diacetyl content of Cheddar cheese and that some *Lactobacillus* species are undoubtedly involved in the gradual decrease in the amount of diacetyl noted in Cheddar cheese during curing (3, 4).

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1001

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Metabolism of Volatile Compounds of *Pediococcus cerevisiae* and Their Occurrence in Cheddar Cheese [1, 2]

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Abstract

Quantities of acetaldehyde, ethanol, and diacetyl produced in milk by single-strain cultures of *Pediococcus cerevisiae* were determined. The cultures produced only small amounts of these compounds, even on extended incubation. Other volatile compounds were not detected in milk cultures of these organisms. *P. cerevisiae* strains were capable of reducing acetaldehyde and propionaldehyde but not acetone or butanone to the corresponding alcohols.

Organisms were isolated from a series of Cheddar cheeses and identified as pediococci. These isolated organisms produced only small amounts of diacetyl.

Although *Pediococcus cerevisiae* is widely distributed in fermenting materials such as beer, sauerkraut, and pickles, few reports have been made on the volatile compounds produced and utilized by these organisms (1). In recent years some interest has been shown in the occurrence of pediococci in fermented dairy products. Daere (6, 7) found substantial numbers of organisms in New Zealand Cheddar cheese which he identified as pediococci. Sharpe and coworkers (8, 19) also reported on the isolation of pediococci from Cheddar cheese in England. In a more recent study, Fryer and Sharpe (9) isolated 59 strains of *P. cerevisiae* and indicated that they represented the predominant nonstarter flora in 16 Cheddar cheeses made over a period of five months. The presence of bacteria of the genus *Pediococcus* has not been reported in Cheddar cheese made in the United States.

At the present time some confusion exists concerning the occurrence of *P. cerevisiae* in butter cultures. Garvie (10) is of the opinion that the associative aroma-producing organisms named *Streptococcus citrovorus* (now known as *Leuconostoc citrovorum*) by Hammer (11) might have been *P. cerevisiae*. Garvie (10) suggested that *P. cerevisiae* might be useful in dairy cultures. No evidence has been found in the literature to indicate that pediococci are useful in this regard. Collins (5) stated that Galesloot found no pedicocci in any cultures; he accumulated nine strains of *P. cerevisiae* and found that none of them produced desirable aroma.

Organisms classified as *Leuconostoc dextranicum* are prevalent in lactic dairy cultures (11, 20). However, Garvie (10) found *L. dextranicum* to be a rare species and suggested that many organisms classified as *L. dextranicum* are strains of *P. cerevisiae*. Sandine et al. (20) have proposed the following utilitarian classification for the genus *Leuconostoc*:

L. dextranicum: those Leuconostocs which associatively produce aroma compounds (acetoin and diacetyl) in nonfat milk and dextran on sucrose medium.

L. citrovorum: those Leuconostocs which associatively produce aroma compounds in nonfat milk but do not form dextran on sucrose medium.

L. mesenteroides: those Leuconostocs which do not associatively produce aroma compounds in nonfat fluid milk but do elaborate dextran on sucrose medium.

The Leuconostocs having none of these abilities should be placed in the genus *Pediococcus* (20). This classification scheme is advantageous to the dairy microbiologist, because it takes into account diacetyl formation.

We conducted the present study to determine the qualitative and quantitative aspects of volatile compound metabolism by *P. cerevisiae*, with particular reference to acetaldehyde and diacetyl. The occurrence of *P. cerevisiae* in Cheddar cheese made in the United States was documented.

Experimental Procedure

Cultures and culturing conditions. Single-strain cultures were obtained from the following sources: Strains 8081 and 10791 from the American Type Culture Collection, Strains 990 and 1557 from Dr. T. F. Fryer, National Institute for Research in Dairying, Shinfield, Reading, England, and Strain F-66 from Dr. H. W. Seeley, Section of Microbiology, Cornell University, Ithaca, New York. Stock cultures were maintained in Rogosa broth (Difco). For volatile compound analysis, organisms were cultured

Received for publication July 15, 1968.

[1] Journal Paper no. 3414, Purdue Agricultural Experiment Station.

[2] Partially supported by a grant from the Nutrition Foundation, Inc.

in a reconstituted 11% solids nonfat milk medium containing 0.1% yeast extract. Cultures were incubated at 30 C, except as otherwise noted. All plate counts were made by plating dilutions of cultures with Rogosa agar (Difco).

Isolation and characterization of pediococci. Cheddar cheese samples varying in age from mild through sharp were obtained from commercial outlets. One gram of cheese was ground in a sterile mortar with sterile 2% sodium citrate solution until completely suspended in a total of 9 ml. Dilutions were plated with tomato juice agar containing 0.5% calcium lactate and 0.6% calcium citrate (17). Plates were incubated for 48 hours at 32 C, after which colonies showing clear zones were picked into Rogosa broth. After 24 hours at 32 C, smears were made, stained, and examined. Cultures forming tetrads in Rogosa broth were selected for further study. Ability to ferment various carbohydrates was determined by adding 0.5 or 1% concentrations of each carbohydrate to purple broth base (Difco) and incubating 48 hours at 32 C.

Fermentation of citrate was determined by the method of Overcast and Skeen (17). Lipolysis was detected by the method of Alford and Steinle (1), using both tributyrin and butter oil. Dextran production from sucrose was tested by streaking cultures on agar plates of medium made up as in a previous study (20). Proteolytic ability was assayed by the milk agar method (2). The method of King (14) was used to detect production of acetoin plus diacetyl. For this test, cultures were incubated in milk medium for 36 hours, acidified to pH 4.7 with sterile aqueous 20% phosphoric acid, and incubated for an additional 6 hours at 30 C.

Production of acetaldehyde and ethanol. Cultures were analyzed for acetaldehyde, ethanol, and other volatile compounds after 48, 96, and 168 hours incubation at 30 C and after 28 days incubation at 10 C, a temperature near that used in ripening Cheddar cheese. At the end of the incubation period, cultures were analyzed for volatile compounds by the on-column trapping, gas-liquid chromatographic (GLC) technique described by Morgan and Day (16). Conditions for quantitative GLC analyses were identical with those described previously, except that methyl acetate was not used as an internal standard (13). Cultures used in this study did not produce acetone in any of the trials. Therefore, the indigenous acetone was used as an internal standard to permit compensation for variations in instrument sensitivity and recorder response.

Reduction of carbonyls. Ability of single-strain cultures to reduce carbonyls was evaluated

TABLE 1. Diacetyl production by *Pediococcus cerevisiae* at 30 C in milk supplemented with 0.2% citrate.

Incubation time	*P. cerevisiae* Strain											
	8081			10791			1557			F-66			990		
	pH	Plate count[a]	Diacetyl	pH	Plate count[a]	Diacetyl	pH	Plate count[a]	Diacetyl	pH	Plate count[a]	Diacetyl	pH	Plate count[a]	Diacetyl
(hr)			(ppm)			(ppm)			(ppm)			(ppm)			(ppm)
24	6.52	17	0.18	6.30	22	0.25	6.32	19	0	6.40	27	0.05	6.41	11	0.1
48	6.34	14	0	5.98	42	0.1	6.09	35	0	6.11	40	0	6.02	18	0
72	6.01	25	0	5.45	36	0.1	5.85	33	0	5.89	51	0	5.65	32	0
96	5.72	20	0	5.11	30	0.1	5.78	40	0	5.70	80	0	5.40	20	0
168	5.30	26	0	4.78	29	0.3	5.40	34	0.18	5.22	51	0.2	4.98	38	0.13

[a] Times 10^7.

by the GLC method (13). Milk cultures were incubated with sterile solutions containing 100 ppm acetaldehyde, propionaldehyde, or butanone for 48 and 90 hours at 30 C and for 28 days at 10 C (3). Cultures without added carbonyl compounds were incubated concurrently to ascertain the amount of each alcohol produced through normal fermentation.

Diacetyl production. Milk cultures of these organisms were analyzed for diacetyl production after various intervals of incubation at 30 C by the method described by Pack et al. (18). In some cases cultures were grown in milk containing 0.2% added sodium citrate.

Results and Discussion

During initial trials, milk medium was observed to support only limited growth of this organism. Therefore, all milk media used in this study were supplemented with 0.1% yeast extract. Yeast extract provides folinic acid, an absolute vitamin requirement of *P. cerevisiae* (9).

Results of studies on diacetyl production by five cultures of these organisms when grown in milk supplemented with 0.2% sodium citrate are presented in Table 1. The organisms produced little diacetyl in this medium, even when incubated for as long as 168 hours. Diacetyl was detected in all five of these cultures at some point during this experiment. The decreases in diacetyl content noted in some cultures suggest that these organisms are capable of utilizing diacetyl. The pH data show that these organisms produce acid very slowly in milk culture.

Because these organisms produced acid very slowly, it was of interest to determine if acidification of citrated milk cultures would stimulate diacetyl production. This was studied by acidifying 48-hour milk cultures to a pH of about 4.7 with a sterile aqueous 20% phosphoric acid solution and continuing incubation. Results obtained (Table 2) gave little or no indication of increased diacetyl production on incubation for periods as long as 144 hours. *P. cerevisiae* Strains 10791 and 990 had accumulated 0.46 and 0.31 ppm diacetyl, respectively, after 144 hours incubation, but no diacetyl was detected in cultures of the three other *P. cerevisiae* strains at any time throughout this experiment. In contrast, the *Leuconostoc* species common to lactic dairy cultures are known to produce large amounts of diacetyl under these conditions (15, 21). In a number of trials, these *P. cerevisiae* cultures never accumulated as much as 0.5 ppm diacetyl. This indicates that inclusion of *P. cerevisiae* in butter cultures would not be beneficial in terms of aroma production. Milk cultures of these organisms were generally devoid of any aroma other than that characteristic of heated milk.

Amounts of acetaldehyde and ethanol accumulated in cultures after various incubation times at both 10 and 30 C are given in Table 3. These organisms usually accumulated less than 1 ppm of these compounds on incubation for as long as 168 hours at 30 C. Although plate count data showed that these organisms multiplied slowly at 10 C, a temperature near that used in the ripening of Cheddar cheese, they produced only very small amounts of acetaldehyde and ethanol. Other than acetaldehyde, ethanol, and diacetyl, chromatograms obtained showed little or no evidence for production of other volatile compounds by these strains of *P. cerevisiae*.

The ability of these organisms to reduce added carbonyls to the corresponding alcohols at both 10 and 30 C is shown in Table 4. All of these organisms reduced both acetaldehyde and propionaldehyde and the extent of reduction varied somewhat among strains. Propionaldehyde was reduced to a much greater extent than was acetaldehyde in all cases. With *P. cerevisiae* Strains 8081, 10791, and F-66, propionaldehyde was reduced nearly quantitatively to *n*-propanol on extended incubation at both 10 and 30 C. None of these cultures was capable of reducing added butanone to 2-butanol under either of these in-

TABLE 2. Diacetyl production by *Pediococcus cerevisiae* in acidified milk at 30 C.[a]

Incubation time	*P. cerevisiae* Strain									
	8081		10791		1557		F-66		990	
	pH	Diacetyl	pH	Diacetyl	pH	Diacetyl	pH	Diacetyl	pH	Diacetyl
(hr)		(ppm)		(ppm)		(ppm)		(ppm)		(ppm)
48	6.35	0	5.88	0.23	6.29	0	6.12	0	6.13	0
72	4.89	0	4.74	0.13	4.90	0	4.88	0	4.63	0.06
96	4.88	0	4.68	0.30	4.90	0	4.85	0	4.58	0.15
144	4.78	0	4.52	0.46	4.81	0	4.82	0	4.47	0.31

[a] Acidified after 48 hr incubation with sterile aqueous 20% phosphoric acid.

TABLE 3. Acetaldehyde and ethanol production in milk culture by *Pediococcus cerevisiae*.

P. cerevisiae	Incubation time[a]	Acetaldehyde	Ethanol
		(ppm)	
8081	48 hr	0.18	0.1
	96 hr	0.65	0
	168 hr	0.70	0.7
	4 wk	0.60	0.4
10791	48 hr	0.30	0
	96 hr	0.76	0.7
	168 hr	0.50	1.7
	4 wk	0.40	1.1
990	48 hr	0.15	0
	96 hr	0.46	0.1
	168 hr	0.25	0
	4 wk	0.20	0
F-66	48 hr	0.39	0
	96 hr	0.67	0.4
	168 hr	1.30	1.1
	4 wk	0.50	0.4
1557	48 hr	1.50	0.4
	96 hr	0.27	0.2
	168 hr	0.65	0.4
	4 wk	0.70	0.1

[a] Incubation at 30 C for 48, 96, and 168 hr and at 10 C for 4 weeks.

TABLE 4. Alcohol dehydrogenase activity by *Pediococcus cerevisiae*.

P. cerevisiae	Incubation time[a]	Ethanol[b]	*n*-Propanol[b]
		(ppm)	
8081	48 hr	3.2	50.0
	90 hr	18.8	96.6
	4 wk	19.5	102.0
10791	48 hr	1.6	22.1
	90 hr	4.8	93.0
	4 wk	14.3	99.0
990	48 hr	1.5	9.4
	90 hr	3.2	39.8
	4 wk	1.8	13.0
F-66	48 hr	1.0	40.4
	90 hr	21.5	101.0
	4 wk	10.5	96.0
1557	48 hr	2.2	24.4
	90 hr	9.0	77.6
	4 wk	6.0	93.0

[a] Incubation at 30 C for 48 and 90 hr and at 10 C for 4 weeks.
[b] 100 ppm of respective carbonyl added. Corrected for amount of alcohol in control culture.

cubation conditions. In addition, none of these strains of *P. cerevisiae* was observed to reduce the indigenous acetone to isopropanol, suggesting that their dehydrogenase system is specific for aldehydes. Dehydrogenase activity appears to be widespread among microorganisms associated with lactic cultures and fermenting cheeses (12).

Lipolytic and proteolytic activity of these organisms was evaluated. None of the five *P. cerevisiae* strains showed evidence of either lipolysis of butter oil or tributyrin or proteolysis of milk protein in up to five days at 30 C. These observations, coupled with those on volatile compound production, strongly suggests that *P. cerevisiae* by itself would contribute little to the characteristic flavor of Cheddar cheese. However, these results do not rule out the possibility that *P. cerevisiae* contributes beneficially to the flavor of Cheddar cheese when it grows in symbiosis with other organisms common to the microflora of this cheese. Studies on the symbiotic relationships of microorganisms common to Cheddar cheese are currently in progress in our laboratories.

To confirm the occurrence of pediococci in Cheddar cheese made in the United States, organisms isolated from a number of cheeses were studied in an attempt to characterize them. Initial selection involved isolation of those organisms which fermented citrate in agar medium and formed tetrads in Rogosa broth. Some tetrad-forming organisms could not be separated from rods (probably lactobacilli) by repeated plating and picking of colonies, and were discarded. Most tetrad-forming organisms were pure when first picked. Tetrad-formation is a general feature of *Pediococcus* species (22). Some of the characteristics used to identify the isolated pediococci are presented in Table 5. Several anomalies in sugar fermentations were observed between the known *P. cerevisiae* strains, the isolated organisms, and the characteristics summarized in Bergey's Manual (4). Nevertheless, the isolated organisms appeared to be pediococci and not members of the closely related *Leuconostoc* genus, on the basis of the following results: These organisms all formed tetrads in Rogosa broth, whereas *L. citrovorum*, *L. dextranicum*, and *Streptococcus diacetilactis* did not. None of the isolated organisms formed dextran on sucrose agar, whereas *L. dextranicum* and *L. mesenteroides* do (20). In almost all cases these organisms gave weak positive or negative King's (14) tests for acetoin and diacetyl and produced very little or no diacetyl under incubation conditions which favor strong positive King tests and production of large amounts of diacetyl by *L. citrovorum* and *S. diacetilactis* (15, 20, 21).

Most of our results indicated that the isolated

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TABLE 5. Cultural reactions of isolated and known pediococci.[a]

Organism or source[b]	Dextrose 1%	Maltose 1%	Mannose 1%	Sucrose 1%	Arabinose 0.5%	Raffinose 0.5%	Salicin 0.5%	Slime from sucrose	King's test[c]	Diacetyl[c] (ppm)
1. Sharp	±	+	+	−	−	−	−	−	−	0.05
2. Sharp	+	+	+	−	−	−	+	−	−	0
3. Sharp	+	+	±	−	−	−	+	−	++	0.15
4. Sharp	+	+	+	−	−	−	−	−	−	0.05
5. Sharp	+	+	+	−	−	−	+	−	−	0
6. Mellow	+	+	+	−	+	−	+	−	±	0.23
7. Mellow	+	±	±	−	+	−	−	−	±	0
8. Mellow	+	±	±	−	+	−	−	−	−	0
9. Mellow	+	±	±	−	+	−	−	−	±	0
10. Mellow	±	±	±	−	+	−	+	−	±	0
11. Mild	+	+	+	−	+	−	−	−	−	0.23
12. Mild	+	+	+	±	+	−	−	−	±	0
13. Mild	+	+	+	−	+	−	−	−	±	0
14. Mild	+	+	+	±	+	−	−	−	−	0.13
15. Mild	+	+	+	±	+	−	−	−	−	0.05
1557	+	−	±	±	+	−	−	−	−	0
F-66	+	+	+	−	+	−	±	−	−	0
990	+	−	−	−	−	−	−	−	−	0.06

[a] Organisms grown at 32 C for 48 hr in purple broth base with different carbohydrates added.
[b] Sharp, mellow, and mild refer to age of cheese from which organism was isolated; 1557, F-66, and 990 are known pediococci.
[c] Milk cultures incubated at 30 C for 36 hr, then acidified to pH 4.7, and incubated an additional 6-12 hr.

organisms were pediococci. Because they grew well at 30 C and appeared as spheres in tetrads, they were classed as *P. cerevisiae* and not *P. acidilactici*, the other *Pediococcus* species recognized in Bergey's Manual (4). Because organisms of this type were isolated from a series of Cheddar cheeses, they probably are prevalent in Cheddar cheese made in this country.

Acknowledgment

We are grateful to Drs. H. W. Seeley and T. F. Fryer for supplying cultures used in this study.

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1123

Proc. 17th Int. Dairy Congr., Munich,
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Influence of the Rate of Contamination of Butter by Yeast and *Klebsiella Aerogenes* on the Development of Diacetyl Content During Storage

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During the course of butter manufacture in the dairy, sometimes, accidentally or because of a lack of precaution in the cleaning and disinfecting of material in contact with the pasteurized cream or the butter, a recontamination by foreign bacteria occurs.

In a factory where everything is completely cleaned and disinfected and where great care is taken in the production of leavens, theoretically, only bacteria which survived pasteurization should be found in the butter, and eventually lactic ferments added for the maturation of cream. In practice, very often other bacteria are found - bacteria coming from the contamination of the surfaces of the dairy machinery, from washing water and sometimes, also, from a poorly conditioned leaven. To some authors, these bacteria play a significant part in the spoiling of butter at the time of cold storage (1).

(1) The works in question were done under the auspices of the Committee for the Scientific and Technical Study of Milk, subsidized by the IRSIA, by Mrs. Hote-Baudart and A. Guyot, R. Permanne and H. Pirmez.

Influence of the Number of Yeasts on the Development of Diacetyl Content

The butters were classified in three categories according to their initial amounts of diacetyl (see Table 1).

Each of these categories was divided into two parts, the first comprising butters containing less than 2000 yeast cells per gram and the second comprising butters containing more than 2000 yeast cells per gram of butter.

1. Storage of butter at 13°C (Table 1).

The examination of the various categories of butter according to their diacetyl content indicated to us that the loss of aroma is more rapid in butters contaminated by yeasts than in butters only slightly contaminated, and this can be summarized as follows:

1. For butters slightly contaminated by yeasts, the decrease in diacetyl is perceptible after 14 days of storage; after 28 days, there remains ± 75% of the original diacetyl.

2. For butters strongly contaminated by yeasts, after 7 days the same decrease is found as that in the previous category after 14 days; after storage for 28 days, only ± 50% of the initial diacetyl remains.

2. Storage of butter at 8°C (Table 2).

Here the loss of diacetyl is more perceptible again for the contaminated butter; the lower temperature curbed the process of spoilage.

Influence of the Numbers of Klebsiella Aerogenes (Table 3).

In this table, we have tried to figure out the influence of Klebsiella Aerogenes in butter on the development of the amount of diacetyl.

In the categories which we established for the counting of yeast cells,

Table 1

1 *Résultats globaux des épreuves de détermination du diacétyle sur les beurres en cours de conservation à 13°C et 8°C (expertises organisées par l'I.R.S.I.A.) Mai 1963 à Octobre 1964*

2 Relation avec la détermination des levures	3 Nombre d'essais	4 Frais	5 Conservation à 13°C 7 jours	14 jours	21 jours	28 jours
6 *Diacétyle 0 – 0,5 mg/Kg*						
< 2000 levures/gr	38	0,28	0,32	0,28	0,28	0,25
% du diacétyle initial		100	114	100	100	89
> 2000 levures/gr	31	0,22	0,17	0,17	0,16	0,12
% du diacétyle initial		100	77	72	72	54
7 *Diacétyle 0,5 – 1 mg/Kg*						
< 2000 levures/gr	48	0,75	0,70	0,69	0,58	0,56
% du diacétyle initial		100	93	92	77	74
> 2000 levures/gr	15	0,68	0,53	0,40	0,42	0,33
% du diacétyle initial		100	77	58	61	48
8 *Diacétyle + de 1 mg/Kg*						
< 2000 levures/gr	66	1,57	1,44	1,23	1,15	1,16
% du diacétyle initial		100	91	78	73	73
> 2000 levures/gr	20	1,61	1,49	1,19	0,96	0,75
% du diacétyle initial		100	92	74	59	47
9 *Moyenne générale pour les beurres contenant*						
< 2000 levures/gr	152	0,98	0,92	0,82	0,75	0,74
% du diacétyle initial		100	93	83	76	75
> 2000 levures/gr	66	0,76	0,65	0,53	0,46	0,35
% du diacétyle initial		100	85	69	60	46
10 *Moyenne générale de l'entièreté des résultats*	218	0,92	0,83	0,73	0,66	0,63
% du diacétyle initial		100	90	79	71	68

1 -- Total results of experiments on the determination of diacetyl in butter, done during the course of storage at from 13°C to 8°C (study organized by the I.R.S.I.A.), May 1963 to October 1964.

2 -- Relationship with the determination of the number of yeasts

3 -- Number of experiments

4 -- Fresh

5 -- Storage at 13°C for 7, 14, 21, 28 days

6 -- Diacetyl 0-0.5 mg/kg
<2000 yeast cells /gr.
% of initial diacetyl
>2000 yeast cells /gr.
% of initial diacetyl

7 -- Diacetyl 0.5-1 mg/kg
<2000 yeast cells /gr.
% of initial diacetyl
>2000 yeast cells /gr.
% of initial diacetyl

8 -- Diacetyl more than 1 mg/kg
<2000 yeast cells /gr.
% of initial diacetyl
>2000 yeast cells /gr.
% of initial diacetyl

9 -- General average for butters containing:
<2000 yeast cells /gr.
% of initial diacetyl
>2000 yeast cell /gr.
% of initial diacetyl

10 -- General average for the sum of the results
% of initial diacetyl

Table 2

	1 Nombre d'essais	2 Diacétyle en mg/Kg Conservation à 8 °C Frais	14 jours	28 jours
3 < 2000 levures/gr	136	1,01	1,05	0,89
% diacétyle initial		100	104	88
> 2000 levures/gr	61	0,73	0,81	0,54
% diacétyle initial		100	108	74
Moyenne générale	197	0,93	0,97	0,78
% diacétyle initial		100	104	83

1 -- Number of experiments

2 -- Diacetyl in mg/kg, storage at 8°C, fresh, 14, 28 days

3 -- <2000 yeast cells /gr.
% of initial diacetyl
>2000 yeast cells /gr.
% of initial diacetyl
General average
% of initial diacetyl

Table 3

1 *Influence du nombre de Klebsiella aerogenes sur la perte en diacétyle dans les beurres conservés à 13 °C*

	2 Nombre d'essais	3 Frais	Diacétyle en mg/Kg Conservation à 13 °C 4 7 jours	14 jours	21 jours	28 jours
5 Levures < 2000/gr Klebsiella aerogenes < 10/gr	57	1,09	1,03	0,97	0,78	0,74
% du diacétyle initial		100	95	89	72	68
Levures < 2000/gr Klebsiella aerogenes > 10/gr	57	0,78	0,69	0,64	0,56	0,52
% du diacétyle initial		100	88	82	72	67
Levures > 2000/gr Klebsiella aerogenes < 10/gr	29	0,96	0,87	0,71	0,60	0,46
% du diacétyle initial		100	91	74	62	48
Levures > 2000/gr Klebsiella aerogenes > 10/gr	35	0,63	0,36	0,45	0,59	0,32
% du diacétyle initial		100	94	71	57	51

1 -- Influence of the number of Klebsiella aerogenes on the losses of diacetyl in butter stored at 13°C

2 -- Number of experiments

3 -- Fresh

4 -- Diacetyl in mg/kg,
Storage at 13°C
7, 14, 21, 28 days

5 -- Yeast cells <2000 /gr.
Klebsiella aerogenes <10 /gr.
% of initial diacetyl
Yeast cells <2000 /gr.
Klebsiella aerogenes >10 /gr.
% of initial diacetyl
Yeast cells >2000 /gr.
Klebsiella aerogenes <10 /gr.
% of initial diacetyl
Yeast cells >2000 /gr.
Klebsiella aerogenes >10 /gr.
% of initial diacetyl

we took the case where the number of Klebsiella was less than or greater than 10 per gram of butter, and from the examination of the numbers, it turns out that only the presence of the yeast cells is determining because in butters of similar categories, for the number of yeast cells, the same percentage of

decrease of diacetyl is obtained, the number of Klebsiella having practically no influence.

Method of Analysis for the Counting of Yeast Cells and Klebsiella Aerogenes

Counting of Yeast Cells

The counting was done with Bacto Malt agar Difco 002401 with pH 5.5, after five days of incubation at 30°C. We identified the doubtful colonies under a microscope.

Counting of Klebsiella aerogenes

Counting done on Bacto green Bile 2% Difco B7, incubation of 48 hours at 37°C.

Setting up the Samples

In a batch of 500 gr. of butter, we took out with a sterile probe a quantity of ± 10 gr., eliminating the outside portions. Melt the butter as quickly as possible at 40°C, shake it in order to completely homogenize it, the dilutions taking place in Ringer's solutions of 1/4 capacity at 40°C.

Conclusion

This study showed the interest in the production of a bacteriologically pure butter and the influence of recontamination by yeasts on the diacetyl content of butter during storage. The number of Klebsiella aerogenes did not seem to have a determing influence on the loss of diacetyl during storage at 13° to 8°C.

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1125

Influence du taux de contamination des beurres en levures et Klebsiella Aerogenes sur l'évolution de leur teneur en diacétyle en cours de conservation

Int. Dairy Congr. Proc. 3 : 317-20, (1966)

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Au cours de la fabrication des beurres de laiterie, il se produit parfois accidentellement ou par un manque de précautions lors du nettoyage et de la désinfection du matériel en contact avec la crème pasteurisée ou le beurre une réinfection par des bactéries étrangères.

Dans une usine où le matériel est parfaitement nettoyé et désinfecté et où l'on apporte beaucoup de soins à la fabrication du levain, on ne devrait théoriquement retrouver dans le beurre que les bactéries ayant résisté à la pasteurisation, et éventuellement les ferments lactiques ajoutés pour la maturation des crèmes. Dans la pratique on dénombre trés souvent outre ces bactéries, des bactéries de contamination apportés par les surfaces des appareils de laiterie, l'eau de lavage et parfois aussi, apportées par un levain mal conditionné. Pour certains auteurs ces bactéries peuvent jouer un grand rôle dans la dégradation des beurres lors de la conservation en frigo (1).

INFLUENCE DU DÉNOMBREMENT DES LEVURES SUR L'ÉVOLUTION DE LA TENEUR EN DIACÉTYLE (2,3)[1]

Les beurres ont été classés en trois catégories suivant leur teneur initiale en diacétyle (voir tableau 1).

Chacune de ces catégories étant divisée en deux parties, la première comprenant les beurres dont le dénombrement est inférieur à 2000 levures gramme et la seconde comprenant les beurres dont le dénombrement est supérieur à 2000 levures par gramme de beurre.

[1] Les travaux en question ont été effectués dans le cadre du Comité pour l'Etude Scientifique et Technique du Lait subsidié par l'IRSIA, par Mme Hôte-Baudart, et M. M. Guyot A., Permanne R. et Pirmez H.

TABLEAU 1

Résultats globaux des épreuves de détermination du diacétyle sur les beurres en cours de conservation à 13 °C et 8 °C (expertises organisées par l'I.R.S.I.A.) Mai 1963 à Octobre 1964

Relation avec la détermination des levures	*Nombre d'essais*	*Frais*	*Conservation à 13 °C* 7 jours	14 jours	21 jours	28 jours
Diacétyle 0 -0,5 mg/Kg						
< 2000 levures/gr	38	0,28	0,32	0,28	0,28	0,25
% du diacétyle initial		100	114	100	100	89
> 2000 levures/gr	31	0,22	0,17	0,17	0,16	0,12
% du diacétyle initial		100	77	72	72	54
Diacétyle 0,5--1 mg/Kg						
< 2000 levures/gr	48	0,75	0,70	0,69	0,58	0,56
% du diacétyle initial		100	93	92	77	74
> 2000 levures/gr	15	0,68	0,53	0,40	0,42	0,33
% du diacétyle initial		100	77	58	61	48
Diacétyle + de 1 mg/Kg						
< 2000 levures/gr	66	1,57	1,44	1,23	1,15	1,16
% du diacétyle initial		100	91	78	73	73
> 2000 levures/gr	20	1,61	1,49	1,19	0,96	0,75
% du diacétyle initial		100	92	74	59	47
Moyenne générale pour les beurres contenant						
< 2000 levures/gr	152	0,98	0,92	0,82	0,75	0,74
% du diacétyle initial		100	93	83	76	75
> 2000 levures/gr	66	0,76	0,65	0,53	0,46	0,35
% du diacétyle initial		100	85	69	60	46
Moyenne générale de l'entièreté des résultats	218	0,92	0,83	0,73	0,66	0,63
% du diacétyle initial		100	90	79	71	68

1. Conservation des beurres à 13°C (tableau 1)

L'examen des diverses catégories de beurre d'après leur teneur en diacétyle nous indique que la perte d'arôme est plus rapide dans les beurres infectés par les levures que dans les beurres peu infectés et on peut résumer la situation de la façon suivante:

1° Pour les beurres peu infectés en levures la diminution du diacétyle est sensible après 14 jours de conservation, après 28 jours il reste ± 75% du diacétyle initial.

2° Pour les beurres assez fortement infectés en levures, on enregistre après 7 jours la même diminution que dans la catégorie précédente après 14 jours, après une durée de conservation de 28 jours on n'enregistre plus que ± 50% du diacétyle initial.

2. Conservation des beurres à 8°C (tableau 2)

La perte en diacétyle est ici plus sensible, également pour les beurres in la température plus basse freine le processus de dégradation.

1123

TABLEAU 2

	Nombre d'essais	Diacétyle en mg/Kg Conservation à 8 °C Frais	14 jours	28 jours
< 2000 levures/gr	136	1,01	1,05	0,89
% diacétyle initial		100	104	88
> 2000 levures/gr	61	0,73	0,81	0,54
% diacétyle initial		100	108	74
Moyenne générale	197	0,93	0,97	0,78
% diacétyle initial		100	104	83

TABLEAU 3

Influence du nombre de Klebsiella aerogenes sur la perte en diacétyle dans les beurres conservés à 13 °C

	Nombre d'essais	Frais	Diacétyle en mg/Kg Conservation à 13 °C 7 jours	14 jours	21 jours	28 jours
Levures < 2000/gr Klebsiella aerogenes < 10/gr	57	1,09	1,03	0,97	0,78	0,74
% du diacétyle initial		100	95	89	72	68
Levures < 2000/gr Klebsiella aerogenes > 10/gr	57	0,78	0,69	0,64	0,56	0,52
% du diacétyle initial		100	88	82	72	67
Levures > 2000/gr Klebsiella aerogenes < 10/gr	29	0,96	0,87	0,71	0,60	0,46
% du diacétyle initial		100	91	74	62	48
Levures > 2000/gr Klebsiella aerogenes > 10/gr	35	0,63	0,36	0,45	0,59	0,32
% du diacétyle initial		100	94	71	57	51

INFLUENCE DE LA TENEUR EN KLEBSIELLA AEROGENES (TABLEAU 3)

Dans ce tableau nous avons essayé de dégager l'influence de la présence de Klebsiella aérogènes dans les beurres sur l'évolution de la teneur en diacétyle.

Dans les classes que nous avions établies pour les dénombrements des levures nous avons repris les cas où le nombre des Klebsiella était inférieur ou supérieur à 10 par gramme de beurre et de l'examen des chiffres il ressort que seule la présence des levures est déterminante car dans les beurres de classes similaires, pour le nombre des levures on obtient le même pourcentage de diminution du diacétyle, le nombre de Klebsiella n'ayant pratiquement aucune influence.

MÉTHODE D'ANALYSE POUR LE DÉNOMBREMENT DES LEVURES ET KLEBSIELLA AEROGENES

Dénombrement des levures

Les dénombrements ont été effectués sur Bacto Malt agar Difco 0024 01 à pH 5.5, après 5 jours d'incubation à 30°C. Nous avons identifié les colonies douteuses au microscope.

Dénombrement des Klebsiella aerogenes

Dénombrement sur Bacto green Bile 2 % Difco B 7, incubation 48 heures à 37°C.

PRÉLÈVEMENT DES ÉCHANTILLONS

Dans un paquet de 500 gr de beurre nous avons prélevé au moyen d'une sonde stérile une quantité de ± 10 gr en éliminant les portions extérieures. Fondre le beurre le plus rapidement possible à 40°C, agiter de façon à homogénéiser parfaitement le tout, les dilutions s'opèrent dans la solution de Ringer au 1/4 portée à 40 C.

CONCLUSION

Cette étude montre l'intérêt de la production d'un beurre bactériologiquement pur et l'influence de la réinfection par levures sur la teneur des beurres en diacétyle en cours de conservation. Le nombre de Klebsiella aerogenes ne semble pas avoir une influence déterminante sur la perte en diacétyle en cours de conservation à 13°C et à 8°C.

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1125

The estimation of diacetyl in the presence of other carbonyl compounds

By G. J. Lees and G. R. Jago

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(*Received 7 October 1969*) 1029

Summary. An examination of the spectra of some volatile carbonyl semicarbazones indicated that only compounds containing vicinal carbonyl groups formed semicarbazones which absorbed at 270 nm. This observation was used for the quantitative estimation of diacetyl. The presence of both acetaldehyde and acetoin did not interfere with the estimation of diacetyl by this method.

During a study of the metabolism of acetaldehyde in Group N streptococci, the formation of acetaldehyde was estimated by the Conway microdiffusion technique in which the acetaldehyde was trapped by semicarbazide in the centre well of the Conway unit. The semicarbazone formed was estimated by the method of Burbridge, Hine & Schick (1950). While this method of estimating acetaldehyde was relatively specific under the conditions used, the concurrent trapping in the semicarbazide of other volatile carbonyls, formed in the reaction mixtures, presented a potential source of error.

As Group N streptococci do produce, in addition to acetaldehyde, such volatile carbonyls as diacetyl and acetoin during the metabolism of glucose or pyruvate (Wilssens & Defloor, 1963; Keenan & Bills, 1968; Speckman & Collins, 1968; G. J. Lees & G. R. Jago, unpublished), a study was made of the spectral properties of a number of semicarbazones including those of the above carbonyl compounds. The results indicated that it is possible to estimate diacetyl free of interference by acetaldehyde and acetoin. A correction factor can be applied to adjust for any interference by diacetyl in the estimation of acetaldehyde by the same method.

MATERIALS AND METHODS

Carbonyl semicarbazones were formed by the addition of 1 ml of a solution of the carbonyl compound (approximately 1 mM) to 1 ml of 6·7 mM semicarbazide. The tubes were sealed with parafilm and allowed to stand for 90 min before the addition of distilled water to make a total volume of 10 ml. Absorption spectra were determined against a semicarbazide blank, prepared as above without the addition of carbonyl compounds, and recorded on a Zeiss spectrophotometer, model PMQII or on a Beckman recording spectrophotometer, model DK2.

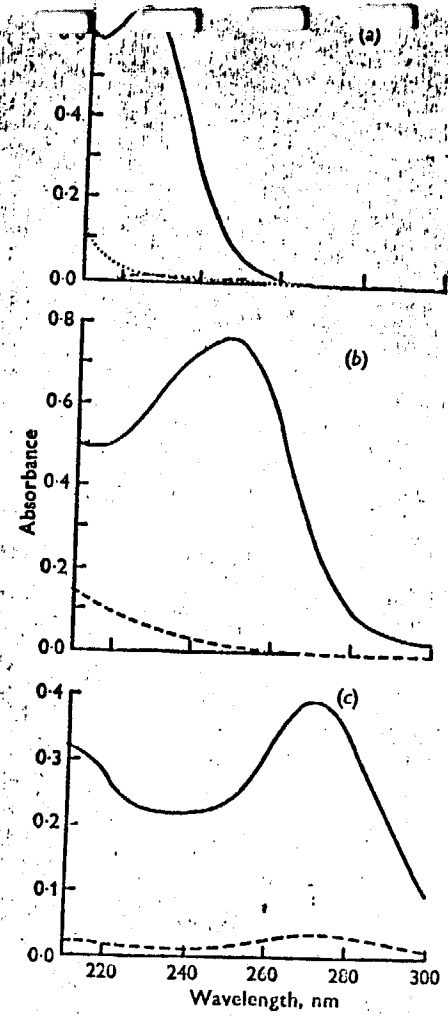

Fig. 1

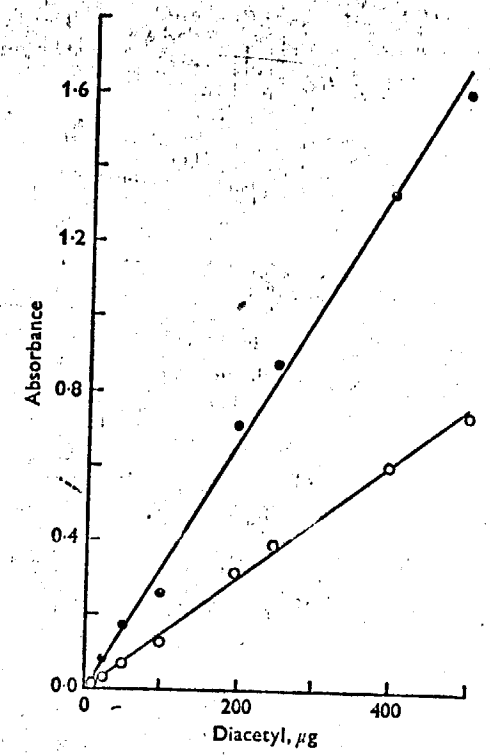

Fig. 2

Fig. 1. Absorption spectra of carbonyl semicarbazones. (*a*), Acetaldehyde; (*b*), pyruvate; (*c*), diacetyl. ——, Semicarbazone; ----, carbonyl compound; ....., semicarbazide (determined against distilled water).

Fig. 2. Standard curve for the estimation of diacetyl. ●, Absorbance at 270 nm; ○, absorbance at 224 nm.

Results and Discussion

The results of the present investigation showed that carbonyl compounds could be classified into 3 groups according to the spectral properties of their semicarbazones. The spectra representative of each group are shown in Fig. 1*a*, *b*, *c*. The carbonyl compounds whose semicarbazones had similar spectral properties are grouped below.

Group 1. Acetaldehyde, n-butyraldehyde, phenylacetaldehyde, glutaraldehyde, acetylacetone, acetone, 2-butanone, acetoin.

1158

Group 2. Pyruvate, α-ketobutyrate, oxaloacetate, glyoxylate.

Group 3. Diacetyl glyoxal, methyl glyoxal.

A list of the wavelengths of the absorption maxima and the difference absorption maxima (where the carbonyl compounds themselves absorbed at these wavelengths) is given in Table 1. It can be seen from this Table that the absorption maximum of a carbonyl semicarbazone depends on the class to which the carbonyl compound belongs. For example, acetaldehyde semicarbazone with one carbonyl group does not absorb at 272 nm, the absorption maximum of diacetyl semicarbazone which has 2 carbonyl groups in the vicinal position. A separation of 2 carbonyl groups by one carbon atom, as in acetylacetone, is sufficient to cause a loss in the absorbance at 270 nm of the semicarbazone.

Table 1. *Spectral properties of carbonyl semicarbazones*

Carbonyl semicarbazone	Absorption peaks, λ max., nm
Monocarbonyl compounds	
Acetaldehyde	224
n-Butyraldehyde	225
Phenylacetaldehyde	230
Acetone	223
Butanone	224
Hydroxycarbonyl compounds	
Acetoin	224
Ketoacids and derivatives	
Pyruvate (sodium salt)	248
α-Ketobutyrate (sodium salt)	236 (245)*
Oxaloacetate (sodium salt)	247
Glyoxylate (sodium salt)	255
Dicarbonyl compounds	
Diacetyl	272
Methyl glyoxal	272
Glyoxal	281
Acetylacetone	228
Glutaraldehyde	227

* λ max of difference spectrum obtained from spectrum of carbonyl semicarbazone minus spectrum of carbonyl compound, at the same concentrations.

As shown in Fig. 2, a linear relationship between the absorbance at either 224 nm or 270 nm and the amount of diacetyl semicarbazone was obtained for diacetyl in concentrations up to 500 μg/ml in the test sample. The ratio of the absorbance at 270 nm to the absorbance at 224 nm did not fall below a value of 2 for any concentration of diacetyl tested. This observation is useful as a means of indicating the absence of acetaldehyde or acetoin in a test sample. Any interference by diacetyl, when estimating acetaldehyde, can be corrected by subtracting half the absorbance at 270 nm from the absorbance at 224 nm.

The use of the Conway microdiffusion technique as described by Burbridge *et al.* (1950) with semicarbazide in the centre well of the Conway units was found to be a convenient method for separating volatile carbonyl compounds from other substances present in the reaction mixtures.

This work was supported by grants from the Australian Dairy Industry Research Fund administered by the Australian Dairy Produce Board. One of us (G. J. L.) acknowledges the receipt of an Australian Dairy Produce Board Senior Studentship and a University of Melbourne Research Scholarship.

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Printed in Great Britain

158

1184

ORIGINAL ARTICLE

(1184)

DIACETYL PRODUCTION IN CREAM BY STREPTOCOCCUS DIACETILACTIS

By LORNA G. LIGHTBODY, Queensland Department of Agriculture and Stock, Brisbane

Australian J. Dairy Technol. 17: 36-40, (1962)

Summary

Cultures of *Streptococcus diacetilactis* produced diacetyl rapidly in cream at 13° to 18°C during the first 16 to 24 hours, but reduced most of it within the next few hours. If incubation was continued until the second day the diacetyl concentration slowly increased again.

The peak of diacetyl production under a variety of conditions was found to occur at a pH of about 5.6. Increasing the temperature of incubation from 13° to 18°C or increasing the percentage inoculum increased the rate of culture growth but did not alter the maximum amount of diacetyl. Variation in the initial pH of the cream within the range 6.0 to 7.4 had practically no effect on the maximum diacetyl level or on the time at which it was reached.

Addition of sodium citrate to the cream markedly increased the maximum amount of diacetyl produced, but did not prevent its subsequent rapid reduction. At the lower pH values cream with added citrate contained only slightly more diacetyl than cream without added citrate.

Introduction

Although a considerable number of investigations have been made concerning the production of diacetyl by butter cultures (Hammer and Babel, 1943, and Villarta, 1958a), relatively few workers have considered the rate of diacetyl production in milk or cream during the first 24 hours. It has been found that there is no relationship between the diacetyl content of the starter and that of the cream before churning (Wiley, Cox and Whitehead, 1939a) or that of the final butter (Prill and Hammer 1939). The greater emphasis should therefore be placed on flavour production in cream rather than in the bulk starter cultures.

Diacetyl production by mixed cultures during growth has been studied by Wiley, Cox and Whitehead (1939b) who found that with these cultures there was a rapid production of diacetyl during the logarithmic growth phase followed by a rapid destruction in the next 12 hours. Cox (1945) showed that a similar rapid production of diacetyl followed by its destruction occurred when *Leuconostoc* strains were inoculated into milk at pH levels between 4.4 and 5.5.

Hoecker and Hammer (1941) suggested the use of pure cultures of *Streptococcus diacetilactis* in butter manufacture. They determined the amounts of diacetyl formed in cream after 16 hours at about 40°F. Villarta (1958b) compared diacetyl production of strains of *S. diacetilactis* and of mixed cultures in skim milk, and determined the amounts produced after 12, 24, 48 and 72 hours at 30°C. This worker did not find any evidence of a sharp peak as had been found by Wiley *et al* (1939b) for mixed cultures. However, as readings were not taken very frequently and the temperature of incubation was high, such an effect could have occurred and not been detected.

The present experiments were designed to obtain further information on the production of diacetyl by *S. diacetilactis* at times and temperatures of incubation similar to those used in the culturing of cream for butter manufacture.

Experimental Methods

(a) Cultures

Cultures used were strains of *Streptococcus diacetilactis* DRC1, DRC2, and DRC4. Fresh 24-hour cultures in skim milk were used for inoculating the cream.

(b) Cream treatment

The cream used in the experiments was low-acid, choice quality cream supplied to a butter factory. It was pasteurised in the laboratory by batch heating to 80°C in a boiling water bath, followed by cooling in cold water. The fat content was reduced to 35 per cent before pasteurization by the addition of sterile skim milk, and the pH was adjusted to the required level by addition of sodium hydroxide solution.

The cream was bulked after pasteurization before distribution to the required number of Erlenmeyer flasks. Two flasks of cream were used

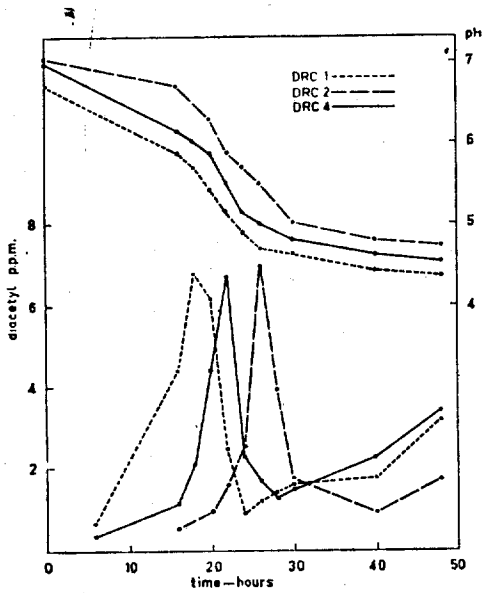

FIG. 1

Diacetyl production and change of pH in cultures of S.diacetilactis in cream at 15·5°C

for each treatment, and these were inoculated at different times so that analyses of the cream could be made after suitable periods of incubation. The cream was adjusted to the temperature of incubation before inoculation. Examinations were made after 6 hours, at 2-hourly intervals from 16 to 30 hours, and after 40 and 48 hours.

(c) Analytical methods

Determinations of pH, titratable acidity, diacetyl and bacterial count were made as in previous experiments (Lightbody 1961).

Results

(a) Growth and diacetyl production at 15.5°C

Cream was inoculated with cultures DRC1, DRC2 and DRC4 at the rate of 1 per cent and incubated at 15.5°C. The results of estimations at intervals for diacetyl and pH are shown in Fig. 1.

The most noticeable feature of all graphs was the peak in diacetyl concentration. There was a rapid increase in diacetyl to about 5-7 p.p.m. followed by a sudden reduction as the pH became lower. On continued incubation at pH below 5, a further increase in diacetyl was observed. The graphs for the 3 curves were similar, although the peaks occurred at slightly different times, related to the activities of the cultures at the time of testing. The curves for pH show these differences in the rates of acid production. The relative rates of acid production of the 3 cultures varied somewhat from time to time.

As the amount of diacetyl in the cream changed rapidly and analyses were made only at 2-hourly intervals, estimations would not always have been obtained of the maximum amount of diacetyl produced. However, the results of this and other similar experiments suggested that there were no practical differences between the 3 cultures as regards diacetyl production.

(b) Effect of temperature on growth and diacetyl production

One per cent of culture DRC2 was inoculated into 3 lots of one cream sample and incubated at 7-8°C, 13°C and 18°C. Estimations for diacetyl, pH, titratable acidity and bacterial count were made at intervals. The results are shown in Table 1.

At 7-8°C growth was very slow and only a small amount of diacetyl was produced in 72 hours. The pH at this time was still high, and little bacterial growth had occurred. At 13°C growth was fairly slow, but a pH of 5.45 and a high diacetyl level was reached after 30 hours incubation. After 40 hours the diacetyl formed had been largely reduced. At 18°C growth and diacetyl production was much faster and a peak value was reached after 18 hours.

(c) Effect of percentage of inoculum on diacetyl production

Culture DRC2 was inoculated into cream at the rate of 2, 5 and 10 per cent and incubated at 13°C. Approximately the same maximum amounts of diacetyl (5-6 p.p.m.) were produced in the creams inoculated with 2, 5 or 10 per cent culture, but after different times of incubation. Peak levels were reached after about 26 hours with 2 per cent inoculum, 20 hours with 5 per cent inoculum and 18 hours with 10 per cent inoculum.

In an experiment in which 1, 2 and 5 per cent DRC2 were inoculated into cream at 15.5°C, the 1 per cent inoculum gave a peak of 3.4 p.p.m. diacetyl at 18 hours, 2% a peak of 3.5 p.p.m. at 16 hours and 5% a maximum of 1.65 p.p.m. with little change from 4 to 28 hours.

(d) Effect of pH of cream after neutralization on growth and diacetyl production

A sample of cream was divided into 3 lots which were neutralized to pH values of 7.4, 6.7 and 6.0. Each lot was inoculated with 1 per cent DRC2 and incubated at 15.5°C. The results of analyses of these creams are shown in Table 2.

The differences in pH after 20 hours were small. At the higher initial pH values growth of organisms was faster, as can be seen by the rate of acid production in the first 20 hours. Diacetyl production was also faster initially, but the peaks were reached at approximately the same time in the 3 creams: after 20 hours for the creams with initial pH values of 6.0 and 6.7, and between 20 and 22 hours for the cream of pH 7.4. The maximum amounts of diacetyl produced in the 3 creams were very similar, as were the subsequent reductions and the later increases at low pH.

(e) Effect of addition of sodium citrate on diacetyl production

A comparison was made of growth and diacetyl production in cream to which sodium citrate had been added. A 25 per cent solution of trisodium citrate was added to the cream to give

1184

TABLE 1.

Effect of Temperature on Growth and Diacetyl Production of DRC2.

Time —hours	7-8°C				13°C				18°C			
	pH	Acidity % lactic acid	Diacetyl p.p.m.	Bact. Count —log.	pH	Acidity % lactic acid	Diacetyl p.p.m.	Bact. Count —log.	pH	Acidity % lactic acid	Diacetyl p.p.m.	Bact. Count —log.
0	6.8	0.08	0.1	6.78	7.1	0.09		6.76	7.1	0.09		6.67
6					7.0	0.10	0.2	6.99	6.9	0.12	0.3	7.24
8	6.7	0.09	0.2	7.03								
16					6.7	0.13	0.4	7.24	5.8	0.31	2.85	7.88
18					6.6	0.16	0.45		5.45	0.35	5.5	
20					6.55	0.17	0.45	7.31	5.0	0.47	1.25	8.06
24	6.75	0.09	0.2	6.82	6.2	0.21	0.65	7.64	4.85	0.48	0.8	8.29
26					5.95	0.26	1.65		4.6	0.55	0.5	
28					5.8	0.31	4.2	7.90				
30					5.45	0.33	4.8		4.6	0.61	0.4	
32	6.65	0.11	0.3	6.88								
40					4.8	0.51	1.05		4.6	0.60	0.6	
48	6.6	0.11	0.35	7.37	4.7	0.51	1.75		4.4	0.60	0.8	
56	6.6	0.11	0.5	7.38								
72	6.5	0.12	1.55	7.42								

citrate concentrations of 0.02, 0.1 and 0.25 per cent.

In all cases addition of sodium citrate increased the maximum amount of diacetyl produced. A maximum level of 13.4 p.p.m. was recorded in cream containing 0.25 per cent sodium citrate, and amounts of up to 16 p.p.m. have been obtained in other creams with this amount of added citrate. However, even these large amounts of diacetyl were rapidly reduced as incubation continued and when the pH of the cream was 5.0 or less the amount of diacetyl was only slightly greater in the citrated cream than in the cream without added citrate. Some detailed results are given in Table 3.

The addition of 0.25 per cent citrate increased slightly the pH of the cream and its buffering capacity. Acid production was similar in cream with and without added citrate, but the amount of diacetyl at each pH level was greater when citrate was added. The peak of diacetyl production occurred a few hours later in the citrated cream than in the cream without citrate.

(f) Relationship between diacetyl production, pH and bacterial count

The maximum amount of diacetyl produced by the same culture under similar conditions has varied considerably from day to day. For example, the maximum amount of diacetyl in cream inoculated with 1 per cent DRC2 and incubated at 15.5°C has varied from 3.5 to 9 p.p.m. and the time taken to reach the maximum under the same conditions has varied from 18 to 26 hours. On

TABLE 2

Effect of pH of Cream at Time of Inoculation on Diacetyl Production by DRC2.

Time —hours	7.4			6.7			6.0		
	pH	Acidity % lactic acid	Diacetyl p.p.m.	pH	Acidity % lactic acid	Diacetyl p.p.m.	pH	Acidity % lactic acid	Diacetyl p.p.m.
0	7.4	.055	0.05	6.7	.095	0.05	6.0	.20	0.05
6	6.85	.075	0.2	6.5	.11	1.0	5.75	.225	1.2
16	6.1	.17	1.2	5.9	.20	2.5	5.6	.28	3.95
18	5.85	.21	3.3	5.7	.24	5.2	5.6	.285	5.30
19	5.75	.225	4.8	5.6	.245	6.8	5.5	.28	7.0
20	5.6	.27	6.8	5.45	.30	7.9	5.5	.295	7.8
22	5.35	.30	6.4	5.2	.33	4.8	5.2	.33	6.4
24	5.3	.31	4.0	5.1	.34	4.6	5.2	.34	6.2
26	5.2	.34	—	5.0	.35	4.0	5.0	.36	4.4
28	4.85	.36	2.4	4.85	.36	2.65	4.8	.37	3.85
30	4.8	.36	—	4.7	.41	2.3	4.7	.37	2.35
40	4.6	.36	2.6	4.6	.39	2.8	4.7	.37	2.8
46	4.5	.38	4.0	4.5	.40	4.3	4.6	.37	4.0

TABLE 3

Effect of Added Citrate on Diacetyl Production of DRC2

Amount of added sodium citrate.

Time hours	Nil I	Nil II	Nil III	Nil IV	0.25% I	0.25% II	0.25% III	0.25% IV
0	6.95	0.10		6.75	7.1	0.10		6.70
6	6.85	0.15	0.4	6.86	7.1	0.13	0.5	6.91
16	6.1	0.25	1.75	7.76	6.5	0.21	1.45	7.89
18	5.9	0.28	2.7		6.25	0.25	2.4	
20	5.6	0.34	4.4	7.95	5.95	0.34	4.8	8.19
22	5.3	0.41	2.9		5.85	0.43	11.2	
24	5.0	0.48	1.55	8.10	5.45	0.48	13.4	8.32
26	4.9	0.54	1.15		5.25	0.54	7.8	
28	4.8	0.57	1.0	7.98	5.1	0.59	2.9	8.40
30	4.8	0.57	1.25		5.05	0.59	2.2	
40	4.55	0.58	1.9		4.75	0.58	2.7	
48	4.6	0.57	2.4		4.75	0.59	3.55	

I—pH
II—Acidity % lactic acid.
III—Diacetyl p.p.m.
IV—Total bacterial count—log.

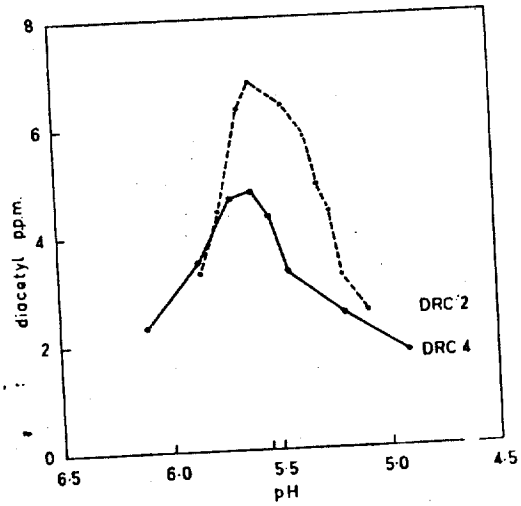

FIG. 2

Relationship between diacetyl concentration and pH for cultures DRC 2 and DRC 4

the other hand in all experiments, including those in which citrate was added to the cream, the peak diacetyl level was recorded at pH values between 5.3 and 5.7. (See Table 4). Similar amounts of diacetyl were present at each pH level in any one cream. The logarithm of the bacterial count at the time the peak diacetyl level was reached varied between 7.9 and 8.4, close to the maximum.

Because analyses of the cream were made only at 2-hourly intervals the values recorded for the maximum amount of diacetyl and for the pH and bacterial counts at the time the maximum was reached are only approximate. In order to determine more accurately diacetyl production at the time of the peak, a few experiments were made in which readings were taken at 20-30 minute intervals when the pH of the cream was between 6.0 and 5.0. The results of 2 such experiments are shown in Fig. 2, in which diacetyl level is plotted against the pH. For the two cultures, DRC2 and DRC4, the greatest amount of diacetyl was recorded at pH 5.6.

TABLE 4.

Relationship between maximum diacetyl level, pH and total bacterial count.

Inoculum %	Temperature °C.	Initial pH	Added citrate %	Approx. max. diacetyl —p.p.m.	Time of peak —hr.	pH at peak	Bacterial count at peak —log.
1	13	7.1	—	5	30	5.45	8.0
1	18	7.1	—	5.5	18	5.45	8.0
2	13	7.05	—	6	26	5.45	8.2
5	13	6.9	—	5.5	20	5.6	8.2
10	13	6.75	—	5.5	18	5.45	8.2
1	15.5	6.9	—	3.5	18	5.4	7.9
2	15.5	6.9	—	3.5	16	5.3	8.0
1	15	7.4	—	7	21	5.5	
1	15	6.7	—	8	20	5.5	
1	15	6.0	—	8	20	5.5	
1	15.5	6.5	—	9	19	5.7	8.0
1	15.5	6.5	0.02	10	19	5.6	8.0
1	15.5	6.6	0.1	13	19	5.6	8.1
1	15.5	6.95	—	4.5	20	5.6	8.0
1	15.5	7.1	0.25	14	23	5.5	8.3
1	15.5	6.7	—	7	19	5.6	8.4
1	15.5	7.0	—	7	22	5.5	8.3
1	15.5	7.2	—	7	26	5.5	8.1

1184

Discussion

The effect of cultures of *Streptococcus diacetilactis* in producing diacetyl rapidly in cream and then partly destroying it was similar to that reported by Wiley, Cox and Whitehead (1939b) for mixed starter cultures. Continued incubation of the inoculated cream at pH values below 5 resulted in a further increase in diacetyl, and in some tests relatively large amounts were present after 48 hours.

The characteristic curves for production of diacetyl by cultures of *S. diacetilactis* must be kept in mind when comparing cultures or noting the effect of different conditions on diacetyl production. Differences in diacetyl concentration after a fixed period of incubation may reflect only differences in the rate of acid production and may not give any indication of diacetyl production unless the pH at the time of testing is also taken into account.

Several workers have reported that the addition of citric acid to mixed starter cultures increased the amount of diacetyl in the culture (Michaelian, Hoecker and Hammer, 1938, Prill and Hammer 1939b). This effect was partly due to the citrate and not only to the increased acidity. Templeton and Sommer (1935) reported that addition of citric acid or sodium citrate to cream or to the lactic culture tended to produce butter of more desirable flavour. Swartling (1951) showed that *S. diacetilactis* also produced acetylmethylcarbinol and diacetyl from citrates, and Krishnaswamy and Babel (1951) found that cultures of citric-acid-fermenting streptococci produced slightly more diacetyl in milk when citric acid had been added.

In the present experiments, although the addition of 0.25 per cent sodium citrate resulted in a marked increase in the maximum amount of diacetyl produced, the subsequent rate of destruction was very rapid. At pH levels above 5.5 there was more diacetyl present in the cream with added citrate, but at the lower pH levels the differences were only slight. This is in line with the conclusion reached by Krishnaswamy and Babel (1951) that increases in diacetyl production in clotted milk cultures due to the addition of citrate were not as conspicuous with the single-strain cultures as with mixed cultures containing *Leuconostoc* strains. It is doubtful whether there would be any practical advantage in adding citrate to cultures or to cream when strains of *S. diacetilactis* are used, because the increased diacetyl due to the presence of the added citrate is appreciable only near the peak of diacetyl production. The amounts normally present at this time in cream are sufficient to provide desirable flavour in the resultant butter.

Acknowledgements

Thanks are due to the manager and staff of Southern Queensland Dairy Co. Ltd., Kingston, for assistance with cream samples and to Mr. D. K. Shackel for technical assistance. This investigation formed part of a project which was aided by funds made available under the Research and Promotion Grant of the Australian Dairy Produce Board.

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CORRIGENDUM

OFFICE-BEARERS, 1961-1962

The name of the Hon. Secretary of the Queensland Division, Mr. P. McCallum, Department of Agriculture and Stock, Brisbane, should be added to the list of office bearers in Vol. 16, No. 4 of this Journal.

5463

1188

FLAVOR CHEMISTRY OF BUTTER CULTURE

(Order No. 66-7116)

Robert Clarence Lindsay, Ph.D.
Oregon State University, 1965

Major Professor: E. A. Day

Numerous investigations have been made on the contribution of butter cultures to the flavor of cultured cream butter but production of uniform cultured cream butter has not been possible in industry. Therefore, it was desirable to investigate in detail the qualitative and quantitative chemistry of the flavor of high quality butter cultures, and to examine more closely some of the aspects of flavor production by butter culture organisms.

Volatile flavor components of high quality butter culture and control heated milk were isolated from intact samples by means of a specially designed low-temperature, reduced-pressure steam distillation apparatus. Most of the flavor compounds present in the resulting distillate fractions were tentatively identified by gas chromatographic relative retention time data. Flavor concentrates obtained by ethyl ether extractions of aqueous distillates were also separated by temperature-programmed, capillary column gas chromatography, and the effluent from the capillary column was analyzed by a fast-scan mass spectrometer. Many of the flavor compounds in the flavor concentrates were positively identified by correlation of mass spectral and gas chromatographic data. In addition, supporting evidence for the identification of some flavor components was obtained through the use of qualitative functional group reagents, derivatives and head-space gas chromatography.

Compounds that were positively identified in butter culture include ethanol, acetone, ethyl formate, methyl acetate, acetaldehyde, diacetyl, ethyl acetate, dimethyl sulfide, butanone, 2-butanol, methyl butyrate, ethyl butyrate, methane, methyl chloride, carbon dioxide and methanol; also included were 2-pentanone, 2-heptanone, acetoin, formic acid, acetic acid, lactic acid, 2-furfural, 2-furfurol, methyl hexanoate, ethyl hexanoate, 2-nonanone, 2-undecanone, methyl octanoate and ethyl octanoate. Compounds that were tentatively identified in butter culture include hydrogen sulfide, methyl mercaptan, n-butanal, n-butanol, 2-hexanone, n-pentanal, n-pentanol, 2-mercaptoethanol, n-butyl formate, n-butyl acetate, 2-methylbutanal, 3-methylbutanal, methylpropanal, methyl heptanoate, n-octanal, 2-tridecanone, methyl benzoate, methyl nonanoate, ethyl nonanoate, ethyl decanoate, methyl dodecanoate, ethyl dodecanoate, delta-octalactone and delta-decalactone.

Compounds that were positively identified in control heated milk include acetaldehyde, ethyl formate, ethyl acetate, 2-heptanone, 2-furfural, 2-furfurol, 2-nonanone, 2-undecanone, ethyl octanoate and methyl decanoate. Compounds that were tentatively identified in control heated milk include dimethyl sulfide, hydrogen sulfide, ammonia, methyl mercaptan, methyl acetate, acetone, methanol, butanone, butanal, n-butanol, methyl butyrate, ethyl butyrate, 2-pentanone, 2-hexanone, 2-mercaptoethanol, 2-furfuryl acetate, ethyl hexanoate, methyl heptanoate, 2-tridecanone, ethyl decanoate, ethyl dodecanoate, delta-octalactone and delta-decalactone. The data indicated that the qualitative flavor composition of control heated milk and butter culture were very similar. Diacetyl, ethanol, 2-butanol and acetic acid were noted to be consistently absent in the data for the control heated milk. Other compounds were not observed in the heated milk fractions, but were also absent from some of the culture fractions. This was attributed to their presence in low concentrations, chemical instability or inefficient recovery.

A modified 3-methyl-2-benzothiazolone hydrazone spectrophotometric procedure was adapted for the determination of acetaldehyde produced in lactic starter cultures. The procedure was applied in conjunction with diacetyl measurements in studying single- and mixed-strain lactic cultures. The diacetyl to acetaldehyde ratio was found to be approximately 4:1 in desirably flavored mixed-strain butter cultures. When the ratio of the two compounds was lower than 3:1 a green flavor was observed. Acetaldehyde utilization at 21°C by Leuconostoc citrovorum 91404 was very rapid in both acidified (pH 4.5) and non-acidified (pH 6.5) milk cultures. The addition of five p.p.m. of acetaldehyde to non-acidified milk media prior to inoculation greatly enhanced growth of L. citrovorum 91404 during incubation at 21°C. Combinations of single-strain organisms demonstrated that the green flavor defect can result from excess numbers of Streptococcus lactis or Streptococcus diacetilactis in relation to the L. citrovorum population.

Diacetyl, dimethyl sulfide, acetaldehyde, acetic acid and carbon dioxide were found to be "key" compounds in natural butter culture flavor. Optimum levels of these compounds in butter culture were ascertained by chemical or flavor panel evaluations. On the basis of these determinations, a synthetic butter culture prepared with heated whole milk and delta-gluconolactone (final pH 4.65) was flavored with 2.0 p.p.m. of diacetyl, 0.5 p.p.m. of acetaldehyde, 1250 p.p.m. of acetic acid, 25.0 p.p.b. of dimethyl sulfide and a small amount of sodium bicarbonate for production of carbon dioxide. The resulting synthetic butter culture exhibited the typical aroma, flavor and body characteristics found in natural high quality butter cultures, except that the delta-gluconolactone was found to contribute an astringent flavor.

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J. Dairy Sci. 48(12): 1566-1574, 1965

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IDENTIFICATION OF VOLATILE FLAVOR COMPONENTS OF BUTTER CULTURE [1, 2]

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ABSTRACT

The volatile flavor components of high quality butter culture and control heated milk medium were isolated from intact samples by means of a specially designed low-temperature, reduced-pressure steam distillation apparatus. The volatile flavor fractions were separated by gas chromatography on packed columns containing polar and nonpolar phases and by programmed-temperature capillary-column gas chromatography. The effluent from the latter column was admitted directly to the inlet of a rapid-scan mass spectrometer enabling concurrent recordings of mass spectra for each chromatographic peak. The volatile fractions from butter culture and heated milk exhibited similar gas chromatographic patterns. By correlation of gas chromatographic and mass spectral data, most of the major components were characterized. These included aldehydes, methyl ketones, primary and secondary alcohols, methyl and ethyl esters of the normal aliphatic acids, and sulfur compounds.

Butter cultures normally contain a rather complex mixture of species and strains of lactic streptococci and aroma bacteria. The cultures are propagated in milk media containing varying amounts of milk fat and, in addition, the milk medium is subjected to some degree of heat treatment. Therefore, the aroma and flavor ultimately exhibited in finished cultures can originate from several sources.

In 1943 Hammer and Babel (9) published a compresensive review on the bacteriology of butter cultures. Their review has adequately surveyed the numerous early research efforts that have led to the identification of certain compounds classically associated with the flavor of butter cultures. It was early recognized that diacetyl was the keynote of butter culture flavor and aroma. The partially reduced analogs of diacetyl, acetoin and 2,3-butanediol, have received considerable attention, but these compounds possess no odor and probably are never present in concentrations sufficient to affect the flavor. Lactic acid, the principal end product of homofermentative metabolism, does not contribute to the aroma, but is largely responsible for the acid taste of butter cultures. Volatile acids, especially acetic acid, contribute to the mildly acid aroma and are considered important in the flavor.

In a preliminary report from this laboratory (3), a number of compounds from the aroma fraction of butter cultures were tentatively identified by gas chromatography. The purpose of this paper is to relate details of further investigations on the identification of volatile flavor compounds isolated from butter cultures.

EXPERIMENTAL PROCEDURE

Culture. A mixed-strain butter culture of commercial origin, selected for its ability to produce a good cultured cream butter flavor, was used as the source of flavor volatiles. The culture contained strains of *Streptococcus lactis*, *Streptococcus cremoris*, *Streptococcus diacetilactis*, and *Leuconostoc sp.* Both mother cultures and bulk cultures were propagated in a whole milk medium prepared by adding 0.2% of sodium citrate to raw whole milk and heating for 1 hr in a boiling water bath. Mother cultures were propagated in 500-ml long-necked culture flasks containing 300 ml of milk medium. Cultures were transferred every-other-day, using from 0.6 to 3.0% of inoculum, and the incuba-

Received for publication October 8, 1965.

[1] Technical Paper no. 2029, Oregon Agricultural Experiment Station.

[2] Partially supported by funds granted by the American Dairy Association.

[3] Submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy.

tion time at 21 C was controlled so that the titratable acidity of the cooled culture was from 0.68–0.78%. Bulk culture was prepared in 10-gal stainless steel cans, in the same manner as the other cultures except that the titratable acidity was allowed to develop to 0.90–0.96%. Bulk culture was held for 24 hr at 5 C to allow maximum flavor development prior to distillation.

Recovery of volatiles. Twenty gallons each of butter culture and control heated milk medium were steam-distilled in a specially designed low-temperature, reduced-pressure glass apparatus. A diagram of the distillation apparatus is shown in Figure 1. The total length of

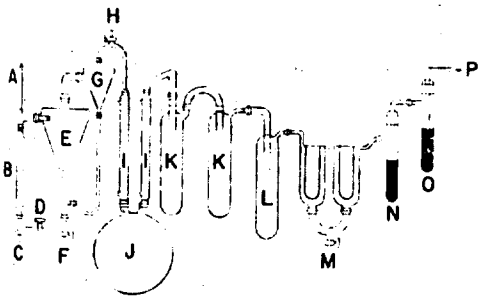

FIG. 1. Diagram of vacuum steam distillation apparatus for collection of volatile flavor components from butter culture and heated milk.

A—Thermometer
B—Heat exchanger
C—Sample inlet
D—Cycle control valve
E—Vaporization chamber
F—Sample exit
G—Foam trap
H—Vacuum to residue container
I—Tapwater-cooled condensers
J—Crushed dry ice trap
K—Ethanol-dry ice traps
L, M, N—Liquid nitrogen traps
O—Liquid nitrogen safety trap
P—To vacuum pump

the apparatus was approximately 8 ft and the maximum height about 4 ft. Referring to Figure 1, the pressure was measured near (P) and was maintained at about 3 mm of Hg during the course of the distillations. To remove the residue from the system, a 20-liter Pyrex carboy was connected by pressure tubing at positions (H) and (F), and residue removed by gravity flow through stopcock (F).

The samples were introduced slowly through stopcock (C) and were immediately flash heated to 37–40 C in the heat exchanger (B), which consisted of a condenser with steam circulated through the jacket. The fluid sample then surged into a four-liter vaporization chamber (E) where, during the course of foaming, volatile compounds were vaporized and removed. A continuous flow was maintained in the heat exchanger to prevent adsorption of solids on glass surfaces. The stopcocks at (C), (D), and (F) were adjusted to give a proper intake of new sample, a recycling of partially distilled sample, and an exit of sample residue, respectively. A one-liter chamber (G) served as a foam trap to prevent intact sample from contaminating the steam distillate.

Two tapwater-cooled condensers (I) above trap (J) condensed most of the aqueous vapor. Trap (J), a 12-liter balloon flask, was cooled by crushed dry ice and the distillate contained within it remained liquid during the distillations. Traps designated (K), with a capacity of two liters each, were cooled with ethanol dry ice slush baths. A recent modification of the system has replaced traps (K) and (L) with pear-shaped flasks of equivalent size. The conical shape of the flasks was found to reduce breakage upon thawing of the frozen distillate. Traps (L), (M), and (N) were cooled with liquid nitrogen and after distillation contained limited quantities of water. However, large amounts of carbon dioxide and volatile flavor compounds were stopped in these containers. Trap (O) served as a liquid nitrogen cooled safety-trap to prevent contamination from the vacuum pump. Traps (N) and (O) were packed with glass beads to increase the surface area in the traps.

During normal operation, approximately 20% of the volume of the intact sample was removed and collected as distillate. By measuring diacetyl levels (4) before and after distillation, and correcting for the decrease in volume, an estimate of the distillation efficiency was obtained.

For the culture distillation, the aqueous distillate from traps (J) and (K) was designated as the acidic fraction because it contained a substantial amount of acetic acid. Approximately one-half of the acidic fraction was made distinctly basic to litmus paper immediately after thawing by adding sodium carbonate, and was designated as the basic fraction. The aqueous distillate obtained from traps (L) and (M) contained insignificant amounts of volatile acids and was designated as the neutral fraction.

For the control heated milk medium distilled on the following day, the aqueous distillates from all traps were combined into one fraction.

Extraction of flavor compounds. Ethyl ether (peroxide-free) was used as the extracting solvent for the aqueous distillates. Peroxides were removed in a manner similar to that described by Valseth (15). This was accomplished by swirling three liters of ethyl ether (using a magnetic stirrer) with 600 ml of 10% H_2SO_4

containing 60 g of $FeSO_4 \cdot 7\ H_2O$ in a large Erlenmeyer flask for 1 hr. The ether was then fractionally distilled with a 3- by 100-cm column packed with glass helices.

The aqueous distillate fractions were extracted in a conventional continuous liquid-liquid extracting device designed for a solvent less dense than water. For each extraction, approximately two liters of aqueous distillate was saturated with reagent grade NaCl and the solution extracted continuously for 24 hr with 300 ml of ethyl ether.

The ethereal solutions of flavor compounds were dried over excess Na_2SO_4, then fractionally distilled to remove the bulk of the ethyl ether with a 1- by 60-cm column packed with glass helices.

Analysis of flavor concentrates. The flavor concentrates were analyzed by gas chromatography in conjunction with rapid-scan mass spectrometry. A Perkin-Elmer Model 800 gas chromatograph equipped with a hydrogen flame detector and a 300 ft by 0.01-in. id stainless steel capillary column coated with polypropylene glycol was used. Two sets of operating conditions were employed for the capillary separations. For one set of conditions, the column was operated isothermally at 73 C for 8 min, then temperature-programmed at 25 C/min to 174 C and operated at 174 C until the separation was complete. For the other set of conditions, the column was temperature-programmed at 2.5 C/min from 30 C for 8 min, then at 25 C/min to 170 C and operated at 170 C until the separation was complete. Retention data were obtained for the butter culture, heated milk, and authentic compounds under the above conditions. The same conditions were employed for analyses in conjunction with fast-scan mass spectrometry. These analyses were conducted as described by McFadden et al. (11), except that the positive ions occurring at m/e 43 (m/e is the mass-to-charge ratio) were monitored by one gate of the electron multiplier to obtain a concurrent strip-chart recording of the gas chromatogram. The other electron multiplier gate was used to scan m/e 12 to 250 in two or six sec, and the spectra recorded by an oscillograph. The spectra were observed concurrently on an oscilloscope, which provided an important means of monitoring the effluent from the capillary column.

Additional gas chromatographic data were obtained by using packed nonpolar (20% Apiezon M on 100-120 mesh Celite 545) and polar (20% diethylene glycol succinate on 100-120 mesh Celite 545) columns operated isothermally at 70 C. The columns were 1/8-in. od by 12 ft stainless steel. A β-ionization detector in a Barber-Colman Model 20 gas chromatograph was employed, and the retention data for unknowns were compared with those of authentic compounds. The ethyl ether extract fractions from the aqueous distillates were analyzed by injection of liquid samples. The highly volatile fractions obtained from the cold traps immediately after distillation of butter culture or heated milk medium were evaluated by the direct concentrated vapor injection procedure described by Libbey et al. (10).

RESULTS AND DISCUSSION

Due to large amounts of carbon dioxide produced by the butter culture organisms, subsequently collected in the liquid nitrogen cold traps, certain manipulations of the cold traps were necessary to separate the major amount of carbon dioxide from the volatile flavor compounds. This was accomplished by connecting clean traps with sample traps (immersed in liquid nitrogen) and reducing the pressure of the system to ca. 3 mm of Hg, closing the system to the vacuum pump, and replacing the liquid nitrogen bath on the sample trap with an ethanol–dry ice slush bath while placing the liquid nitrogen on the clean trap. Manipulation of temperature and pressure effectively separated the carbon dioxide after several transfers.

A typical gas chromatogram depicting the separation of the culture aroma concentrate, using the vapor injection technique of Libbey et al. (10) and a packed Apiezon M column, is shown in Figure 2. The tentative identifications of peaks shown in Figure 2, based on coincidence of relative retention times, are listed in Table 1. The gas chromatograms for the ether extracts of the aqueous fractions gave qualitatively similar patterns to those for the direct vapor injections.

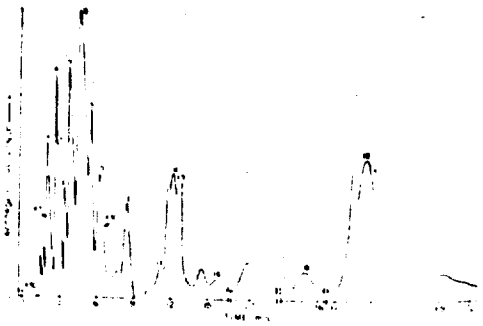

FIG. 2. Gas chromatogram of butter culture volatiles analyzed by the vapor injection techniques of Libbey et al., using an Apiezon M column at 70 C (see Table 1 for peak designations).

TABLE 1

Relative retention times of compounds tentatively identified from butter culture volatiles analyzed by vapor injection technique of Libbey et al. using an Apiezon M column; gas chromatogram shown in Figure 2

Peak No.	Compound	t_R/t_R^a Butter culture	Authentics
1	Hydrogen sulfide	0.269	0.253
2		0.312	.....
3	Acetaldehyde	0.355	0.364
4	Methyl mercaptan	0.477	0.466
5	Ethanol	0.520	0.541
6	Ethyl formate	0.624	0.634
7	Methyl acetate	0.675	0.686
8	Dimethyl sulfide	0.830	0.846
9	Diacetyl	1.000	1.000
10	Ethyl acetate	1.140	1.140
	Butanone		1.140
11	2-Mercaptoethanol	1.398	1.480
12	.....	1.548	.....
13	n-Butanol	2.054	2.120
14	2-Pentanone	2.236	2.240
15	n-Pentanal	2.656	2.510
16	Methyl butyrate	2.892	2.880
17	Ethyl butyrate	4.921	5.020
18	.....	6.365	.....
19	2-Heptanone	11.354	11.700
20	Methyl heptanoate	14.688	14.000

[a] t_R/t_R = relative retention time based on diacetyl = 1.000. Packed column, 11 ft by ⅛-in. od, 5% Apiezon M on 100-120 mesh alkali-acid-treated Chromosorb 545; column temperature: 70 C; Barber-Colman Model 20.

Figure 3 is a gas chromatogram showing the separation of volatile flavor compounds in the acidic ether extract fraction of the butter culture distillate by temperature-programmed capillary column gas chromatography. The separation and resolution of the flavor components are much greater by this technique than by isothermal-packed column gas chromatography. The identifications of peaks shown in Figure 3 are given in Table 2. Identification of components by capillary column gas chromatography are based on the coincidence of relative retention times and comparison of fast-scan mass spectral fragmentation patterns of unknowns with those for authentic compounds. In some instances, such as those for methane and methyl chloride in Table 2, the mass spectral data were sufficient for tentative or positive identifications without corroborating gas chromatographic retention data. In these cases, retention data for authentic compounds were not available. All identifications of compounds based on coincidence of gas chromatographic retention data only are considered as tentative identifications. It should be noted that the relative retention data and the mass spectral data did not allow identification of all of the components.

To illustrate the type of data obtained by fast-scan mass spectrometry of the capillary column effluents, mass spectral charts representing spectra for gas chromatographic peaks 7, 8, and 9, respectively, in Figure 3 are shown in Figure 4. Spectrum (a) in Figure 4 shows

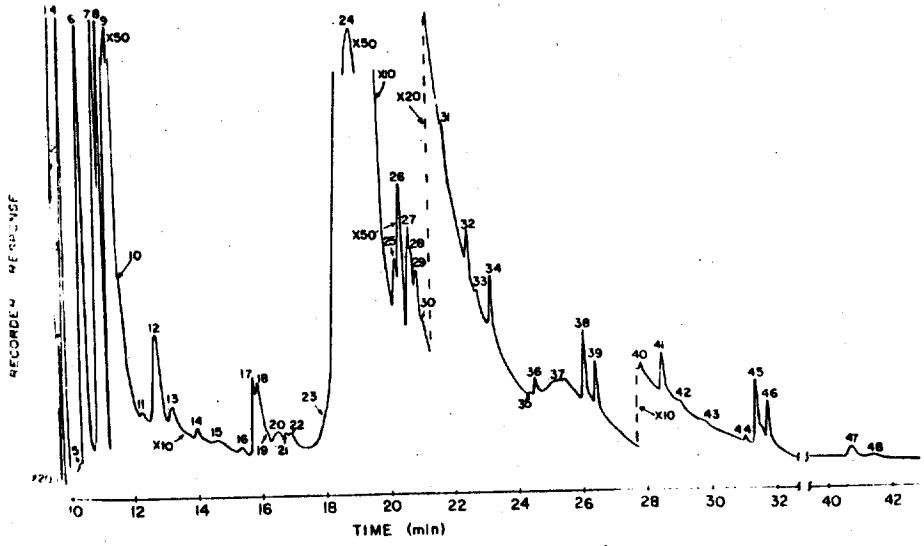

FIG. 3. Gas chromatogram of butter culture distillate acidic fraction extract using a capillary column coated with polypropylene glycol; isothermal at 73 C for 8 min, then temperature-programmed at 25 C/min to 174 C (see Table 2 for peak designations).

TABLE 2

Gas chromatographic and mass spectral identification of components of butter culture acidic extract fraction; gas chromatogram shown in Figure 3[a]

Peak no.	Identity	$t_R/t_{R'}$[b] Butter culture	$t_R/t_{R'}$ Authentics	Confirmed by packed column GLC	Mass spectral identification	Reference for M.S.
1	Methane	0.859	.....	.....	Positive	(1)
2	Methyl chloride	0.863	.....	.....	Positive	(1)
3	Acetaldehyde	0.873	0.876	yes	Positive	(1)
4	Ethyl ether	0.887	0.883	yes	Positive	(1)
5	Acetone	0.923	0.917	yes	Positive	(1)
6	Ethyl formate	0.937	0.924	yes	Positive	(1)
	Methyl acetate		0.928	yes	Positive	(2)
7	Ethanol	0.979	0.962	yes	Positive	(1)
8	Ethyl acetate	1.000	1.000	yes	Positive	(1)
9	Diacetyl	1.028	1.007	yes	Positive	(1)
10	2-Butanol	1.056	1.034	yes	Positive	(5)
	3-Methylbutanal			yes	Tentative	(7)
11	n-Pentanal	1.106	1.145	yes	.....	.....
12	2-Pentanone	1.148	1.151	yes	Positive	(13)
13	Methyl butyrate	1.190	1.200	yes	Positive	(1)
14	Butyl formate	1.254	1.214	.....	.....	.....
15	.....	1.310			.....	.....
16	n-Butanol	1.380	1.403	yes	.....	.....
17	Ethyl butyrate	1.415	1.414	yes	Positive	(1)
18	.....	1.430		.....	.....	.....
19	n-Butyl acetate	1.451	1.455	.....	.....	.....
20	.....	1.486		.....	.....	.....
21	.....	1.507		.....	.....	.....
22	.....	1.521		.....	.....	.....
23	.....	1.620		.....	.....	.....
24	Acetoin	1.711	1.680	yes	Positive	(2)
25	.....	1.824		.....	.....	.....
26	2-Heptanone	1.838	1.834	yes	Positive	(1)
27	n-Pentanol	1.859	1.841	.....	.....	.....
28	.....	1.866		.....	.....	.....
29	Methyl hexanoate	1.884	1.879	.....	Positive	(14)
30	.....	1.894		.....	.....	.....
31	.....	1.958		.....	.....	.....
32	2-Furfural	2.021	2.007	.....	Positive	(2)
33	.....	2.049		.....	.....	.....
34	Ethyl hexanoate	2.049	2.055	.....	Positive	(14)
35	.....	2.190		.....	.....	.....
36	Methyl heptanoate	2.204	2.148	.....	.....	.....
37	2-Furfurol	2.275	2.345	.....	Positive	(2)
38	2-Nonanone	2.338	2.359	.....	Positive	(13)
39	Methyl octanoate	2.373	2.376	.....	Positive	(2)
40	.....	2.500		.....	.....	.....
41	Ethyl octanoate	2.563	2.510	.....	Positive	(2)
42	Methyl nonanoate	2.613	2.607	.....	.....	.....
43	.....	2.676		.....	.....	.....
44	Ethyl nonanoate	2.789	2.766			.....
45	.....	2.820				
46	2-Undecanone	2.859	2.886	.....	Positive	(13)
47	δ-Octalactone	3.655	3.400			
48	2-Tridecanone	3.725	3.848			

[a] Compounds not identified by mass spectrometry are tentative identifications.

[b] $t_R/t_{R'}$ = Relative retention time based on ethyl acetate = 1.000.

Stainless steel capillary, 300 ft by 0.01 in. id coated with polypropylene glycol; operated at 73 C for 8 min, then at 25 C/min to 174 C.

an intense peak at m/e 31; this fragment is ($CH_3OH_2^+$), and is quite characteristic of low molecular weight primary alcohols. The base peak for ethanol is m/e 31 and its parent ion is m/e 46. Comparison of spectrum (a) with the spectrum for ethanol in the API tables (1) shows that the ion fragments at m/e 45 ($C_2H_5O^+$), 43 ($C_2H_3O^+$), and 42 ($C_2H_2O^+$) are present in both spectra and are present at approximately the same relative intensity. The identification can be verified by comparing the relative retention time for Peak 7 in Figure 3

with the retention time for authentic ethanol in Table 2.

Spectrum (b) in Figure 4 shows the appearance of heavier ion fragments while certain characteristic peaks for ethanol are still apparent. The parent ion of the new component is m/e 88, and its base peak is m/e 43; the ion at m/e 73 indicates an oxygenated compound

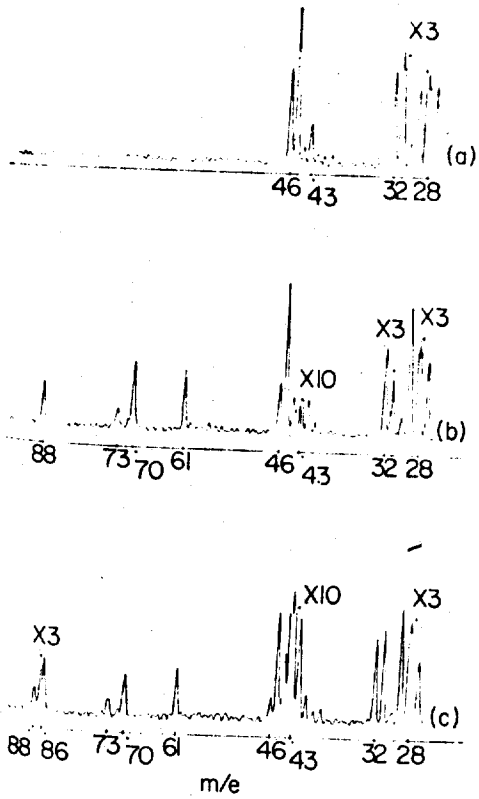

FIG. 4. Mass spectral charts for chromatographic fractions shown in Figure 3: (a) corresponds to chromatographic Peak 7; (b) = Peak 8; (c) = Peak 9.

and, furthermore, the ion at m/e 61 is quite characteristic of acetate esters (14). That the unknown (b of Figure 4) is ethyl acetate is evident by comparing the spectrum (b) with the spectrum of authentic ethyl acetate in the API tables (1). The gas chromatographic data (Table 2) confirm this identification. Spectrum (c) of Figure 4 shows the presence of a new parent ion at m/e 86; it is evident that the base peak is m/e 43 and that this peak is greatly increased in magnitude over spectrum (b). The parent ion for diacetyl is m/e 86 and the base peak is m/e 43 (1). The gas chromatographic relative retention time of Peak 9 (Figure 3) agrees with that for authentic diacetyl. The series of mass spectral charts in Figure 4 show the manner by which the volatile flavor compounds of butter culture were identified using capillary gas chromatography combined with fast-scan mass spectrometry. It also demonstrates that complete separation of compounds is not absolutely necessary for identification of relatively simple organic compounds. This is best illustrated in spectrum (c) of Figure 4, where ion fragments originating from ethanol, ethyl acetate, and diacetyl are all observed.

The absence of large amounts of acetic acid and carbon dioxide in the heated milk control made it possible to combine all of the aqueous distillate collected in the cold traps into one fraction. Both packed column and capillary column gas chromatographic analyses showed that the heated distillate contained most of the compounds found in the butter culture distillates. Table 3 summarizes the compounds identified in butter culture and heated milk distillates by gas chromatographic and mass spectral methods. Since many of the compounds are present in both distillates, the data suggest that at least part of normal butter culture flavor can be attributed to compounds present in the heated milk medium. Some compounds, such as diacetyl, 2-butanol acetoin, and acetic acid were observed only in the culture distillates. Although the qualitative data for heated milk and butter culture are similar in many respects, quantitative gas chromatographic differences were observed for many of the compounds found in the two distillates. In some cases differences in relative concentrations of certain compounds appeared to be significant. However, caution must be exercised in interpreting such data, because the distillation apparatus used for the isolation of flavor compounds was found to give 45% diacetyl removal when 20% by volume of culture was distilled.

In comparing gas chromatographic data obtained from culture distillate with that from heated milk distillate, it was observed that 2-pentanone and 2-heptanone were present in much greater amounts in the culture distillate. Both the heated milk and the culture were prepared from whole milk. The origin of odd-carbon methyl ketones in milk fat has been found to be a result of decarboxylation of β-ketoacids hydrolyzed from milk glycerides (12). Although spores of *Penicillium roqueforti* have been found to convert octanoic acid to β-keto-octanoic acid (6), butter culture organisms apparently do not act similarly, because they do

TABLE 2

Gas chromatographic and mass spectral identification of components of butter culture acidic extract fraction; gas chromatogram shown in Figure 3[a]

Peak no.	Identity	t_R/t_R[b] Butter culture	t_R/t_R Authentics	Confirmed by packed column GLC	Mass spectral identification	Reference for M. S.
1	Methane	0.859			Positive	(1)
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3	Acetaldehyde	0.873	0.876	yes	Positive	(1)
4	Ethyl ether	0.887	0.883	yes	Positive	(1)
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9	Diacetyl	1.028	1.007	yes	Positive	(1)
10	2-Butanol	1.056	1.034	yes	Positive	(5)
	3-Methylbutanal			yes	Tentative	(7)
11	n-Pentanal	1.100	1.145	yes		
12	2-Pentanone	1.148	1.151	yes	Positive	(13)
13	Methyl butyrate	1.190	1.200	yes	Positive	(1)
14	Butyl formate	1.254	1.214			
15		1.310				
16	n-Butanol	1.380	1.403	yes		
17	Ethyl butyrate	1.415	1.414	yes	Positive	(1)
18		1.430				
19	n-Butyl acetate	1.451	1.455			
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22		1.521				
23		1.620				
24	Acetoin	1.711	1.680	yes	Positive	(2)
25		1.824				
26	2-Heptanone	1.838	1.834	yes	Positive	(1)
27	n-Pentanol	1.859	1.841			
28		1.866				
29	Methyl hexanoate	1.884	1.879		Positive	(14)
30		1.894				
31		1.958				
32	2-Furfural	2.021	2.007		Positive	(2)
33		2.049				
34	Ethyl hexanoate	2.049	2.055		Positive	(14)
35		2.190				
36	Methyl heptanoate	2.204	2.148			
37	2-Furfurol	2.275	2.345		Positive	(2)
38	2-Nonanone	2.338	2.359		Positive	(13)
39	Methyl octanoate	2.373	2.376		Positive	(2)
40		2.500				
41	Ethyl octanoate	2.563	2.510		Positive	(2)
42	Methyl nonanoate	2.613	2.607			
43		2.676				
44	Ethyl nonanoate	2.789	2.766			
45		2.820				
46	2-Undecanone	2.859	2.886		Positive	(13)
47	δ-Octalactone	3.655	3.400			
48	2-Tridecanone	3.725	3.848			

[a] Compounds not identified by mass spectrometry are tentative identifications.
[b] t_R/t_R = Relative retention time based on ethyl acetate = 1.000.
[c] Stainless steel capillary, 300 ft by 0.01-in. id coated with polypropylene glycol; operated at 73 C for 8 min, then at 25 C/min to 174 C.

an intense peak at m/e 31; this fragment is (CH_3OH^+), and is quite characteristic of low molecular weight primary alcohols. The base peak for ethanol is m/e 31 and its parent ion is m/e 46. Comparison of spectrum (a) with the spectrum for ethanol in the API tables (1) shows that the ion fragments at m/e 45 ($C_2H_5O^+$), 43 ($C_2H_3O^+$), and 42 ($C_2H_2O^+$) are present in both spectra and are present at approximately the same relative intensity. The identification can be verified by comparing the relative retention time for Peak 7 in Figure 3

bonding to the keto group, where it aids the decarboxylation reaction. West (16) lists the pKa of acetoacetic acid as 3.8 (Ka = 1.6 × 10^{-4}), and it can be reasonably assumed that other short-chain β-ketoacids have similar pKa values. The heated milk control had a pH of near 6.0 and, thus, more of the β-ketoacids would exist in the anion form than in butter culture where the pH was near 4.5. Therefore, methyl-ketone production should be enhanced in the butter culture, due to the more acid nature of the medium.

Immediately after the apparatus was disassembled after distillation of the control heated milk, a very pungent ammonia odor was observed in some of the cold traps. The organic acids produced in butter cultures normally lowered the pH of the medium to a level where practically all amines would exist in the form of salts and in this state would not be expected to contribute significantly to the flavor of butter culture. Whereas the butter culture extracts exhibited an odor characteristic of commercial starter distillate, the odor of the heated milk extract did not resemble cooked milk aroma. An interesting odor, described as a hot buttered popcorn aroma was observed in the heated milk extract. Attempts to isolate and identify the compound (or compounds) responsible for the odor were unsuccessful.

The methyl and ethyl esters of the shorter-chain fatty acids observed in the distillates are to be expected, in view of the potential availability of both free acids and alcohols. To preclude the possibility of ester formation as artifacts during the isolation procedures, part of the butter culture distillate from the first cold trap (Trap J of Figure 1) was made distinctly basic to litmus paper with sodium carbonate, immediately after thawing. If esters were not already formed, the existence of the acid components as salts during the preparative procedures should have prevented esterification. On examination of the gas chromatographic data it was found that the same esters were present in the alkaline fraction extract as in the acidic fraction extract. The existence of short-chain aliphatic esters in intact butter culture has subsequently been demonstrated through use of gas chromatographic headspace analyses techniques.

The origin of some of the compounds observed in the culture distillates, such as methane and methyl chloride, is not easily explained. The extracting solvent, ethyl ether, was treated to remove peroxides and fractionally distilled. It showed only one gas chromatographic peak when handled alone in a manner similar to the culture extracts. However, the possibility of peroxide formation and reaction after extraction and concentration of flavor materials cannot be ruled out.

In summary, most of the compounds either positively or tentatively identified in the butter culture and heated milk distillates have been previously observed in the volatile flavor fractions isolated from dairy products. It appears that the major flavor compounds produced by butter culture organisms have been isolated and identified. Results of investigations on the formulation of synthetic butter culture flavor concentrates based on this work will appear in a subsequent communication.

ACKNOWLEDGMENT

The authors gratefully acknowledge the invaluable assistance of W. H. McFadden, Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, California, in obtaining and interpreting the mass spectra.

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1191

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J. Dairy Sci. 50(1) 25-31, (1967)

1192

Preparation and Evaluation of Butter Culture Flavor Concentrates[1,2]

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Abstract

Synthetic butter culture concentrates, based on the analyses of natural high-quality products, were prepared for butter, Cottage cheese, sour cream, and buttermilk. Flavor compounds included in the formulations were diacetyl, acetaldehyde, dimethyl sulfide, acetic acid, and lactic acid. Products flavored with the culture flavor concentrates were found to possess a culture flavor very similar to that produced by natural butter cultures. A student preference panel showed equal preference for high-quality naturally cultured products and artificially flavored products.

The development of sufficient amounts of desirable culture flavor and aroma is one of the major problems facing the cultured dairy product industry today. In recent years a number of methods for providing enhanced culture flavor in Cottage cheese, sour cream, cultured butter, and cultured buttermilk have been used.

The general approach involves the use of special cultures and culturing techniques. A degree of success has been achieved by incorporating high diacetyl-producing strains of *Leuconostoc citrovorum* or *Streptococcus diacetilactis* into general-purpose mixed-strain cultures specifically formulated for use in the manufacture of cultured products. However, experience with such mixed-strain cultures has shown that it is difficult to realize consistency of flavor production under routine plant production programs. Alternate natural culturing methods involve the use of pure cultures of *L. citrovorum* (12, 19), or *S. diacetilactis* (18). These methods improve flavor, but the attainment of uniform, desirable culture flavor is not always easily achieved.

Starter distillates have been used with some degree of success for both producing and enhancing culture flavor. However, it is well accepted that they yield a harsh diacetyl flavor and do not duplicate the balanced flavor obtained through use of successful natural culturing procedures. Numerous studies have been conducted on the use of diacetyl as a flavoring agent in cultured dairy products (1, 2, 22). The general conclusion from these studies has been that diacetyl alone imparts a definite culture flavor, but at the same time gives an undesirable harsh flavor.

In previously reported work from this laboratory on the identification of volatile flavor fraction from butter cultures (16), it was noted that most compounds found in butter culture were also present in control heated milk used for culture preparation. The data suggested that the quantitative relationships of the flavor compounds were extremely important in defining culture flavor. This paper relates results of quantitative studies on key culture flavor compounds and the subsequent development of synthetic butter culture flavor formulations.

Experimental Procedure

Preparation of butter cultures. A mixed-strain butter culture of commercial origin, selected for its ability to produce a good cultured cream butter flavor, was employed for culture flavor development. Cultures were propagated in a whole milk medium containing 0.2% added sodium citrate. The milk medium was heated in a boiling-water bath for 1 hr. Mother cultures were transferred every other day, using from 0.6 to 3.0% of inoculum, and the incubation time at 21 C was controlled so that the titratable acidity of the cooled culture was from 0.68–0.78%. Bulk culture was prepared in a similar manner, except that the titratable acidity was allowed to develop to 0.90–0.96%. Bulk cultures were held at 5 C for 24 hr prior to use, to allow maximum flavor development.

Preparation of cultured cream butters. Approximately 1,000 lb of fresh cream (38% fat) was pasteurized at 80 C for 30 min, cooled to 21.1, and inoculated with 1–5% of ripened bulk culture. The cream was incubated at 21.1 C for 1–3 hr, depending upon the activity of the culture and the amount of developed acid de-

Received for publication July 25, 1966.

Technical Paper no. 2150, Oregon Agricultural Experiment Station.

Partially supported by funds granted by the American Dairy Association.

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1192

sired. Following incubation, the cream was cooled to 4.4 C and held 14 hr prior to churning. The cream was churned at 10 to 13 C, depending upon the season of the year, and the butter granules were not washed. Salt content of the butters ranged from 0.5 to 1.5% and chilled buttermilk was used to adjust the moisture content during working. The finished butter was tempered at 4.4 C, printed into 1-lb blocks and samples taken for immediate analysis. Aliquots were wrapped and stored at −5 C for a maximum of 3 wk, until used for flavor panel evaluations.

Determination of diacetyl. The method of Elliker (8), with a few modifications, was used to determine diacetyl. The method was altered by using a 50-g sample and by collecting 50 ml of distillate into a 50-ml volumetric flask. A 10-ml aliquot of distillate was then reacted with 0.5 ml of concentrated hydrochloric acid and 0.1 ml of freshly prepared *o*-diaminobenzidine-HCl reagent. Absorbance was determined at 345 mμ instead of 400 mμ and the concentration of diacetyl taken from a standard curve. Recovery of added diacetyl from acidified milk and butter was 90% and all quantitative data were corrected on this basis.

Determination of short-chain acids. The acids in butter culture and cultured cream butter were determined by the procedure of Wiseman and Irvin (24). Samples of butter culture were analyzed intact. For butter, the acids were analyzed in the serum, isolated by warming butter to 40 C and centrifuging. Three grams of sample were acidified with 50% H_2SO_4 and mixed with sufficient dry silicic acid to give a free-flowing powder. This was added to the column as a cap and the column developed.

Since the acids were measured in the serum phase of butter, it was necessary to determine their distribution in the milk fat–serum binary phase system. The distributions of lactic, formic, and acetic acids between 80 g of milk fat and 17 ml of 6% aqueous NaCl solution held at 50 C were determined by adding known amounts of acids, shaking the mixture, and titrating the aqueous phase with standard base. The percentages of acids partitioned into the aqueous phase were lactic, 100; formic, 91; acetic, 86. These values were used to correct the amounts of acids found in the butter serum.

The pH of all products was determined with a Beckman Zeromatic instrument.

Determination of desirable levels of culture flavor compounds. The flavor panel data reported by Day et al. (5) was used as a guide for desirable levels of dimethyl sulfide in cultured cream butters.

For determination of a desirable level of diacetyl in cultured butter, samples were evaluated by an eight- to ten-member experienced flavor panel. One-pound butter samples were coded, tempered at 5 C, and evaluated in a room free of extraneous odors. A maximum of seven samples were displayed simultaneously for evaluation, and panel members could make comparisons. Over-all flavor desirability was scored on a seven-point hedonic ballot, with a scale ranging from 7, extremely desirable, to 1, extremely undesirable. One ballot was used by each judge for each sample and the data from three replications were averaged. Diacetyl in the samples was measured as previously described.

The data reported by Lindsay et al. (15) was used as a guide for desirable levels of acetaldehyde in relation to diacetyl content in butter cultures.

Preparation of synthetic culture flavor formulations. Initial formulations were prepared by adding aliquots of accurately prepared aqueous dilutions of individual culture flavor compounds to products. Flavor chemicals used were: Eastman-grade (Distillation Products Industries) diacetyl, acetaldehyde, and dimethyl sulfide; reagent-grade glacial acetic acid (DuPont). Lactic acid (food grade—50%) was supplied by Monsanto Company. After basic formulations were established, complete culture flavor concentrates were prepared in propylene glycol carrier (Matheson Coleman and Bell).

Preparation of products for culture flavor formulation evaluations. Initial culture flavor formulations were evaluated in H_3PO_4-acidified raw whole milk heated for 1 hr in a boiling-water bath. The heated milk was acidified to pH 4.6 with 20% H_3PO_4 and the culture formulations added. These samples lacked desirable body and texture characteristics, and in later trials δ-gluconolactone (Fisher Scientific Company) was used as the acidogen (6), to obtain a more viscous product. However, use of δ-gluconolactone as the sole acidogen yielded products with an astringent flavor, similar to that described by Deane and Thomas (7). To overcome this defect in the latter stages of this investigation, the lactone was further purified by recrystallization from ethanol, and partially replaced by citric acid in the artificial buttermilk preparations.

Artificial buttermilk for large panel evaluations was prepared by adding citric acid (20 ml of 10% solution per kilogram) and δ-gluconolactone (8 g per kilogram) to whole milk heated in a boiling-water bath for 1 hr and cooled to

The samples were held without agitation hr at 21 C to develop a firm coagulum, stored for 12 hr at 5 C to complete hy- of the lactone (ca. pH 4.4). The acetic was then added (ca. pH 4.2) and the final was adjusted to 4.6 with $NaHCO_3$ (ca. Salt (0.15%) and the remainder of the compounds were then added, to complete preparations.

Sour cream was prepared from cream con- 18.5% fat and 2% added nonfat dry- solids was pasteurized at 80 C for 30 min homogenized at 2,500 psi through the first of a Manton-Gaulin homogenizer. Citric 20 ml of 10% solution per kilogram) and crystallized δ-gluconolactone (6 g per kilo- were added to the cream (7), which was held initially for 12 hr at 21 C and sub- tly for 12 hr at 5 C to develop acidity pH 4.6). Flavor formulations were then to the coagulated cream and mixed to the preparation. A good body and was obtained in this manner.

Cottage cheese curd (farmer style) was man- in the University creamery with a conventional long-set method, using fresh skim- a mixed-strain cheese culture of com- origin. The cream dressing was stan- to 14.5% milk fat and 22% total milk and contained 0.3% stabilizer (Dariloid was pasteurized at 80 C for 30 min and tly homogenized at 2,500 psi through stage of a Manton-Gaulin homogenizer. flavor formulations and salt (1.1%) added to the cream dressing, which was combined with the curd (3:4). Finished Cottage cheese was held 12 hr at 5 C evaluation, to allow flavor equilibration pH 5.2).

Preparation of synthetic culture flavored fresh cream (38% fat) was pasteurized for 30 min, then churned in the pre- described manner. Culture flavor formulations were added at the time of moisture adjustment, and finished butter samples obtained at the time of printing.

Flavor panel evaluations of culture flavor formulations. Initial culture flavor formulations and subsequent formulation modifications were evaluated by a small panel (eight to ten members) of experienced judges.

Samples of synthetic culture flavored buttermilk, butter, Cottage cheese, and sour cream, prepared as previously described, were presented to a preference panel composed of approximately 175 University students. The samples were scored on a nine-point hedonic scale from 9, like extremely, to 1, dislike extremely.

In preliminary preference panel trials, panel members expressed displeasure with eating butter and sour cream alone. A trial conducted to determine the influence of serving butter with a warm yeast roll showed insignificant differences between mean flavor scores of butter samples served with or without rolls. However, the absolute scores were higher when a roll was included. On this basis, butter samples were served with a roll and sour cream with a 2-oz bag of potato chips.

Butter samples were tempered to 4.4 C, cut into patties, then tempered at 10 C on the day prior to testing. The butter patties were served directly from the refrigerator on coded institutional-style butter-chip paper plates. A warm yeast roll, plastic knife, napkin, and ballot were included on each serving tray. Sour cream, buttermilk, and Cottage cheese samples were served in coded 3.5-oz paper cups.

Each serving tray for sour cream, Cottage cheese, and buttermilk contained two synthetically flavored samples—one high-quality natural product manufactured in the University creamery and one commercial sample obtained from a local supermarket. Cultured butter was presented in the same manner, except that no commercial sample was included.

TABLE 1

The pH, short-chain organic acid, and diacetyl content of butter cultures

	Acetic acid[1]	Formic acid[a]	Lactic acid[a]	Diacetyl ($\times 10^{-3}$)[a]	pH
1	1.33	0.20	8.80	3.68	4.70
2	1.48	0.29	9.03	1.20	4.60
3	1.16	0.16	8.50	0.87	4.50
4	1.27	0.18	8.27	1.58	4.60
5	1.12	0.17	7.14	2.51	4.90
6	1.09	0.13	7.68	1.89	4.50
7	1.11	0.11	7.28	2.78	4.90
8	1.23	0.14	7.21	1.58	4.45
Average	1.22	0.17	7.99	2.01	4.64

[1] Grams/kilogram culture.

TABLE 2

Short-chain organic acid and diacetyl content of cultured butter and pH of corresponding butter serum

Butter no.	Acetic acid[a]	Formic acid[a]	Lactic acid[a]	Diacetyl ($\times 10^{-3}$)[a]	pH of butter serum
1	0.08	0.004	0.75	2.32	5.40
2	0.07	0.006	0.53	2.71	5.10
3	0.02	0.003	0.42	2.84	5.50
4	0.02	0.004	0.61	3.42	4.90
5	0.03	0.002	0.50	1.82	5.15
6	0.01	0.002	0.70	2.13	5.20
7	0.01	0.001	0.37	2.04	5.31
8	0.01	0.002	0.49	2.35	5.15
Average	0.03	0.003	0.54	2.45	5.21

[a] Grams/kilogram butter.

Results and Discussion

Results of analyses of eight high-quality butter cultures for acetic, formic, and lactic acids, diacetyl, and pH are shown in Table 1. Each culture was used in the manufacture of a lot of cultured cream butter and results of analyses of corresponding butter samples are shown in Table 2. Rather large variations in the concentrations of compounds and pH values of both butter cultures and butters are apparent. However, the average values obtained for the organic acids and pH determinations were useful in developing subsequent culture flavor formulations.

The diacetyl concentrations and average over-all flavor scores by the experienced panel for 14 butter samples are given in Table 3. Since judges directly compared all samples, the flavor scores tended to magnify differences. Previous reports (2, 22) have indicated that levels of 2.0 to 2.5 ppm of diacetyl in butter were too high and have undesirably harsh flavors. However, the butter samples evaluated in the present investigation were highly acceptable to the flavor judges. Apparently, the proper balance of other flavor compounds and individual flavor preferences are very important in determining desirable levels of diacetyl.

TABLE 3

Relationship of diacetyl content to over-all flavor score of cultured cream butter

Sample no.	Diacetyl	Average over-all flavor score[a]
	(ppm)	
1[b]	0.05	4.06
2	0.50	4.36
3	0.72	4.72
4	1.82	5.50
5	2.04	5.25
6	2.13	5.63
7	2.14	5.72
8	2.32	5.00
9	2.35	5.07
10	2.62	5.45
11	2.71	5.31
12	2.75	5.15
13	2.84	5.14
14	3.42	5.72

[a] From a seven-point hedonic scale where 7.0 = extremely desirable and 1.0 = extremely undesirable; 30 observations per sample.
[b] Sweet-cream butter.

Volatile esters have been long considered to contribute to the flavor of butter cultures and cultured cream butter (2, 11). In view of the presence of a large number of esters in culture distillates (16), this point was investigated further. The volatile ester contents of four high-quality butter cultures were determined by a spectrophotometric, hydroxamate procedure (14). Less than 1.0 ppm of esters expressed as ethyl acetate were found in the cultures. The flavor threshold value (21) of ethyl acetate was found to be approximately 10 ppm. Thus, the short-chain esters, such as ethyl formate and ethyl acetate, probably do not play a major role in culture flavor, and were not included in formulations. However, this observation does not preclude the possibility that low levels of some esters may contribute to the over-all flavor balance of high-quality butter cultures and cultured cream butters.

Earlier work in this laboratory (5) had shown that dimethyl sulfide could smooth out harsh diacetyl flavors. Another paper (15) reported that acetaldehyde contributed to the over-all full balanced flavor of butter cultures. The diacetyl-to-acetaldehyde ratio in full flavored cultures was found to be in the range of 4.4:1 to 3.2:1 and these data were used in initial culture flavor formulations.

On the basis of the foregoing data, culture flavor formulations were prepared and evaluated by trained judges. When a culture flavor formulation containing 2 ppm of diacetyl,

0.5 ppm of acetaldehyde, 1,250 ppm of acetic acid, and 25 ppb of dimethyl sulfide was added to acidified heated whole milk, it yielded a product that possessed an aroma very similar to natural butter cultures. The same formulation added to heated skimmilk was judged to have a fairly desirable culture aroma and flavor, but lacked the full flavor of the heated whole milk product. Artificial culture prepared from pasteurized skimmilk had the poorest flavor, and that prepared from homogenized milk was only slightly better. These results indicated that the added flavor compounds plus the flavor compounds present in heated whole milk were necessary for the typical aroma of high-quality butter cultures.

It was found also that the final pH of the medium exerted a strong influence on the overall culture flavor balance. Undesirable acetic acid-like flavors were observed in artificial butter cultures when the medium was below pH 4.5. However, when the pH was adjusted to the range of 4.6 to 4.8, a typical full aroma was obtained. Use of sodium bicarbonate to adjust the pH of these cultures introduced carbon dioxide and was found to markedly improve flavor. This observation is in agreement with the data reported by Andersen (1), which indicated that addition of 0.05 to 0.15% of sodium bicarbonate improved flavor of natural lactic cultures.

Trials were conducted to determine the effect of altering the amounts of flavor components in the artificial butter culture formulation. It was found that increasing dimethyl sulfide to 25 ppb caused a slight feedy flavor, whereas decreasing the level to 15 ppb resulted in a slightly harsh diacetyl flavor. When acetaldehyde was added at a level of 0.75 to 1.0 ppm, a typically green flavor was observed. It was found that the acetaldehyde concentration could be lowered to about 0.2 ppm without seriously affecting the flavor and even appeared to improve the flavor if the diacetyl concentration was lowered to less than 1.0 ppm. However, if the diacetyl concentration was above 2.0 ppm, reduction of the acetaldehyde content caused a loss of full culture flavor and a somewhat harsh diacetyl flavor predominated. Altering the acetic acid level (± 100 ppm) did not noticeably influence the flavor. Formic acid was not added to the culture formulations, as the levels found in natural cultures were not considered important. Data by Morr et al. (20) indicated that comparable levels of formic acid were present in milk samples that had received heat treatments similar to those used in these studies.

Diacetyl was the predominant flavor note in the culture formulations. It was found that the intensity of culture flavor could be easily manipulated by simply changing the diacetyl concentration in the respective product flavor formulation. In general, levels of diacetyl from 0.5 to 1.00 ppm were found to give mild intensities, from 1.25 to 2.0 ppm intermediate intensities, and from 2.25 to 3.0 ppm pronounced intensities of culture flavor. With adjustments to account for differences in product manufacturing procedures, composition, and physical characteristics, culture flavor formulations were developed subsequently for butter, Cottage cheese, and sour cream. Recent work by Bennett et al. (3) has shown that fat, pH, and volatile acids affect the flavor perception of diacetyl. This is undoubtedly true for other flavor compounds, as well.

Flavor formulations that have given best results are presented in Table 4. Lactic acid was added only to butter, as the necessary acidity for buttermilk and sour cream was obtained from citric acid and δ-gluconolactone. Cottage cheese did not require added lactic acid, because sufficient acid was present in the curd to attain an adequate pH in the finished product. The data obtained for natural cultured butters (Table 2) showed the presence of an average of 540 ppm of lactic acid. However,

TABLE 4

Synthetic culture flavor formulations for dairy products

Compound	Cultured butter	Sour cream	Cottage cheese	Buttermilk[a]
	Concentration—mg/kg of product			
Lactic Acid	250.0	.....	.....	.....
Acetic Acid	30.0	30.0	30.0	1,250.0
Acetaldehyde	0.2	0.2	0.2	0.5
Dimethyl sulfide	0.08	0.025	0.025	0.025
Diacetyl (low level)	0.5	1.0	1.0	1.0
(high level)	2.0	2.0	2.0	2.0
pH of product	5.2	4.6	5.2	4.6

[a] Prepared from heated whole milk and containing ca. 0.15% of added sodium bicarbonate.

this amount of lactic acid added to sweet cream butter at the time of moisture adjustment caused the pH of the butter serum to drop to near 4.8. The resulting low pH yielded a harsh, unbalanced culture flavor in the butter. When the amount of lactic acid was reduced to 250 ppm, a desirable flavor was obtained and pH values from 5.1 to 5.4 were observed, depending on the buffer capacity of the serum. The cause of the discrepancy in measured lactic acid in natural cultured butters and that required for synthetically flavored butters is not clear. Some acid buffering system effective in cream and absent in churned butter may be involved.

It can be noted in Table 4 that the same culture flavor formulation was used for both sour cream and Cottage cheese. Some experienced judges objected to the flavor of the 25 ppb of dimethyl sulfide in Cottage cheese, but they did not object to its influence when the concentration was lowered to 20 ppb. Much higher concentrations of dimethyl sulfide were required in butter to give a fresh, full culture flavor than in the other products. Dimethyl sulfide is quite nonpolar, and naturally occurring dimethyl sulfide would be expected to be concentrated in the fat phase of milk or cream. Therefore, dimethyl sulfide is probably associated more with the fresh flavor of butter than with other cultured products. The acetic acid concentration of 30 ppm found as an average for cultured butters proved satisfactory for butter, Cottage cheese, and sour cream. In these products it was also found that a better flavor was obtained with 0.2 ppm of acetaldehyde than with the 0.5 ppm for buttermilk.

The experienced judges used in this study were in general agreement that the flavor formulations very closely simulated naturally produced high-quality butter culture flavor. Most found it difficult to distinguish between synthetically flavored and naturally cultured butter, Cottage cheese, or sour cream and all liked the smooth culture flavor produced by the formulations. The experienced judges did observe that the artificial buttermilk lacked the exact character of natural buttermilk. This was the most difficult product to work with and problems associated with tactile properties may have been at least partly responsible.

Samples of synthetic culture flavored products, containing the flavor formulations given in Table 4, were presented to large student preference panels shown to compare favorably with household consumer panels (4). To compare the synthetically flavored products with natural products, two synthetically flavored products, one high-quality natural product and one commercially obtained product, were included on each respective serving tray. A summary of the flavor panel evaluations is given in Table 5. No significant preference was found for either the synthetically flavored products or the high-quality natural products. The only samples that scored significantly lower than other samples in a group were the commercial sour cream and Cottage cheese.

It is of interest to note that no preference was shown for either the high or low level of diacetyl in the synthetically flavored products. Recent data reported by Hempenius et al. (13) showed that their consumer panel could not detect differences between creamed Cottage cheese samples unless the diacetyl content differed by 1.4 ppm or more. This suggests that considerable variations in diacetyl may not strongly influence consumer preferences.

The culture flavor concentrates used in the latter part of this work for flavoring butter, Cottage cheese, and sour cream were prepared in a propylene glycol carrier. The flavor compounds are very volatile and difficult to manip-

TABLE 5

Flavor preference scores for culture flavored dairy products

Sample identification	Cultured butter	Sour cream	Cottage cheese	Buttermilk
	Mean score[a]			
Commercial	....[b]	5.25	6.02	3.72
OSU natural	6.38	5.73	6.22	3.69
Synthetic (low level diacetyl)	6.27	5.80	6.37	3.81
Synthetic (high level diacetyl)	6.29	5.75	6.41	3.71
LSD .01[c]	.34	.44	.37	.35
LSD .05	.26	.36	.28	.27

[a] Nine-point hedonic scale from 9, like extremely, to 1, dislike extremely; 175 observations per sample.
[b] Sample not available.
[c] Least-significant difference at the .01 level.

[illegible] accurately, and extreme care is required [illegible] preparing formulations, to achieve desired [illegible]

[illegible] potential use of the butter culture fla[illegible] concentrates in the dairy industry is un[illegible] at the present time. Recent em[illegible] the development of methods for the [illegible] of Cottage cheese (9, 23), sour [illegible] and buttermilk (7, 10, 12, 17) without [illegible] of starter cultures has resulted in a [illegible] for a source of culture flavor in these [illegible]. In addition, there has been a long-[illegible] need for a uniform desirable culture [illegible] in the butter industry. The culture fla[illegible] concentrates described in this paper were [illegible] on the basis of chemical analysis [illegible] naturally cultured products and subsequent [illegible] panel evaluations. The final flavor pres[illegible] in the synthetically flavored products re[illegible] from a blending of those natural flavors [illegible] in milk-based products and those added [illegible] culture flavor concentrates. The culture [illegible] concentrates will not mask defective fla[illegible] a product.

[illegible] storage stability tests indicate that the [illegible] is reasonably stable. Work is contin[illegible] this aspect, as well as on other related [illegible] influencing the application of culture [illegible] concentrates.

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1193

RESEARCH NOTE

Further Investigations on the Properties of the 2,4-Dinitrophenylhydrazine Derivatives of Diacetyl, α-Acetolactic Acid, and Acetoin*

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J. Dairy Sci. 29(3): 266-268, (1964)

An earlier communication (Lindsay *et al.*, 1962) reported some of the properties of the 2,4-dinitrophenylhydrazine (DNP) derivatives of diacetyl, α-acetolactic acid, and acetoin. Subsequent work has revealed that the compound originally reported as the DNP-hydrazone of acetoin is 2,4-dinitroaniline. This communication corrects the earlier data and relates some additional properties of the derivatives that enable their identification.

The revised and corrected properties of authentic derivatives are given in Table 1. The properties for diacetyl DNP-hydrazone and diacetyl bis-(DNP-hydrazone) are as reported previously. It should be noted, however, that the data presented for 2,4-dinitroaniline were previously reported as that for acetoin DNP-hydrazone. The properties of the acetoin DNP-hydrazone are in agreement with those reported by other workers (Patton *et al.*, 1958; Day *et al.*, 1960), and contrary to our earlier communication.

Infrared spectra are useful for distinguishing the derivatives provided special care is taken in purification and drying of materials. After chromatography of the DNP-hydrazones as described previously, the acetoin DNP-hydrazone was further purified by a hexane-nitromethane partition column (Day *et al.*, 1960) and recrystallized from hexane. Diacetyl bis-(DNP-hydrazone) was purified by chromatography on a benzene-ethanol amine partition column (Schwartz, 1962). To circumvent the interfering OH stretch due to water, the DNP-hydrazones were dried at 60°C at pressure of 30 mm Hg for 24 hr prior to analysis. The KBr used in making pellets was ground for 1 min in a stainless-steel capsule and then dried 24 hr at 360°C. All samples were handled rapidly immediately prior to analysis to prevent adsorption of excessive moisture.

The distinguishing characteristics of the IR spectrum for the diacetyl bis-(DNP-hydrazone) (Fig. 1a) are the absence of significant OH or NH stretch and C=O stretch. Diacetyl DNP-hydrazone (Fig. 1b) shows the absence of the significant OH or NH stretch, but shows a relatively intense C=O stretch band near 1685 cm^{-1}. Acetoin DNP-hydrazone (Fig. 1c) shows an intense band near 3380 cm^{-1}, but does not show a C=O stretch band. The band near 3380 cm^{-1} for acetoin DNP-hydrazone is a rather broad band, which is to be expected where hydrogen-bonding through a hydroxyl group

* Supported by funds granted by the American Dairy Association.

Table 1. Properties of 2,4-dinitrophenyl hydrazine derivatives.

Derivative	Melting point (°C)	λ_{max} Chloroform	λ_{max} Alcoholic KOH	Color in Alcoholic KOH
Diacetyl bis-(DNP-hydrazone)	318d	430,394	540	Purple
Diacetyl DNP-hydrazone	175	355	500	Reddish pink
Acetoin DNP-hydrazone	114	357	500	Red
2,4-Dinitroaniline *	178–9	327,259	510,382	Bright pink

* Soluble in water.

1193

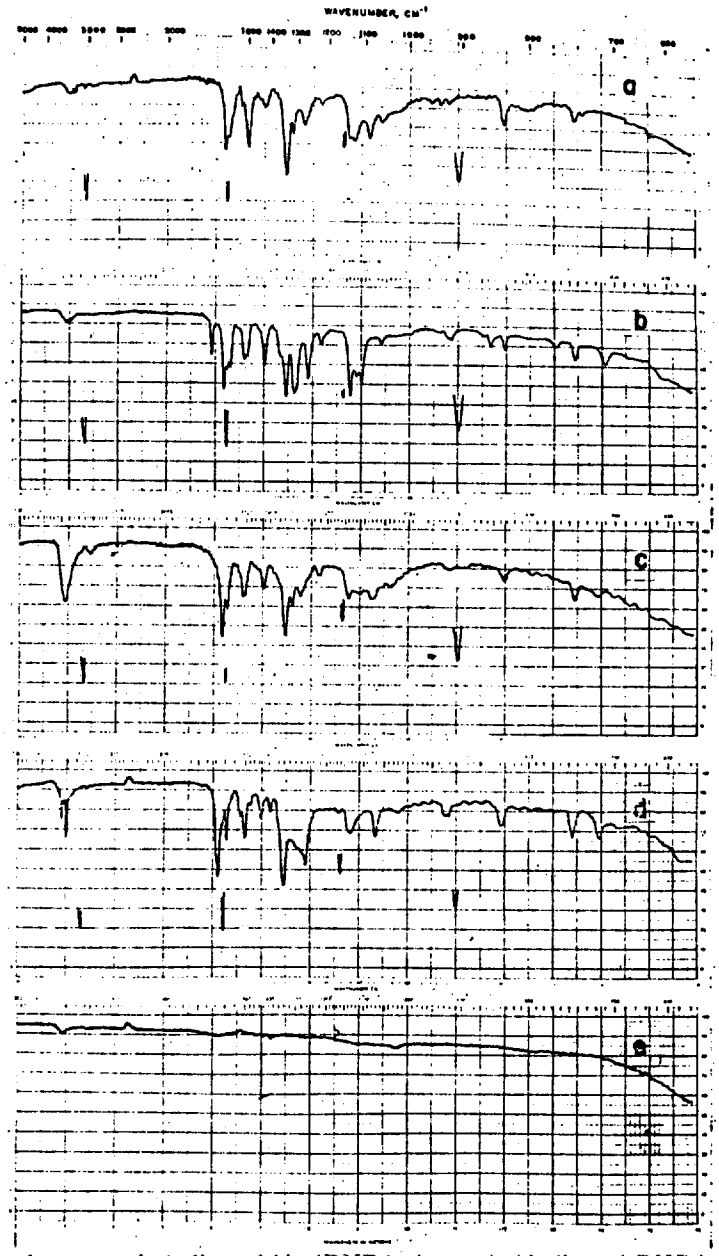

Fig. 1. Infrared spectra of a) diacetyl bis-(DNP-hydrazone), b) diacetyl DNP-hydrazone, c) acetoin DNP-hydrazone, d) 2,4-dinitroaniline, e) blank KBr pellet.

be effected. The IR spectrum for 2,4-dinitroaniline (Fig. 1d) shows two sharp bands near 3333 cm^{-1} and 3460 cm^{-1}. The two bands are characteristic of the NH stretch resulting from the two NH bonds in the free amine group.

The origin of 2,4-dinitroaniline in carbonyl reaction mixtures is probably through a series of reactions analogous to those for phenylhydrazine reacting with an α-hydroxy ketone (Holleman and Richter, 1953):

$$R\cdot CH(OH)\cdot CO\cdot R' + C_6H_5\cdot NH\cdot NH_2 \xrightarrow[\text{acetic acid}]{\text{dilute}} R\cdot CH(OH)\cdot C(:N\cdot NH\cdot C_6H_5)\cdot R' + H_2O \quad [1]$$

$$R\cdot CH(OH)\cdot C(:N\cdot NH\cdot C_6H_5)\cdot R' + C_6H_5\cdot NH\cdot NH_2 \longrightarrow R\cdot CO\cdot C(:N\cdot NH\cdot C_6H_5)\cdot R' + NH_3 + C_6H_5\cdot NH_2 \quad [II]$$

$$R\cdot CO\cdot C\cdot(:N\cdot NH\cdot C_6H_5)\cdot R' + C_6H_5\cdot NH\cdot NH_2 \longrightarrow R\cdot C(:N\cdot NH\cdot C_6H_5)\cdot C(:N\cdot NH\cdot C_6H_5)\cdot R' + H_2O \quad [III]$$

With DNP-hydrazine and acetoin, reaction I would yield acetoin DNP-hydrazone; reaction II would yield diacetyl DNP-hydrazone and 2,4-dinitroaniline; reaction III would yield diacetyl bis-(DNP-hydrazone).

The rigorous extraction of the reaction mixtures with ethylene chloride to obtain the diacetyl bis-(DNP-hydrazone) undoubtedly recovered considerable quantities of 2,4-dinitroaniline hydrochloride in the samples to be analyzed by column chromatography. Since the magnesium oxide-celite adsorption columns are quite basic, the hydrochloride salt could be readily converted to the free amine, thus accounting for its slight solubility in water and relatively short elution time from the column. Since 2,4-dinitroaniline has appeared as a component in derivatives from several autoxidizing fat samples in this laboratory, it is well that other workers in this area be aware of its potential presence in unknown DNP-hydrazone mixtures.

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1215

Volatile Reducing Substances in Canned Bartlett Pears[a]

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Food Tech. 9(12): 639-642, (1955)
(Manuscript received June 21, 1955)

The influence of ripeness of fresh Bartlett pears on flavor and on the quantity of various volatile reducing substances in the canned product was investigated. As pears ripened at 68° F. (20° C.), methyl alcohol, total carbonyl compounds, acetyl methyl carbinol, diacetyl, and ester content gradually increased. The rate of production of such compounds was especially rapid when the pressure test of the fresh pears dropped below 2 pounds. The presence of an excess of methyl alcohol and diacetyl might be used as a criterion of off-flavor and overripeness. The presence of excess methyl alcohol in overripe Bartlett pears was thought to be caused by de-esterification of the methyl ester group of the pectin molecule by the enzyme pectin-esterase. The importance of these constituents to flavor of canned pears is discussed.

In a previous study it was reported that ripeness of fresh Bartlett pears as measured by pressure test is related to flavor acceptance of the canned product *(19)*. Fresh Bartlett pears with an average pressure test of 2 to 3 pounds produced the highest quality canned product. There was also a correlation between the ripeness of fresh pears, as measured by pressure test, and the amount of volatile reducing substances (VRS) in the canned product. Canned Bartlett pears with high VRS content were scored high in flavor and aroma. The presence of acetaldehyde in pears has been reported *(21)*. Tindale, Trout, and Huelin *(25)* found an increase in alcohol and acetaldehyde content during ripening of Bartlett pears at 65° F. (18° C.). Ezell and Diehl *(7)* reported on the relation of maturity and handling of Bartlett pears grown in the Pacific Northwest to quality of the canned product but gave no information on the volatile substances. The chemistry of fruit and vegetable flavors has been reviewed by Kirchner *(14)*, but information on flavor constituents of pears is very scarce.

In the study reported here, some of the volatile compounds in canned Bartlett pears that bear a close relationship with the pressure test of the fresh fruit and the flavor and aroma score of the canned product are identified. Thus, it may be possible to develop chemical tests that will supplement or possibly replace subjective organoleptic methods used in quality evaluation of canned Bartlett pears. Likewise, the effects of climatic conditions, cultural practices, and processing variables on the quality of canned Bartlett pears may be investigated more objectively.

MATERIALS AND METHODS

Bartlett pears. The pears used in this investigation were harvested on September 2, 1953, from the Homer Henderson Orchard, Lake County, California. Immediately after harvest, tons of pears were hauled to Davis and stored at 32° F. (0° C.). The firmness of the fresh pears at harvest was 16 to 18 pounds, and the soluble solids content as measured by a refractometer was 11.12 ± 0.51% at 68° F. (20° C.) *(19)*. The pears were kept at 32° F. (0° C.) for 7 days and then ripened in a 68° F. (20° C.) room at 85% relative humidity until they reached the desired firmness. It takes about 4 to 5 days at 68° F. (20° C.) for pears to reach canning ripeness. Pears of desired firmness were taken out periodically for canning and chemical analyses.

Canning. Bartlett pears of proper pressure tests were peeled and cored in a Food Machinery and Chemical Corporation pear-peeler and then dipped into tap water for 3 to 5 minutes. The pear halves were trimmed and sorted before canning. Eighteen ounces (± 0.05 oz.) of pears and 11 ounces of 25° Brix sucrose syrup were placed in each No. 2½ can. All cans were sealed under 15 inches of vacuum and cooked for 30 minutes at 212° F. (100° C.) in a rotary cooker. The processed cans were cooled rapidly in a rotary cooler and then stored at room temperature.

Flavor evaluation. Three months after canning, a panel of 15 selected persons evaluated the pears for flavor, using a balanced incomplete block design method as described in a previous paper *(18)*. An aliquot of each sample was used for chemical analyses.

Pectin-esterase activity. The enzyme pectin-esterase was extracted from pears of 2 to 3 pounds firmness, which had been previously frozen at 0° F. (−17.7° C.). The frozen pears were thawed and mixed with an equal weight of 10% sodium chloride solution at pH 7.0. The pectin-esterase activity in the extract was determined by measuring the rate of liberation of carboxyl groups in a 0.5% solution of pectin N. F. at pH 7.5 and a temperature of 84.9° F. (29.4° C.) *(12)*. One unit of pectin-esterase activity (PEU) is the amount that releases 1 milliequivalent of acid per minute.

Physical and chemical tests. The tests for pressure reading, pH, soluble solids, total titratable acids, and ascorbic acid in the pears were the same as those described in a previous paper *(19)*. For canned pears, syrup and fruit were blended together in a Waring blender for 30 seconds prior to chemical analyses.

Reducing sugars. The content of reducing sugars was determined by an alkaline-ferricyanide method modified by Hassid *(9)*. The results were reported as dextrose equivalents.

Volatile reducing substances (VRS). The amount of VRS in canned pears was determined by steam distilling 200 g. of the sample in an all-glass apparatus. The sample was obtained by mixing the juice and pears from 3 cans of chilled pears in a Waring blender for 30 seconds. The distillate was collected through an ice-water-circulated spiral condenser directly into an ice-cooled receiver. Any uncondensed volatile compounds were sucked through a sintered glass tube into a receiver containing 20 ml. of distilled water, which was also chilled in an ice-water bath. Distillation was stopped after about 170 ml. of the distillate had been collected, which required about 30 minutes. The distillation was done under a slight negative pressure created by a water aspirator. The contents were diluted to 200 ml. with distilled water, and 75 ml. aliquots in duplicate were used for determination of total VRS as described in a previous paper *(18)*. The remainder of the sample was used for analyses of methyl alcohol, total carbonyl compounds, acetyl methyl-carbinol, and diacetyl.

Methyl alcohol. A 2 ml. aliquot of the 200-ml. distillate was used for the determination of methyl alcohol by permanganate oxidation and chromotropic acid reagent as described by Boos *(4)*.

Total carbonyl compounds. A 1 ml. aliquot of the distillate was analyzed for total carbonyl compounds by measuring the color developed with 2,4-dinitrophenylhydrazine in alkaline solution at 480 mμ in a Beckman Model DU spectrophotometer as described by Lappin and Clark *(17)*.

[a] Presented at the Fifteenth Annual Meeting of the Institute of Food Technologists, Columbus, Ohio, June 15, 1955.

Voges-Proskauer reaction. Twenty-ml. aliquots of the distillate were analyzed for acetyl-methyl-carbinol and diacetyl content by the Voges-Proskauer reaction *(2)* as described by Byer *(5)*. In each analysis 5 ml. of 5% alpha-naphthol in 99% isopropyl alcohol was used instead of 10 ml.

Determination of esters. Ester content of the canned pears was determined essentially by the colorimetric method originated by Hill *(10, 11)* and used by Thompson *(21)* for esters in apples. This method is based on the conversion of the esters to hydroxamic acid, which forms red complexes with ferric ion. The method was slightly modified as follows: 2 cans of Bartlett pears were first chilled in an ice-water bath. The pears and syrup were then mixed in a Waring blender for 30 seconds. A 250-g sample of slurry was weighed into a 600-ml. beaker, and 100 ml. of distilled water was added. The mixture was brought to pH 7.5 with N NaOH while being stirred vigorously. A Coleman pH meter was used to determine the end point. Twenty-five ml. of freshly prepared 5% mercuric acetate solution in water was added to precipitate tannins and other interfering substances. The resulting mixture was readjusted to pH 7.5 with N NaOH to convert the organic acids into anions, and then diluted with distilled water to 500 ml. in a volumetric flask. The solution was mixed well and centrifuged. About 330 ml. of the supernatant was accurately measured and quantitatively transferred into a Kötsdorfer-type ether extractor for liquids *(13)* with 70 ml. of distilled water. The solution was extracted with 250 ml. of reagent grade ethyl ether for 24 hours. A 5-ml. portion of the ether extract was pipetted into a 25-ml. graduated cylinder with glass stopper. To this was added 1.5 ml. of a freshly prepared and centrifuged solution of hydroxylamine, made by mixing equal volumes of 5% hydroxylamine hydrochloride and 12.5% sodium hydroxide in methanol. The mixture was allowed to stand at 77° F. (25° C.) for 30 minutes, and 12.5 ml. of ferric perchlorate reagent solution was added. The ferric perchlorate reagent solution was made by mixing 2 ml. of stock ferric perchlorate solution (1.93 g. $FeCl_3 \cdot 6H_2O$, 5 ml. conc. HCl, and 5 ml. 70% perchloric acid diluted to 100 ml. with water) with 7.5 ml. of 70% perchloric acid, and then diluting to 100 ml. with ethanol. The solution was mixed thoroughly and allowed to stand for 10 minutes. The color was measured in a Klett photocolorimeter, using a 540 mμ filter. A blank was prepared using all the reagents and 5 ml. of reagent grade ethyl ether instead of the ether extract. A standard curve was constructed in the same manner as above, using redistilled reagent grade ethyl acetate.

Identification of acetaldehyde. Because of their higher VRS content, pears canned at 1.2 pounds pressure test in 25° Brix sucrose syrup were used to identify acetaldehyde. Three successive batches of 200 g. pear pulp each were steam distilled into the same ice-cooled receiver for 30 minutes. The total amount of distillate from 600 g. pear pulp was about 170 ml.

The 2,4-dinitrophenylhydrazone was prepared according to the method described by Lappin and Clark *(12)*. Sixty ml. of saturated solution of twice recrystallized 2,4-dinitrophenylhydrazine in carbonyl-free methanol and 12 ml. of conc. HCl were added to the distillate. The flask containing the reaction mixture was loosely stoppered and heated in a water bath at 118.4° F. (48° C.) for 30 minutes. The phenylhydrazone was extracted with 150 ml. of ethyl ether and separated from the reaction mixture in a separatory funnel. The ether was evaporated in a current of air at room temperature. The residue containing the derivative was dried overnight in a vacuum oven at 82.4° F. (28° C.) and finally in a desiccator containing $CaCl_2$. The dried residue containing the derivative and excess reagent was dissolved in a mixture of 1 part benzene and 2 parts petroleum ether. The solution was poured into a 19 mm. x 80 mm. column packed dry with 2 parts of Merck reagent grade silicic acid and 1 part by weight of Super-cel. The column was eluted with petroleum ether containing 4% ethyl ether. A large and strong yellowish-orange colored band (fraction 1) moved down with this solvent followed by another yellow band (fraction 2). The whole column was extruded, and the yellow bands were cut and extracted separately with methanol. After filtration by suction, the solvents were evaporated in a current of air. Fraction 1 was dissolved in 25 ml. of hot ethyl alcohol, and then about 5 ml. of water was added until the mixture became turbid. After 12 hours at room temperature, the crystals were filtered and recrystallized twice by the same procedure. The crystals were separated and dried in a vacuum oven at 131° F. (55° C.) and finally in a $CaCl_2$ desiccator overnight.

Paper chromatography. The 2,4-dinitrophenylhydrazone of acetaldehyde prepared from the pear distillate was also identified by comparing its *Rf* value with that from a known sample by paper chromatography on Whatman No. 1 filter paper using the epiphase of heptane-methanol as developing agent *(22)*.

RESULTS

Chemical analyses and flavor score of canned Bartlett pears. Pears canned at different stages of ripeness, as judged by pressure tests, were canned in 25° Brix sucrose syrup and then stored at room temperature for 3 months to allow exchange of constituents between fruit and syrup. The canned fruits were then evaluated for quality both by chemical analyses and flavor scoring (taste tests). The results are shown in Table 1. The soluble solids and sugar contents of the samples differed very little. Thus any difference in flavor was not due to sweetness. Riper pears had lower ascorbic acid content. Flavor scores of the canned pears increased with a decrease in pressure test of the fresh fruit. Samples canned at 1.5 pounds pressure test scored highest in flavor. The results agree in general with a previous report on Bartlett pears *(19)*, even though the fruits were grown at two different districts in different years.

Volatile reducing substances. Results (Table 2) indicate that the total VRS of canned pears increased with a decrease in firmness of the fresh fruit. The amount of total VRS correlated well with the flavor score of the canned product down to a firmness of 1.5 pounds in this test. A coefficient of correlation of 0.90, significant at a probability level of 2%, was found. Below 1.5 pounds there was a reduction in flavor score, although VRS continued to increase. This would indicate that qualitative as well as quantitative determinations of VRS are important in evaluating flavor acceptance.

The amounts of various types of VRS in Bartlett pears canned at different stages of ripeness are shown in Table 2.

TABLE 1

Chemical analysis and flavor score of canned Bartlett pears of different stages of ripeness

Sample	Texture of fresh pears prior to canning in lbs.	pH	Soluble solids in ° Brix at 20° C.	Total titratable acids as % citric monohydrate	Ascorbic acid in mg./100 g.	Sugars: Before inversion, as % Dextrose	Sugars: After inversion, as % Dextrose	Average flavor score [1,2]
A	3.8 ± 0.3	4.00 ± .06	18.46 ± 0.23	.301 ± .014	1.69 ± .11	6.53	16.33	4.98
B	3.4 ± 0.4	4.03 ± .04	18.31 ± 0.31	.291 ± .018	1.69 ± .16	6.96	16.75	5.85
C	3.2 ± 0.3	4.06 ± .08	18.24 ± 0.29	.276 ± .026	1.30 ± .17	6.22	16.52	6.83
D	2.4 ± 0.5	4.05 ± .06	18.32 ± 0.31	.281 ± .024	1.51 ± .15	6.48	17.10	6.80
E	1.9 ± 0.2	4.02 ± .06	18.21 ± 0.14	.302 ± .012	1.53 ± .15	6.50	16.09	6.92
F	1.5 ± 0.3	3.99 ± .06	18.34 ± 0.21	.286 ± .028	1.28 ± .13	6.52	16.58	8.35
G	1.2 ± 0.4	4.06 ± .05	18.34 ± 0.16	.290 ± .018	1.21 ± .08	6.22	16.40	7.90

[1] Samples are scored for flavor by balanced incomplete block design method by a panel of 15 people. A scale of 1 to 10 was used. An excellent sample was scored 9 to 10 points: good, 7 to 8; fair, 5 to 6; poor, 3 to 4; and very poor, 1 to 2.

[2] Differences in flavor score between samples C:D, C:E, D:E and F:G are not significant at 2% level. The rest of the combinations are significant to 2% level or better.

1215

TABLE 2

Volatile reducing substances in canned Bartlett pears (juice and pears combined)

Pressure test of fresh pears in lbs. (5/16" plunger)	Total volatile reducing substances as microeq. $KMnO_4$/50 g.	Total carbonyl compounds as acetaldehyde mg. per kg.	Methyl alcohol mg. per kg.	Acetyl methyl carbinol by Voges-Proskauer reaction mg. per kg.	Diacetyl mg. per kg.	Esters micromoles per 100 g.
14.4 ± 1.0	99.7	2.6	6.5	0.2	0	6.5
5.5 ± 0.5	147.0	3.8	8.4	0.5	0	6.0
3.8 ± 0.3	157.0	5.0	9.4	0.6	0	6.6
3.4 ± 0.4	140.0	4.0	10.5	0.7	0	7.2
3.2 ± 0.3	165.8	5.7	12.5	1.5	0	7.9
2.4 ± 0.3	195.0	6.0	13.3	1.3	0	8.9
1.9 ± 0.2	288.0	4.6	23.8	1.1	0	19.3
1.5 ± 0.3	356.0	10.4	40.0	6.2	1.6	21.1
1.2 ± 0.4	377.0	12.4	96.0	7.4	6.2	24.9

The methyl alcohol content increased with a decrease in pressure test of the fresh pears. It increased very rapidly as the pressure test dropped to 2 pounds or less. The coefficient of correlation was − 0.74, significant at a probability level of 5%. The amount of carbonyl compounds increased as the pressure test decreased with a coefficient of correlation of − 0.78, significant at a probability level of 5%. Ester content increased as the firmness decreased. The rate of increase in ester content was most rapid as the pressure test dropped below 2.4 pounds. Since the flavor of the canned product scored highest if the fresh pears tested 1.5 pounds, it might be inferred that the rapid increase in methyl alcohol, acetyl-methyl-carbinol, and carbonyl compounds has a certain optimum range. When these compounds are present in excessive amounts, off-flavor may result. It might also be possible that at 1.5 pounds, these volatile compounds are not at levels injurious to eating quality, and the improvement in flavor was caused by other aromatic compounds which were not investigated in this work.

Diacetyl, which may be an undesirable constituent, began to show up as the firmness of the fresh pears dropped to 1.5 pounds. It is important to point out that the various VRS did not increase at the same rates as the firmness decreased, although they all increased during ripening. Apparently they accumulated as end products of various metabolic processes. Although the presence of diacetyl was demonstrated by the Voges-Proskauer reaction, further work is needed to identify the compound chemically.

Identification of acetaldehyde. Acetaldehyde was identified in the steam distillate from pears canned at 1.2 pounds pressure test as crystalline 2,4-dinitrophenylhydrazone melting at 329° F. (165° C.). When the derivative was mixed with that of known acetaldehyde, no drop in melting point was observed. The 2,4-dinitrophenylhydrazone was also identified by paper chromatography by comparing its Rf value with that prepared from acetaldehyde, using heptane-methanol as irrigating agent in the descending direction *(22)* on Whatman No. 1 filter paper. Both samples have an Rf value of 0.21 at 71° F. (21.7° C.).

Pectin-esterase activity in Bartlett pears. Since methyl alcohol content increases during ripening of Bartlett pears, one can assume that the enzyme pectin-esterase might have de-esterified the pectin in pears to form methyl alcohol and pectic acid. It was therefore decided to demonstrate the presence of pectin-esterase in pears and to extract the enzyme for quantitative study. Results indicate that pears at a pressure test of 2 to 3 pounds have an activity of 8.4 x 10^4 pectin-esterase units (PEU) per g. of pear tissue, whereas green pears at 14 pounds pressure test have only 4.2 x 10^4 per g. of tissue. Thus it might be postulated that ripening of Bartlett pears is accompanied by de-methylation of the ester groups in the pectin molecule by pectin-esterase. This enzymic action caused the formation of a large amount of methyl alcohol, with simultaneous formation of pectic acid or lower oligouronic acids through the action of polygalacturonase *(26, 27)*.

DISCUSSION

One of the main observations that may be made on the above data is that the determination of the VRS content may be useful in evaluating quality of canned Bartlett pears. By this procedure a quantitative measure of the changes in VRS during ripening might be obtained. Once the range of volatile constituents for normal samples has been established and correlated with quality, it may be possible to objectively evaluate unknown samples for quality. The data presented here are of a rather cursory nature. However, they serve to illustrate that the quantity of VRS in Bartlett pears varies greatly with the ripeness of the fresh fruit before processing. A large amount of total VRS composed of an excess of certain volatile constituents, such as alcohols, acetyl-methyl-carbinol, carbonyl compounds and others, might be used as criteria of overripeness and off-flavor. In the past, the majority of the work on volatile constituents of fruits has been on only one particular stage of ripeness of a certain fruit *(8, 15, 16, 28)*. It is advisable to investigate the same fruits at different stages of ripeness.

Only a few of the many possible VRS of Bartlett pears are reported in this paper. However, the quantities of these constituents correlated well with changes in firmness of the fresh fruit and flavor acceptance of the canned product. In this test pears canned at 1.5 pounds firmness scored highest in flavor, with off-flavors developing in softer fruit. Studies with fresh pears indicate that firmness alone is not always a reliable indication of off-flavor development. Further work is needed to determine these relationships.

Tindale, Trout, and Huelin *(25)* studied the storage, ripening, and respiration of 9 varieties of Australian pears. They indicated that during ripening of Bartlett pears at 65° F. (18.1° C.) there was a considerable and continuous increase in the alcohol content. Acetaldehyde content increased during cold storage and subsequent ripening and reached its maximum concentration at the onset of core breakdown. They postulated that this was due to the progressive replacement of normal oxidative metabolism by anaerobic respiration. The work reported on here showed similar trends although the quantities of carbonyl compounds found were lower than those of the Australian Bartlett pears. This variation could be caused by differences in climate, or cultural factors, and specificity of the methods used in analyses.

It has been reported by Kirchner *et al. (10)* that there is only a small difference in the composition of fresh and freshly canned grapefruit juices. During storage, however, there was a considerable increase in the volatile acids, methanol, and other constituents. In the work reported on here, the difference in methanol, carbonyl compounds, acetyl-methyl carbonyl, and ester content observed among the canned pears of different ripeness was not due to storage effect, since the pears were canned and stored under the same conditions. Nevertheless, it is still possible that the volatile constituents of fresh pears may differ slightly from those of the canned product because of the heating process during canning.

The gradual increase in free methyl alcohol content during ripening possibly may be explained by the hydrolysis of pectin by the enzyme pectin-esterase. It is

1215

not known why pectin-esterase did not act on the pectin in green pears even though it was found to be present. Probably the pectin in green pears was present as protopectin, which is not available to pectin-esterase. Another possible explanation is the presence of an inhibitor for the pectic enzyme in green pears *(26, 27)*.

Although the general trends of changes in chemical constituents during ripening as reported here were the same as those for pears from another district *(19)*, there were differences in acid contents, soluble solids, and total VRS. These might be due to differences in climate and cultural practices. It is not known whether pears grown at different localities or under different cultural practices exhibit the same relationships between firmness and the amount of various volatile reducing substances. This point needs further investigation.

Because of the importance of texture in marketability of canned Bartlett pears, it is desirable to use fresh fruit with a pressure test of 2 to 3 pounds for canning, even though pears at 1.5 pounds scored highest in flavor. Pears below 2 pounds pressure test will be too soft to maintain a desirable texture, as shown in a previous paper *(19)*.

SUMMARY

The influence of ripeness of fresh Bartlett pears on flavor and on the quantity of volatile reducing substances in the canned product has been reported. It was found that during ripening there was an increase in methyl alcohol, carbonyl compounds, acetyl-methyl-carbinol, diacetyl, and ester content. The rate of production of these volatile compounds was especially rapid as the pressure reading dropped below 2 pounds. The presence of a large excess of total volatile reducing substances, methyl alcohol and diacetyl in the canned product might be indicative of off-flavor and over-ripeness. Pears canned at an average pressure test of 1.5 pounds scored highest in flavor. Because of the importance of both texture and flavor to quality of the canned product, it is desirable to use fresh fruit at a pressure test range of 2 to 3 pounds for canning.

The presence of pectin-esterase is ripe Bartlett pears has been demonstrated. The gradual increase in methyl alcohol content as pears ripen may possibly be explained by the de-esterification of pectin by pectin-esterase. The possibility of using a chemical method to supplement or replace the organoleptic method in quality evaluation of canned Bartlett pears is discussed.

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German Translation

Microchemical Detection of Aldehydes and Ketones

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Mikrochemie ver. Mikrochim. Acta 32: 6-10 (1944)

A simple micro method for the detection of volatile aldehydes and ketones has recently been reported by R. Opfer-Schaum (1). The material to be tested is placed in a "micro beaker" and the reagent is placed in the form of a "hanging drop" on a specimen holder located above the sample [Griebel (2, 3)]. The reagent used is an acetic acid solution of p-nitrophenylhydrazine. Identification of the p-nitrophenylhydrazones obtained in the hanging drop is done by determining the eutectic point (temperature) in the mixture containing excess reagent and(or) in the acetone hydrazone obtained therefrom by treatment with an acetone-water mixture.

This method offers considerable advantages over the improvement of Griebel's method proposed by R. Fischer (4, 5, 6). Fischer used for identification of the p-nitrophenylhydrazones, instead of the not always well defined crystalline form and color, the determination of the micro melting point after previous washing between the specimen holder and the glass cover. However, according to his own report, he was not always able to obtain a product with a sharp melting point by this procedure.

The eutectic points (temperatures) obtained by the method of R. Opfer-Schaum are sharp and easily reproducible, and, in addition, the working procedure is considerably quicker. The eutectic points are determined according to L. and A. Kofler (7, 8) in a micro melting point apparatus. Already when 1% of one of the

components is present, the start of melting can be determined in a good reproducible way, when the solid phases are miscible or the liquid phases are immiscible. Identification can be achieved with maximum certainty in view of the fact that the mixed melting point of mixtures can be determined under a microscope (9).

A rapid and simple working procedure has been developed by me for the case considered here. It is not necessary to prepare the p-nitrophenylhydrazone of the analyzed aldehyde or ketone in pure form. As will be described later and as can be done in a few minutes, it is sufficient to prepare a mixture of the hydrazone with acetone hydrazone from the aldehyde or ketone in question. A small portion of this mixture is then added to the hydrazone mixture obtained from the tested material, and the start of melting is again determined. When the p-nitrophenylhydrazone obtained from the tested material is identical with the one obtained from the comparison product, the eutectic point remains unchanged. If this is not the case, a ternary mixture is present, the start of melting of which is considerably lower, as shown in Table II.

Working Procedure

The material to be tested for volatile aldehydes or ketones is placed in a small micro beaker 1.5-2 cm high and 1-1.5 cm wide. The beaker is covered with a specimen holder carrying a hanging drop of reagent. This "hanging drop" is prepared by placing on the specimen holder a drop (3 mm in diameter) of about 15% acetic acid ("aldehyde-free per analysis"). Into this drop a few crystals of p-nitrophenylhydrazine are ground with a thin glass rod; the amount of p-nitrophenylhydrazine is selected in such a way that the total area is equal to about 1-2 sq. mm. and part of it remains undissolved. The micro beaker with the

hanging drop is left standing for some time at room temperature or, when the substance is less volatile, at 40-50°C on a heated table. At the same time, formation of the hydrazone in the hanging drop can be observed under the microscope. When a sufficient amount of reaction product has been formed, the specimen holder carrying the reaction product is removed and a drop of an acetone-water mixture (4:1) is added. The mixture is ground with a thin glass rod until the reaction product formed and the p-nitrophenylhydrazine crystals are completely dissolved in acetone and both hydrazones precipitate again after evaporation of the excess acetone. In order to be sure that all p-nitrophenylhydrazine has been converted into its acetone compound, a drop of acetone-water mixture is added a second time and the mixture ground again. The crystal slurry is then packed not too tightly with a 0.5 cm-wide strip of hardened filter paper, and the liquid on top of the slurry is sucked off as completely as possible. Since hardened filter paper has a low absorbency, it is advisable to smoothly cut off several times with a sharp scissors that portion of the filter paper strip which has fully absorbed the liquid. The product is then placed on a heating plate and dried at 80-100°C. The eutectic point (temperature) is then determined.

In case the acetic acid in the reagent drop has evaporated or has been too strongly diluted, it should be replaced before the acetone treatment, since otherwise conversion to the acetone hydrazone is not complete.

The sensitivities listed in Table I were determined when the reagent drop was allowed to stand for not more than 10-15 minutes on top of the tested materials. The sensitivity could probably be increased further by lengthening the reaction time or by reducing the size of the reagent drop.

Table I.

Aldehyd bzw. Keton	1 Fp. des p-Nitrophenylhydrazons	2 Eut. Temp. des p-Nitrophenylhydrazons mit Aceton-p-nitrophenylhydrazon	3 Empfindlichkeit in γ in 1 ml Wasser
Isobutyraldehyde...	132°	99°	50 γ
Crotonaldehyde....	185°	124°	50 γ
Bourbonal.....	184—185°	129°	2 γ in 0,05 g Milchzucker
Acrolein[10].....	162°	122°	5 γ
Diacetyl.....	229,5°	143°	10 γ
Acetophenone.....	184°	127°	20 γ

1 - M.p. of p-nitrophenylhydrazone
2 - Eutectic point of p-nitrophenylhydrazone with acetone p-nitrophenylhydrazone
3 - Sensitivity, in γ, in 1 ml water

Other aldehydes or ketones investigated either give no usable reaction product with p-nitrophenylhydrazine whatsoever, or the melting points of their p-nitrophenylhydrazones lie below 100°C, and therefore cannot be mistaken with the aldehydes and ketones listed in the table. Only small drop-shaped reaction products were obtained with n-octylaldehyde, n-nonylaldehyde, methyl isobutyl ketone, pinacoline, carvone and methone. Benzaldehyde, cinnamaldehyde, methyl propyl ketone, ethyl-n-propyl ketone, p-methylacetophenone, monochloroacetone, monochloracetophenone, and monobromoacetophenone gave no sharp and well-reproducible eutectic points. The p-nitrophenylhydrazones of n-butyraldehyde, n-valeraldehyde, isovaleraldehyde, caprylaldehyde, enanthole (heptanol), methyl butyl ketone, methyl-n-hexyl ketone, methyl isohexyl ketone, methyl-n-nonyl ketone and methylheptenone had melting points below 100°C.

Some of the aldehydes and ketones processed here and by Opfer-Schaum (11) exhibit similar eutectic points. When the corresponding aldehydes and ketones are available, it is possible to achieve a positive (certain) differentiation by means of the mixed melting point of the mixtures, as was already stated above. The hydrazone-acetone hydrazone mixture is prepared from the comparison (reference)

Table II.

Hydrazone (2)	Eutektische Temperatur (1)	
	binäre der Hydrazone mit Acetonhydrazon	ternäre der beiden Hydrazone mit Acetonhydrazon (3)
Isobutyraldehyde...	99°	89°
Propionaldehyde[11] ..	101°	
Formaldehyde[11]	124°	110°
Crotonaldehyde....	124°	
Formaldehyde[11]	124°	112°
Acetophenone.....	127°	
Crotonaldehyde....	124°	116°
Acetophenone.....	127°	
Bourbonal.....	129°	125°
Piperonal-[11].....	133°	

1 - Eutectic point
2 - Binary, of hydrazones with acetone hydrazone
3 - Ternary, of both hydrazones with acetone hydrazone

product according to the working procedure described above. A small amount of this mixture is added to the product obtained from the tested material, the two are mixed by sliding the cover glass back and forth, and the start of melting is determined again. If the aldehyde or ketone present in the tested material are identical with the reference product, no depression of the melting point is noted. If this is not the case, a ternary mixture is present, and the eutectic point of this mixture is then recorded. Table II shows ternary eutectic points of aldehydes and ketones, the binary eutectic points of which exhibit similar values. As this table shows, their ternary eutectic points are considerably lower, so that a confusion (mistake) is impossible by the procedure described. The ternary eutectic points in many cases also represent leads (clues), when the detection of two aldehydes and ketones side by side must be carried out.

Summary

A micro method reported by R. Opfer-Schaum for the detection of volatile aldehydes and ketones is extended and used for the detection of diacetyl, acetophenone, bourbonal, isobutyraldehyde and crotonaldehyde. The reagent is p-nitrophenylhydrazine in acetic acid solution, used as a "hanging drop" located above the tested material. The p-nitrophenylhydrazones obtained are identified by their eutectic points with excess reagent and(or) with the acetone hydrazone obtained from the latter by treatment with acetone-water mixture. The detection is made with greater certainty when the hydrazone mixture is prepared in the same manner from a reference product and added to the hydrazone mixture obtained from the tested material. If the aldehyde and(or) ketone in the tested material is identical with the reference product, the eutectic point of the mixture remains unchanged. In the opposite case (when the aldehyde or ketone are not the same), the eutectic point exhibits a definite drop.

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Received for publication on
14 March 1944.

Translated by
A. Schidlovsky (JITCO)
9/10/73

Mikrochemie ver. Mikrochim. Acta 32: 6-10 (1944)

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Zum mikrochemischen Nachweis von Aldehyden und Ketonen.

Von

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(Eingelangt am 14. März 1944.)

Vor kurzem gab *R. Opfer-Schaum* ein einfaches Mikroverfahren zum Nachweis flüchtiger Aldehyde und Ketone an.[1] Das Untersuchungsmaterial befindet sich in einem „Mikrobecher" und das Reagens als „hängender Tropfen" an einem darüberliegenden Objektträger (*Griebel*[2,3]). Als Reagens dient p-Nitrophenylhydrazin in essigsaurer Lösung. Die Identifizierung der im Hängetropfen erhaltenen p-Nitrophenylhydrazone erfolgt durch Bestimmung der *eutektischen Temperatur im Gemisch mit dem überschüssigen Reagens*, bzw. dem aus diesem durch Behandeln mit einer Aceton-Wasser-Mischung gewonnenen Acetonhydrazon.

Das Verfahren hat wesentliche Vorzüge auch gegenüber der von *R. Fischer*[4,5,6] angegebenen Verbesserung der *Griebel*schen Methode. *Fischer* benützte an Stelle der nicht immer eindeutigen Kristallform und -farbe zur Erkennung der p-Nitrophenylhydrazone die Bestimmung des Mikroschmelzpunktes nach vorherigem Waschen zwischen Objektträger und Deckglas. Er gelangt jedoch nach seinen eigenen Angaben nach dieser Arbeitsweise nicht immer zu einem scharf schmelzenden Präparat.

Die nach dem Verfahren von *R. Opfer-Schaum* erhaltenen eutektischen Temperaturen sind scharf und gut reproduzierbar. Darüber hinaus arbeitet es wesentlich schneller. Die Bestimmungen der eutektischen Temperaturen erfolgen nach *L.* u. *A. Kofler*[7,8] auf dem Mikroschmelz-

[1] *R. Opfer-Schaum*, Arch. Pharmaz. Ber. dtsch. pharmaz. Ges. (im Druck).
[2] *C. Griebel*, Z. Unters. Nahrungs- u. Genußmittel 47, 438 (1924).
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punktapparat. Schon bei 1%igem Anteil der einen Komponente läßt sich der Schmelzbeginn gut reproduzierbar bestimmen, wenn nicht Mischbarkeit der festen oder Nichtmischbarkeit der flüssigen Phasen vorliegt.

Größte Sicherheit der Identifizierung wird dadurch erreicht, daß man unter dem Mikroskop *den Mischschmelzpunkt von Gemischen bestimmen kann.*[9]

Für den vorliegenden Fall entwickelte ich eine schnelle und einfache Arbeitsweise. Es ist nicht notwendig, das p-Nitrophenylhydrazon des nachzuweisenden Aldehyds oder Ketons rein darzustellen. Es genügt, wie weiter unten beschrieben und wie in wenigen Minuten durchführbar ist, aus dem in Frage kommenden Aldehyd oder Keton ein Gemisch des Hydrazons mit dem Acetonhydrazon herzustellen. Von diesem gibt man etwas zu dem aus dem Untersuchungsmaterial erhaltenen Hydrazongemisch und bestimmt wieder den Schmelzbeginn. War das aus dem Untersuchungsmaterial erhaltene p-Nitrophenylhydrazon mit dem aus der Vergleichssubstanz gewonnenen identisch, so bleibt die eutektische Temperatur unverändert. War dies nicht der Fall, so liegt jetzt ein Dreistoffgemisch vor, dessen Schmelzbeginn, wie Tabelle II zeigt, wesentlich erniedrigt ist.

Arbeitsvorschrift.

Das auf flüchtige Aldehyde oder Ketone zu prüfende Material gibt man in ein Mikrobecherchen von etwa 1,5 bis 2 cm Höhe und 1 bis 1,5 cm Weite. Das Mikrobecherchen wird mit einem Objektträger bedeckt, an dem sich ein hängender Reagenstropfen befindet. Der „Hängetropfen" wird so vorbereitet, daß man auf den Objektträger einen Tropfen etwa 15%iger Essigsäure („pro analysi aldehydfrei") von etwa 3 mm Durchmesser bringt. In diesem Tropfen verreibt man mittels eines dünnen Glasstabes ein paar Kriställchen des p-Nitrophenylhydrazins, dessen Menge man so gestaltet, daß die Gesamtfläche ungefähr 1 bis 2 qmm ausmacht und ein Teil davon ungelöst bleibt. Das Mikrobecherchen mit dem Hängetropfen läßt man einige Zeit bei Zimmertemperatur oder — bei geringerer Flüchtigkeit der Substanz — bei etwa 40 bis 50° auf dem Heiztisch stehen. Dabei kann man die Bildung des Hydrazons im Hängetropfen mikroskopisch verfolgen. Wenn sich genug Reaktionsprodukt gebildet hat, nimmt man den Objektträger mit dem Reaktionsgut ab und tropft einen Tropfen einer Aceton-Wasser-Mischung (4:1) auf. Man reibt nun mit einem dünnen Glasstab so lange, bis sich das entstandene Reaktionsprodukt sowie auch die Kristalle des p-Nitrophenylhydrazins im Aceton vollständig gelöst haben und die beiden Hydrazone nach dem Verdunsten des überschüssigen Acetons wieder ausfallen. Um sicher zu sein, daß sich das ganze p-Nitrophenylhydrazin in seine Acetonverbindung

[9] *L. Kofler* und *M. Brandstätter*, Angew. Chem. 54, 322 (1941).

126

umgewandelt hat, tropft man ein zweites Mal einen Tropfen Aceton-Wasser-Mischung auf und verreibt wieder. Dann schiebt man den Kristallbrei mit einem etwa 0,5 cm breiten Streifen gehärteten Filtrierpapiers nicht zu dicht zusammen und saugt die überstehende Flüssigkeit möglichst vollständig ab. Da die Saugfähigkeit des gehärteten Filtrierpapiers gering ist, ist es zweckmäßig, den vollgesogenen Teil des Streifens mit einer scharfen Schere mehrmals glatt wegzuschneiden. Darauf legt man das Präparat auf den Heiztisch und trocknet bei 80 bis 100°. Man bestimmt nun die eutektische Temperatur.

Sollte die Essigsäure des Reagenstropfens verdunstet oder zu stark verdünnt sein, so muß sie vor der Acetonbehandlung ergänzt werden. Die Umwandlung in das Acetonhydrazon gelingt sonst nicht vollständig.

Die in der Tabelle I angegebenen Empfindlichkeiten wurden bei im Höchstfall 10 bis 15 Minuten langem Liegenlassen des Reagenstropfens über dem Untersuchungsmaterial bestimmt. Durch Verlängerung der Reaktionszeit sowie durch Verkleinerung des Reagenstropfens dürfte sich die Empfindlichkeit noch steigern lassen.

Tabelle I.

Aldehyd bzw. Keton	Fp. des p-Nitrophenylhydrazons	Eut. Temp. des p-Nitrophenylhydrazons mit Aceton-p-nitrophenylhydrazon	Empfindlichkeit in γ in 1 ml Wasser
Isobutylaldehyd....	132°	99°	50 γ
Crotonaldehyd....	185°	124°	50 γ
Bourbonal.....	184—185°	129°	2 γ in 0,05 g Milchzucker
Acrolein[10].....	162°	122°	5 γ
Diacetyl.....	229,5°	143°	10 γ
Acetophenon.....	184°	127°	20 γ

Die anderen untersuchten Aldehyde oder Ketone geben entweder mit p-Nitrophenylhydrazin überhaupt kein brauchbares Reaktionsprodukt oder es liegen die Schmelzpunkte ihrer p-Nitrophenylhydrazone unterhalb 100° und daher sind Verwechslungen mit den in der Tabelle genannten Aldehyden und Ketonen unmöglich. Nur tröpfchenförmige Reaktionsprodukte wurden erhalten beim n-Octylaldehyd, n-Nonylaldehyd, Methylisobutylketon, Pinakolin, Carvon und Menthon. Benzaldehyd, Zimtaldehyd, Methylpropylketon, Äthyl-n-propylketon, p-Methylacetophenon, Monochloraceton, Monochloracetophenon und Monobromacetophenon gaben kein scharfes und gut reproduzierbares Eutektikum. Unterhalb 100° liegen die Schmelzpunkte der p-Nitrophenylhydrazone des n-Butylaldehyds, n-Valeraldehyds, Isovaleraldehyds,

[10] *R. Opfer-Schaum*, Mikrochem. 31, 324 (1944).

Caprylaldehyds, Önanthols, Methylbutylketons, Methyl-n-hexylketons, Methylisohexylketons, Methyl-n-nonylketons und n-Methylheptenons.

Tabelle II.

Hydrazone	Eutektische Temperatur: binäre der Hydrazone mit Acetonhydrazon	Eutektische Temperatur: ternäre der beiden Hydrazone mit Acetonhydrazon
Isobutylaldehyd-....	99°	89°
Propionaldehyd-[11]..	101°	
Formaldehyd-[11]....	124°	110°
Crotonaldehyd-....	124°	
Formaldehyd-[11]....	124°	112°
Acetophenon-.....	127°	
Crotonaldehyd-....	124°	116°
Acetophenon-.....	127°	
Bourbonal-.....	129°	125°
Piperonal-[11].....	133°	

Ein Teil der hier, bzw. von *Opfer-Schaum*[11] bearbeiteten Aldehyde und Ketone weist ähnliche eutektische Temperaturen auf. Ist man im Besitz der betreffenden Aldehyde und Ketone, so kann man, wie schon oben erwähnt, mit Hilfe des Mischschmelzpunktes der Gemische in wenigen Minuten eine sichere Unterscheidung treffen. Man stellt sich aus der Vergleichssubstanz nach obiger Arbeitsvorschrift das Gemisch Hydrazon-Acetonhydrazon dar. Von diesem gibt man eine kleine Menge zu dem aus dem Untersuchungsmaterial erhaltenen Präparat, vermischt durch Hin- und Herschieben des Deckglases und bestimmt wieder den Schmelzbeginn. Waren der im Untersuchungsmaterial vorhandene Aldehyd oder das Keton und Vergleichspräparat identisch, so tritt keine Depression auf. War dies nicht der Fall, so liegt jetzt ein ternäres Gemisch vor, dessen eutektische Temperatur nun beobachtet wird. In Tabelle II sind die ternären eutektischen Temperaturen von Aldehyden und Ketonen zusammengestellt, deren binäre eutektische Temperaturen ähnliche Werte aufweisen. Wie die Tabelle zeigt, liegen ihre ternären eutektischen Temperaturen wesentlich tiefer, so daß eine Verwechslung bei der beschriebenen Arbeitsweise ausgeschlossen ist. Die ternären eutektischen Temperaturen bieten in manchen Fällen auch Anhaltspunkte, wenn es sich um den Nachweis zweier Aldehyde und Ketone nebeneinander handelt.

[11] *R. Opfer-Schaum*, Arch. Pharmaz. Ber. dtsch. pharmaz. Ges. (im Druck).

Zusammenfassung.

Es wird ein von *R. Opfer-Schaum* angegebenes Mikroverfahren zum Nachweis flüchtiger Aldehyde und Ketone erweitert und auf den Nachweis von Diacetyl, Acetophenon, Bourbonal, Isobutylaldehyd und Crotonaldehyd ausgedehnt. Als Reagens dient p-Nitrophenylhydrazin in essigsaurer Lösung, das sich als „Hängetropfen" über dem Untersuchungsmaterial befindet. Die erhaltenen p-Nitrophenylhydrazone werden durch ihre eutektischen Temperaturen mit dem überschüssigen Reagens, bzw. dem aus diesem durch Behandeln mit einer Aceton-Wasser-Mischung erhaltenen Acetonhydrazon gekennzeichnet.

Größte Sicherheit des Nachweises erreicht man, wenn man in der gleichen Weise aus einer Vergleichssubstanz das Hydrazongemisch herstellt und dem aus dem Untersuchungsmaterial erhaltenen Hydrazongemisch beimengt. Ist der Aldehyd bzw. das Keton im Untersuchungsmaterial mit der Vergleichssubstanz identisch, so zeigt die Mischung eine unveränderte eutektische Temperatur. Im anderen Fall wird die eutektische Temperatur deutlich erniedrigt.

Résumé *(W)*.

L'auteur étend un procédé microchimique indiqué par *R. Opfer-Schaum* pour l'identification d'aldéhydes et de cétones volatils à la recherche du diacétyle, de l'acétophénone, du bourbonal, de l'isobutylaldéhyde et du crotonaldéhyde. Comme réactif, on emploie la p-nitrophénylhydrazine en solution acétique; la réaction s'effectue en exposant une goutte du réactif en dessus de la substance à analyser. On caractérise les p-nitrophénylhydrazones obtenues en déterminant leurs températures eutectiques (eutectiques dues à l'excès de réactif, ou fournis par l'acétonehydrazone produite par traitement de l'excès de réactif par un mélange acétone-eau).

On arrive à de meilleurs résultats, si l'on fait de la même façon l'hydrazone d'une substance de contrôle que l'on ajoute à l'hydrazone obtenue avec la substance à analyser. Si l'aldéhyde ou la cétone de contrôle est identique à celle de la substance à analyser, la température eutectique du mélange ne diffère pas. Si les deux substances (substance de contrôle et substance à analyser) diffèrent, la température eutectique sera nettement abaissée.

1276

A METHOD FOR STANDARDIZING THE BIACETYL CONTENT OF CREAMED COTTAGE CHEESE[1]

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J. Dairy Sci. 42(6): 1045 56

SUMMARY

The addition of 0.15% citric acid to creamed cottage cheese held at 45° F. did not increase the biacetyl content; whereas, addition of *Streptococcus citrovorus* gave a slight increase and addition of both citric acid and *S. citrovorus* gave still more of an increase. No biacetyl was formed in a 12% fat creaming mixture containing *S. citrovorus* or *S. citrovorus* plus 0.15% citric acid. Considerable biacetyl was produced in a creaming mixture containing large numbers of *S. citrovorus* after it was adjusted to pH 4.2 with citric acid and held at 70° F. Cottage cheese creamed with such a preparation had a high initial biacetyl content.

The most satisfactory method of preparing a creaming mixture which contains sufficient biacetyl and volatile acid to influence the flavor of creamed cottage cheese is the following: Heated skimmilk is inoculated with a pure culture of *S. citrovorus* and, after sufficient growth, adjusted to pH 4.3 with sterile citric acid. Further incubation at 70° F. results in the formation of considerable biacetyl and volatile acid. The skimmilk culture thus prepared is used to standardize the fat content of cream to about 12%. Standardization of biacetyl is accomplished by varying the fat content of the cream in the creaming mixture and, therefore, the amount of skimmilk culture to standardize the fat content. Cheese creamed with such a preparation has an appreciable amount of flavor immediately after creaming.

Dairy products manufactured with lactic culture commonly contain some biacetyl. Such products are butter, cultured buttermilk, cottage cheese, and cultured sour cream. A considerable amount of information has been published on methods for increasing the biacetyl content of butter (1) and cultured buttermilk (3, 9), but very little information of this type is available on cottage cheese and cultured sour cream.

Nelson and Breuer (6) applied some of the methods known to increase volatile acid and biacetyl in lactic cultures to cottage cheese. However, cultures which contained considerable volatile acid did not yield cottage cheese with a high volatile acid content. No relationship was found between the flavor of a culture and the flavor of cottage cheese made with it. They noted that the addition of 0.008 to 0.05% citric acid to the cheese milk increased the volatile acid content of the resulting cheese and generally improved its flavor.

Mather and Babel (5) found that many samples of commercially creamed cottage cheese contained less than 0.5 p.p.m. biacetyl. The addition of citric acid to a creaming mixture for cottage cheese did not increase the biacetyl content

Received for publication March 7, 1959.

[1] Published with the approval of the Director of the Purdue Agricultural Experiment Station as Journal Series Paper No. 1399.

[2] Portion of a thesis prepared by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: Breakstone Foods, Inc., Walton, New York.

when the cheese was held at 45° F. Addition of both citric acid and lactic culture to the creaming mixture increased the biacetyl content of the creamed cottage cheese when the holding temperature was 45° F., but a sour flavor was evident after several days of storage.

The object of this study was to develop a method for increasing and standardizing the biacetyl content of creamed cottage cheese.

METHODS

Biacetyl and acetylmethylcarbinol. The colorimetric method of Prill and Hammer (7) was used for the estimation of biacetyl and acetylmethylcarbinol in cottage cheese, creamed cottage cheese, and cultures. Color intensity was measured with a Beckman spectrophotometer (Model B) at a wave length of 430 mμ. Dimethylglyoximate was used in constructing the standard cure.

Volatile acid. A 50-g. sample was acidified with 3 ml. of 1.0 *N* sulfuric acid and steam-distilled. The first 100 ml. of distillate was titrated with 0.1 *N* sodium hydroxide, using phenolphthalein as indicator.

Determination of pH. Samples of cheese were prepared and tested by the method of Sanders (8). A Leeds and Northrup Type K potentiometer fitted with a quinhydrone electrode and saturated calomel cell was used for all pH measurements on cheese. A Beckman Laboratory Model pH meter was used to adjust the pH of cultures and creaming mixtures.

Cottage cheese. The long-set method outlined by Hales (4) was used for cottage cheese manufacture. When creamed, a 12% fat creaming mixture was added at the rate of one part creaming mixture to two parts cheese. The creamed cottage cheese was salted at the rate of 1%.

RESULTS

Effect of addition of citric acid and a culture of S. citrovorus to a creaming mixture on the biacetyl content of creamed cottage cheese. The rather low pH of well-mixed samples of creamed cottage cheese suggested that a pure culture of a citric acid–fermenting *Streptococcus*, of the type commonly associated with *Streptococcus lactis* in lactic cultures, might produce biacetyl from citric acid when added to a creaming mixture. The culture used in this trial was selected on the basis of its ability to produce biacetyl from citric acid at a relatively low pH.

One batch of cottage cheese was divided into five lots and treated as follows: (1) No treatment, (2) creamed and salted, (3) creamed, salted, and 0.15% citric acid added, (4) creamed, salted, and 1% of *S. citrovorus* culture added, (5) same as Lot 4 except that 0.15% citric acid was added. Samples of each lot were held at 45° F. and examined at intervals for biacetyl, acetylmethylcarbinol, and pH.

Table 1 shows that the biacetyl content of this batch of cottage cheese decreased slightly over a nine-day period; the carbinol content and pH remained about the same. Creaming the cottage cheese decreased the biacetyl content and a further decrease occurred during holding. The addition of 0.15% citric acid to creamed cottage cheese did not increase the biacetyl and carbinol contents, but

TABLE 1

Biacetyl, acetylmethylcarbinol, and pH values of cottage cheese and creamed cottage cheese without and with addition of citric acid and/or a culture of *Streptococcus citrovorus*

Product and treatment	Days held at 45° F.														
	Biacetyl					Acetylmethylcarbinol					pH				
	0	2	4	6	9	0	2	4	6	9	0	2	4	6	9
	(p.p.m.)					(p.p.m.)									
Cottage cheese	1.6	1.6	1.4	1.3	1.0	11.3	18.2	16.2	18.5	17.3	4.73	4.80	4.75	4.73	4.72
Creamed cottage cheese	1.1	1.0	1.0	0.9	0.6	11.5	18.8	8.3	7.7	7.5	4.90	5.03	5.03	5.00	4.98
Creamed cottage cheese plus citric acid	1.1	1.0	1.0	1.0	1.0	9.7	11.9	9.8	13.7	9.2	4.71	4.75	4.75	4.75	4.71
Creamed cottage cheese plus *S. citrovorus*	1.1	1.3	1.4	1.6	1.8	11.5	17.6	19.6	28.8	21.0	4.90	5.00	4.93	4.83	4.57
Creamed cottage cheese plus citric acid and *S. citrovorus*	1.1	1.3	1.7	2.3	2.7	9.4	17.0	27.3	41.8	63.7	4.68	4.72	4.73	4.73	4.59

1216

seemed to stabilize the amounts initially present; such an addition gave a definite decrease in pH. Addition of *S. citrovorus* to creamed cottage cheese resulted in a progressive increase in biacetyl throughout the holding period. This addition

seemed to stabilize the amounts initially present; such an addition gave a definite decrease in pH. Addition of *S. citrovorus* to creamed cottage cheese resulted in a progressive increase in biacetyl throughout the holding period. This addition also gave increases in carbinol for six days, a slight decrease occurring at nine days. The addition of citric acid and *S. citrovorus* culture to creamed cottage cheese gave progressive increases in biacetyl and carbinol throughout the holding period, and the increases were greater than those obtained without added citric acid.

Formation of biacetyl and acetylmethylcarbinol in a creaming mixture for cottage cheese. Since previous experiments indicated that biacetyl could be produced in creamed cottage cheese inoculated with *S. citrovorus*, but that some holding time was necessary, trials were conducted to determine whether biacetyl could be formed in the creaming mixture prior to its addition to cottage cheese. The creaming mixture used for these studies was heated in flowing steam for 30 min. and then cooled to 45° F.

Data presented in Table 2 show that a 12% fat creaming mixture with no additions (Lot 1), and held at 45° F., contained no biacetyl or carbinol during four days. Also, addition of a culture of *S. citrovorus* to the creaming mixture (Lot 2) did not increase the biacetyl or carbinol content at 45° F. The addition of *S. citrovorus* plus 0.15% citric acid to the creaming mixture (Lot 3) caused a very slight increase in both biacetyl and carbinol after holding three days at 45° F. A creaming mixture inoculated with *S. citrovorus* and held at 70° F. (Lot 4) did not contain biacetyl after holding for three days. However, when this lot was acidified with citric acid to pH 4.24 on the third day, a conspicuous increase in both biacetyl and carbinol occurred within 24 hr. at 70° F. Much smaller amounts of these compounds were formed when the acidified creaming mixture was held at 45° F. (Lot 5). No biacetyl was detected in a creaming

TABLE 2

Effect of holding temperature, pH, and citric acid on the production of biacetyl and acetylmethylcarbinol in a cottage cheese creaming mixture inoculated with *Streptococcus citrovorus*

Lot No.[a]	Storage temperature	Holding time (days)											
		0	1	3	4	0	1	3	4	0	1	3	4
		Biacetyl				Acetylmethylcarbinol				pH			
		(p.p.m.)				(p.p.m.)							
1	45	0.0	0.0	0.0	0.0	0.4	0.4	0.0	0.2	6.65	6.62	6.70	6.50
2	45	0.0	0.0	0.0	0.0	0.3	0.8	0.4	0.2	6.50	6.54	6.25	6.20
3	45	0.0	0.0	0.3	0.4	0.3	1.1	7.3	7.1	5.78	5.92	5.85	5.75
4	70	0.0	0.0	0.0	6.5	0.3	0.7	0.2	149.5	6.48	6.40	6.25	4.18
5	45				2.3				56.9				4.08
6	70	0.0	0.0	0.0	6.0	0.2	26.0	20.0	139.8	5.64	5.89	5.88	4.12
7	45				1.4				126.6				4.02

[a] Lot 1. Creaming mixture (12% fat); Lot 2. Creaming mixture plus *S. citrovorus*; Lot 3. Creaming mixture plus *S. citrovorus* and 0.15% citric acid; Lot 4. Creaming mixture plus *S. citrovorus*; acidified to pH 4.24 on third day with citric acid; Lot 5. Same as Lot 4, but held at 45° F. after acidifying to pH 4.24; Lot 6. Creaming mixture plus *S. citrovorus* and 0.15% citric acid; acidified to pH 4.22 on third day with citric acid; Lot 7. Same as Lot 6, but held at 45° F. after acidifying to pH 4.22.

mixture containing *S. citrovorus* plus 0.15% citric acid and held at 70° F. for three days (Lot 6), but when this lot was acidified with citric acid to pH 4.22 on the third day, a large increase in biacetyl and carbinol occurred. The increase was larger with holding at 70° F. (Lot 6) than at 45° F. (Lot 7).

Effect of an acidified creaming mixture containing S. citrovorus on the biacetyl content of creamed cottage cheese. A creaming mixture containing 12% fat was heated in flowing steam for 1 hr., cooled to 70° F., and inoculated with 4% of a culture of *S. citrovorus*. The mixture was held at 70° F. for 48 hr. and acidified with sterile citric acid solution (10%) to pH 4.38. After the pH adjustment, the mixture was held for 21 hr. at 70° F.

Table 3 gives the biacetyl, acetylmethylcarbinol, and pH values of cottage cheese (Lot 1), regularly creamed cottage cheese (Lot 2), and cottage cheese creamed with the special creaming mixture containing *S. citrovorus* (Lot 3). The cottage cheese and regularly creamed cottage cheese showed only small variations in biacetyl throughout a seven-day holding period at 45° F. Creaming cottage cheese with the regular creaming mixture decreased the biacetyl content and increased the pH. The cottage cheese creamed with the special creaming mixture contained 3.6 p.p.m. of biacetyl immediately after creaming and 5.7 p.p.m. after holding for seven days at 45° F. The carbinol content of this lot also was considerably greater than either of the two other lots. Regularly creamed cottage cheese had the highest pH and the uncreamed product the lowest pH.

Production of biacetyl, acetylmethylcarbinol, and volatile acid in a skimmilk culture of S. citrovorus adjusted to various pH levels with citric acid. In the experiments which follow, skimmilk is used as the growth medium for *S. citrovorus* and also for the development of fermentation products from citric acid. Therefore, an experiment was conducted to determine the most favorable pH for the production of biacetyl and volatile acid, compounds which contribute to the flavor of creamed cottage cheese.

A large lot of skimmilk was heated in flowing steam for 1 hr. and cooled to 70° F. One portion was removed to serve as a control. The remainder was inoculated with 2% of a culture of *S. citrovorus*, divided into seven portions, and incubated at 70° F. for 48 hr. After incubation, sterile citric acid solution (15%) was added in varying amounts to give a pH range of 5.97 to 3.72. The acidified samples were held at 70° F. for 24 hr. and analyzed for biacetyl, acetylmethylcarbinol, and volatile acid. Results of these analyses are given in Table 4.

Only small amounts of biacetyl were presented in the skimmilk samples having a pH above 5.01. The difference between pH 5.01 and 5.50 was striking, since the former yielded about a fivefold increase over the latter. Further increases occurred as the pH was decreased, but the amount of increase was not great at pH values less than 4.31. Acetylmethylcarbinol and volatile acid increased as the pH was decreased and maximum amounts were obtained at pH 4.31. Further lowering of the pH resulted in slightly smaller amounts of these compounds.

Preparation of a creaming mixture for standardizing the biacetyl content of creamed cottage cheese. The development of biacetyl, acetylmethylcarbinol, and

TABLE 3

Biacetyl, acetylmethylcarbinol, and pH values of cottage cheese and creamed cottage cheese, using a regular creaming mixture and a creaming mixture inoculated with *Streptococcus citrovorus* and adjusted to pH 4.38 with citric acid

Lot No.[a]	Days held at 45° F.														
	0	2	4	6	7	0	2	4	6	7	0	2	4	6	7
	Biacetyl					Acetylmethylcarbinol					pH				
	(p.p.m.)					(p.p.m.)									
1	0.9	1.2	1.1	1.2	0.9	9.7	2.9	7.5	9.9	15.9	4.67	4.60	4.60	4.60	4.60
2	0.6	0.7	0.6	0.7	0.6	9.3	10.7	9.9	9.6	8.2	5.06	4.99	4.98	4.99	5.00
3	3.6	4.7	4.8	5.0	5.7	84.6	97.3	102.0	114.0	128.3	4.62	4.78	4.77	4.75	4.80

[a] Lot 1. Cottage cheese; Lot 2. Cottage cheese creamed with regular creaming mixture (12% fat); Lot 3. Cottage cheese creamed with a special creaming mixture containing *S. citrovorus* and adjusted to pH 4.38 with citric acid.

TABLE 4

Biacetyl, acetylmethylcarbinol, and volatile acids content of a skimmilk culture of *Streptococcus citrovorus* adjusted to various pH levels with citric acid

pH	Biacetyl	Acetylmethylcarbinol	Volatile acids
	(p.p.m.)	*(p.p.m.)*	*(ml. N/10 NaOH)*
6.41*	0.2	0.8	0.2
5.97	0.4	1.6	1.2
5.50	0.7	2.1	1.3
5.01	3.4	49.6	2.1
4.62	6.1	119.3	2.4
4.31	10.7	274.1	3.1
4.02	11.1	230.1	2.9
3.72	12.0	222.0	2.5

* No pH adjustment. No citric acid added.

volatile acid in a 12% fat creaming mixture presented certain problems, particularly with regard to standardization of the biacetyl content. Therefore, the following method was adopted.

Skimmilk is heated to 190° F. and held for 1 hr., or it can be heated in an autoclave at 250° F. for 10 min. After cooling to 70° F., it is inoculated with 2 to 4% of a culture of *S. citrovorus* and incubated at 70° F. for about 24 hr. Sterile citric acid solution (15%) is added to lower the pH to 4.3, and the material is further incubated at 70° F. for 20 to 24 hr. Considerable biacetyl, acetylmethylcarbinol, and volatile acid is formed. The skimmilk thus prepared is used to adjust the fat content of cream to about 12%. The mixture is homogenized at 500 to 1,000 p.s.i. Standardization of biacetyl is accomplished by varying the fat content of cream used in the creaming mixture and, therefore, the amount of skimmilk culture to standardize the fat content. When 20% fat cream is used for preparing a mixture containing 12% fat, the ratio of cream to fermented skimmilk is 1.5 to 1.0, by weight.

Table 5 gives the biacetyl, acetylmethylcarbinol, and pH values of a batch of cottage cheese treated as follows: (1) No treatment, (2) creamed with a regular creaming mixture containing 12% fat, (3) creamed with a mixture made up of 60 parts 20% cream and 40 parts of the specially treated skimmilk containing *S. citrovorus* (when added to cheese, the specially treated skimmilk culture constituted 13.5% of the creamed cottage cheese), and (4) creamed with a creaming mixture made up of 80 parts cream and milk containing 15% fat and 20 parts of the specially treated skimmilk containing *S. citrovorus* (when added to cheese, the specially treated skimmilk culture constituted 6.75% of the creamed cottage cheese).

The cottage cheese used in this experiment contained about 0.5 p.p.m. biacetyl initially and it remained at this level throughout an 11-day holding period at 45° F. Creaming with a regular creaming mixture decreased the biacetyl content to 0.3 p.p.m. When creamed with the special creaming mixture so that the *S. citrovorus* culture constituted 13.5% of the creamed cottage cheese, the biacetyl content was 1.6 p.p.m. initially and it increased to 2.8 p.p.m. during 11 days of storage at 45° F. The lot in which *S. citrovorus* culture constituted

TABLE 5

Biacetyl, acetylmethylcarbinol, and pH values of cottage cheese, and creamed cottage cheese using a regular and special creaming mixture

Product	Days held at 45° F.														
	0	4	6	8	11	0	4	6	8	11	0	4	6	8	11
	Biacetyl					Acetylmethylcarbinol					pH				
	(p.p.m.)					(p.p.m.)									
Cottage cheese	0.5	0.6	0.5	0.5	0.5	8.6	10.0	8.3	9.0	8.6	4.68	4.66	4.68	4.69	4.65
Regularly creamed cottage cheese	0.3	0.3	0.3	0.4	0.3	6.2	6.4	7.1	6.4	6.0	4.99	5.05	5.05	5.02	5.03
Specially creamed cottage cheese[a]	1.6	2.2	2.2	2.4	2.8	39.6	52.2	43.4	51.2	50.6	4.94	5.00	4.98	4.98	4.92
Specially creamed cottage cheese[b]	0.9	1.4	1.4	1.7	1.9	22.3	24.8	27.1	28.9	35.1	5.01	5.00	4.98	4.97	5.00

[a] *Streptococcus citrovorus* culture constituted 13.5% of the creamed cottage cheese.
[b] *Streptococcus citrovorus* culture constituted 6.75% of the creamed cottage cheese.

1276

0.75% of the creamed cottage cheese had a biacetyl content of 0.9 p.p.m. initially and it increased to 1.9 p.p.m. during 11 days of storage at 45° F. The largest amount of acidified *S. citrovorus* culture decreased the pH of the creamed cottage cheese by 0.04 to 0.11 of a pH unit.

This experiment was repeated in another trial, in which the cottage cheese and creamed cottage cheese were held at 50° F. The data obtained are reported in Table 6.

The cottage cheese used in this trial contained 0.3 p.p.m. of biacetyl initially and only slight variations were noted during the eight-day holding period. Also, the acetylmethylcarbinol content and pH varied only slightly. The cottage cheese had a bitter and yeasty flavor at eight days. The regularly creamed cottage cheese contained 0.2 to 0.3 p.p.m. biacetyl throughout the holding period. The carbinol content and pH remained quite stationary for five days, but decreased considerably at eight days. This lot also had a bitter and yeasty flavor at eight days. Cottage cheese creamed with the largest amount of acidified *S. citrovorus* culture had an initial biacetyl content of 1.4 p.p.m., which increased to 2.6 p.p.m. in five days, followed by a decrease to 1.8 p.p.m. in eight days. The carbinol content followed the same general trend as the biacetyl content and the pH remained quite constant throughout the holding period. This lot did not develop an off-flavor within eight days. Cottage cheese creamed with the smaller amount of acidified *S. citrovorus* culture showed the same general trend in biacetyl and carbinol as the lot creamed with the larger amount. However, a decrease in pH was noted at eight days and at this time the cheese had a bitter and yeasty flavor.

DISCUSSION

In previous experiments (5), the addition of lactic culture to a creaming mixture resulted in the formation of some biacetyl, but gave the cheese a sour flavor after holding at 45° F. Therefore, the use of a culture of *S. citrovorus* seemed to offer some possibilities. *S. citrovorus* does not form lactic acid from lactose but does produce biacetyl from citric acid in pure culture at a favorable pH.

The addition of *S. citrovorus* to a creaming mixture for cottage cheese caused definite increase in biacetyl (Table 1), but approximately nine days at 45° F. was required to obtain a desired level of flavor. Acetylmethylcarbinol, the precursor of biacetyl, began to decrease at nine days. The addition of 0.15% citric acid to the creamed cottage cheese, plus *S. citrovorus*, resulted in a desired flavor level being attained in about four days. The carbinol content of this lot increased throughout the nine-day holding period.

The time required to attain a desired level of flavor when *S. citrovorus* and citric acid were added to creamed cottage cheese was considered a definite disadvantage. However, the results suggested that it might be possible to develop the flavor compounds (biacetyl and volatile acid) in a cottage cheese creaming mixture before its addition to cottage cheese.

Little or no biacetyl was formed in a creaming mixture containing *S. citrovorus* or *S. citrovorus* plus 0.15% citric acid and held four days at 45° F. (Table 2).

1216

This temperature was unfavorable for the growth of *S. citrovorus* and the pH of the creaming mixture was too high for citric acid fermentation. Although 70° F. was a satisfactory growth temperature for *S. citrovorus*, no biacetyl was formed by the organism at this temperature until the pH of the creaming mixture was lowered. Decreasing the pH to 4.24 with citric acid resulted in the accumulation of much biacetyl (6.5 p.p.m.) in 24 hr. When 0.15% citric acid was added to the creaming mixture before the addition of *S. citrovorus*, followed by incubation and acidification with citric acid to pH 4.22, slightly less biacetyl was produced. This decrease was probably due to less growth of *S. citrovorus* because of the lower initial pH.

A creaming mixture inoculated with *S. citrovorus*, incubated 48 hr. at 70° F., then acidified with citric acid to pH 4.38 and further incubated for 21 hr. at 70° F., contained about 9 p.p.m. of biacetyl. This creaming mixture, added to cottage cheese containing 0.9 p.p.m. biacetyl, increased the biacetyl content to 3.6 p.p.m. (Table 3). Such an addition resulted in the creamed cottage cheese having considerable flavor immediately after creaming. Additional flavor was formed in the creamed cottage cheese as it was held at 45° F. Cottage cheese creamed in this manner has a lower pH than regularly creamed cottage cheese, but the product did not taste sour because of the masking effect caused by biacetyl and volatile acid.

Heating a creaming mixture containing 12% fat in flowing steam for 1 hr., or in an autoclave at 250° F. for 10 min., caused some fat separation. Acidification of the creaming mixture with citric acid caused precipitation of the casein. Although homogenization partially overcame these physical changes, a creaming mixture undergoing such treatment gave a creamed cottage cheese that was quite dry in appearance, and in texture when eaten. To eliminate these undesirable features, skimmilk was used as the growth medium for *S. citrovorus* and was later acidified with citric acid. With this procedure, the flavor compounds were formed in skimmilk by a pure culture of *S. citrovorus* and the skimmilk was mixed with cream and homogenized to form the creaming mixture. This method eliminated the physical defects noted earlier and also permitted some standardization of the biacetyl content, since the amount of skimmilk culture used could be varied by the use of cream with different fat contents.

A 20% fat cream standardized to 12% with the acidified skimmilk culture of *S. citrovorus* yielded creamed cottage cheese with a pleasing flavor and a fairly high initial biacetyl content (Table 5). The biacetyl content of cottage cheese creamed in this manner also increased during an 11-day holding period at 45° F.

The storage temperature of cottage cheese has considerable effect on the time required to produce certain bacteriological defects (2). Trials were made to determine what effect a storage temperature of 50° F. would have on the biacetyl content of cottage cheese creamed with the special creaming mixture containing *S. citrovorus*. A representative trial reported in Table 6 shows that the biacetyl content increases for about five days at 50° F. and then decreases. It should be noted that flavor defects were evident sooner in cottage cheese, regularly creamed

TABLE 6

Biacetyl, acetylmethylcarbinol, and pH values of cottage cheese, regularly creamed cottage cheese, and specially creamed cottage cheese held at 50° F.

Product	Days held at 50° F.											
	0	2	5	8	0	2	5	8	0	2	5	8
	Biacetyl				Acetylmethylcarbinol				pH			
	(p.p.m.)				(p.p.m.)							
Cottage cheese	0.3	0.4	0.4	0.2*	8.7	8.6	8.4	8.0	4.61	4.58	4.63	4.55
Regularly creamed cottage cheese	0.2	0.2	0.3	0.2*	6.5	7.0	6.1	0.7	4.97	4.96	4.95	4.86
Specially creamed cottage cheese[a]	1.4	2.1	2.6	1.8	43.4	53.9	63.4	46.2	4.87	4.88	4.85	4.86
Specially creamed cottage cheese[b]	0.9	1.4	1.7	1.3*	27.1	31.0	37.5	22.7	4.93	4.93	4.93	4.88

*A bitter and yeasty flavor was noted at this time.

[a] *Streptococcus citrovorus* culture constituted 13.5% of the creamed cottage cheese.

[b] *Streptococcus citrovorus* culture constituted 6.75% of the creamed cottage cheese.

1216

cottage cheese, and cottage cheese creamed with a small amount of *S. citrovorus* culture, than in cottage cheese creamed with a larger amount of this culture.

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1298

STEAM DISTILLATION OF TAINTS FROM CREAM.

VI. BUTTERFAT/LACTOSE-SOLUTION DISTRIBUTION COEFFICIENTS OF SOME REFERENCE SUBSTANCES

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(With 3 Figures)

J. Dairy Res. 26(1): 39-45 (1959)

The vapour pressure of volatile tainting substances in cream is related primarily to the concentration of the substance in the aqueous portion of the cream ((1), p. 325). For any given concentration of the taint in a cream of given fat content, the concentration in the plasma is dependent on the distribution coefficient between butterfat and cream plasma for the particular substance at the temperature obtaining (2). Since the tainting substances normally present in commercial cream have not been identified, it is necessary in the meantime in investigations on this subject to use appropriate reference substances for study of the influence of different factors on the effectiveness of cream deodorization equipment (1, 3).

Distribution equilibrium relationships have been extensively studied in connexion with investigations on the purification or isolation of substances by multiple and counter-current extraction processes (4). There is, however, little information on these relationships for substances in very dilute solution. For the two substances earlier used as reference substances, acetoin and diacetyl, the butterfat/water distribution coefficients were found to be relatively small (1), and therefore the effect of presence of butterfat in the cream on the vapour/liquid equilibrium coefficient was not of major significance (2, 5). It seemed likely that the distribution coefficients for the higher diketones would be greater, and that therefore they would cause a more important change in the vapour/liquid equilibrium relationship for cream as compared with water or lactose solutions.

In cream deodorization equipment the vapour/liquid equilibrium of the tainting substances between steam and cream is normally established at elevated temperatures, although in most types of apparatus there is provision for application of some degree of reduced pressure. In the Vacreator vacuum-pasteurizer of the design that has been in common use for upwards of 30 years (6) the main deodorization occurs at 6–15 in. of mercury reduction in pressure; but in the final, or flash-cooling, section, the partition of the substances between steam and cream takes place at a relatively high vacuum, viz. 26–28 in. of mercury, corresponding to temperatures of 126–101° F. In some types of equipment the only steam available as carrier vapour to effect the deodorization is obtained by flash ebullition as the result of transfer of the cream to a region of lower pressure. The quantity of deodorization steam produced is then directly related to the pressure change, and the lower the temperature of deodorization the greater is the quantity of carrier vapour released. Since the concentration of the tainting substance in the vapour is directly proportional to the concentration in the aqueous phase at time of partition, the effect of change of temperature on the solubility distribution equilibrium of

a substance between butterfat and cream plasma can be expected to influence the efficiency of taint removal from the cream at the temperature of steam distillation applied. Reference has been made to this aspect in previous papers in this series ((1), p. 325; (5), pp. 54–6).

Some results of direct measurement of the butterfat/water distribution coefficients for diacetyl and acetoin at 40° and 60° C. have already been presented ((1), p. 325). These measurements were made using a centrifuge for separation of the mixture into two layers. In the centrifugal method, however, there is difficulty in maintaining the temperature during the centrifuging, and the results are somewhat unreliable. It was found that with care to avoid vigorous agitation a satisfactory separation of a reasonably clear aqueous layer could be obtained without centrifuging. It was then possible to ensure separation at approximately the desired temperature. When the substance under investigation has a high vapour/liquid equilibrium coefficient, however, there is at high temperatures danger of loss in vapour form during separation of the two phases. Measurements by the direct separation method were therefore done only at 40° and 60° C. Since the aqueous phase in cream is a slightly diluted skim-milk, the measurements of distribution coefficient by the direct method were made with 4·5% lactose solutions in contact with the butterfat (7).

In milk the butterfat is present as globules of average diameter 3–5 μ. Because of the fineness of this dispersion of butterfat, it may be assumed that in milk the distribution equilibrium of a substance between butterfat and plasma is rapidly established. Based on this assumption an indirect method was utilized for measuring the coefficient of distribution of some of the reference substances between butterfat and skim-milk, and the effect of change in temperature on this coefficient. It required use of a plate pasteurizer for continuously heating whole milk to the desired temperature and an hermetic cream separator for separating the preheated milk into cream and skim-milk. Estimations by this method were made only with acetoin, diacetyl and acetylpropionyl, other diketones not being available in sufficient quantity for investigation by this method.

EXPERIMENTAL

Measurement of butterfat/water distribution coefficient

(*a*) *Direct method.* Butterfat (50 g.) and 4·5% lactose solution (50 g.) were placed in each of two conical separating funnels with short outlet tubes, and the substance under investigation was added in aqueous solution to give a suitable over-all concentration. The actual concentration used was varied with variation in the distribution coefficient for the substance under investigation, to give a suitable concentration in the aqueous phase for direct colorimetric estimation. The over-all concentrations ranged from 2 to 250 p.p.m. One of the funnels was placed in a water-bath at 40° C. and the other in a water-bath at 60° C. They were allowed to stand for 30 min. to permit heating of the contents to bath temperature and were then inverted several times at frequent intervals for 30 min. For substances with a very high distribution coefficient in favour of the butterfat it was an advantage to add only a portion of the lactose solution to begin with, and after mixing to stand at the desired temperature for some time to permit approximate establishment of equilibrium before adding the remainder of the water (establishment of the final equilibrium is more rapid when the substance is required to diffuse from the more con-

centrated to the more dilute solution). The funnels were finally allowed to stand in the water-bath until the aqueous layer had reached maximum clarity, complete clarity being seldom obtained. The diketone was estimated directly in the aqueous layer by use of the diaminobenzidine reagent of Pien, Baisse & Martin(8). The concentration in the butterfat layer was calculated by difference, on the assumption that there was no loss during the period of establishment of equilibrium. The butterfat/water distribution coefficient was derived from the results for the concentrations in the two phases at equilibrium.

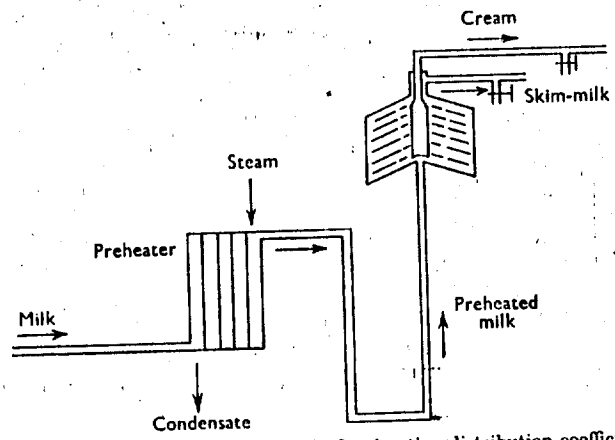

Fig. 1. Apparatus for indirect method of estimating distribution coefficients.

(*b*) *Indirect method.* A 660 gal./hr. Alfa-Laval hermetic cream separator was connected by a $1\frac{1}{2}$ in. diameter pipe, 15 ft. long, to a plate preheater. The cream and skim-milk outlets from the separator were fitted with side-tubes and milking-machine dropper-taps, and the tap tubulures were fitted with rubber bungs to take the neck of a 160 ml. sample bottle (Fig. 1). Whole milk of good quality was pasteurized and at the same time cleared relatively free from naturally occurring diacetyl by treatment at high temperature and high intensity in a Vacreator vacuum-pasteurizer. Pasteurization was necessary to eliminate bacteria that otherwise might have destroyed diacetyl or other α-diketone used as reference substance. The reference substance was added to the cooled pasteurized milk to give the required concentration. The milk was then pumped at constant rate through the plate heat-exchanger, where it was raised by direct steam to the required temperature. The heated milk was delivered to the separator at about 300 gal./hr., giving a holding time of 13–14 sec. at the final temperature. The outlet control valves were adjusted to give a cream of about 35 % fat content. Samples of the cream and skim-milk were taken simultaneously through the sampling cocks into bottles fitted tightly on to the rubber stoppers on the tubulures, thus avoiding loss of reference substance from the hot liquid to the atmosphere during sampling. The bore of the tubulures was large enough to permit gravity flow of the sample into the bottle by direct displacement of air.

By varying the temperature of preheating a series of samples was obtained representing the equilibrium conditions over the whole required range of temperatures, without interruption of delivery of milk from the vat.

Butterfat was estimated in the milk samples by the Gerber method, in the cream samples by the Babcock method, and in the skim-milk samples by the Roese–Gottlieb

method. Diketones were estimated in the skim-milk and in the cream by the method of Pien *et al.* (8).

The fat contents of the skim-milk were uniformly low, of the order of 0·06–0·08%. The effect of this fat on the distribution coefficient was therefore not taken into account in the calculations. The concentration of diketone in the fat, and hence the distribution coefficient, was computed as in the following example:

% fat and % plasma in cream by estimation	= 37 and 63
Concentration of diketone in skim-milk by estimation	= 0·87 p.p.m.
Wt. of diketone in the plasma of 100 g. cream	$= 63 \times 0{\cdot}87 = 55\,\mu g.$
Concentration of diketone in cream by estimation	= 1·2 p.p.m.
Wt. of diketone in 100 g. of cream	$= 100 \times 1{\cdot}2 = 120\,\mu g.$
Wt. of diketone in fat in 100 g. of cream	$= 120 - 55 = 65\,\mu g.$
Concentration of diketone in fat	$= \frac{65\,\mu g.}{37\text{ g.}} = 1{\cdot}7$ p.p.m.
Hence butterfat/skim-milk distribution coefficient	$= \frac{1{\cdot}7}{0{\cdot}87} = 2{\cdot}0$

The method was subject to considerable error because of error of colorimetric estimations of diketone; moreover, an error in estimation of diketone in the skim-milk led to an error in the opposite direction in the result for content of diketone in the fat. The error thus had a double effect on the result for distribution coefficient. In addition, for substances with a high distribution coefficient, a small error in the estimation of concentration in the liquid introduces a large error into the final experimental value for the coefficient. The results, however, were considered to be sufficiently accurate for the purposes of the present investigation, which is designed to formulate general principles rather than to provide standard values for the characteristics of the diketones used.

RESULTS

Distribution coefficients

(*a*) *Direct method.* The results for estimations on acetoin and a number of α-diketones at 40° and 60° C. are given in Table 1. Except for acetoin and acetylbenzoyl there was an appreciable rise in the coefficient with rise in temperature from 40° to 60° C.

Table 1. *Effect of temperature on the coefficients of distribution between butterfat and 4·5% lactose solution*

(Estimations by direct method)

Substance	Distribution coefficients at 40° C. (103·7° F.)	60° C. (140° F.)	100° C. (by extrapolation)
Acetoin*	0·18	0·19	0·21
Diacetyl	0·67	0·89	1·33
Acetylpropionyl	2·3	3·3	5·3
Acetyl-*iso*-butyryl	11·3	14·3	20·3
Acetylvaleryl	17·4	19·4	23·4
Acetylcaproyl	110	124	152
Acetylbenzoyl	51	50	48

* Results for water solution as quoted in (1), p. 325.

(*b*) *Indirect method.* Results for acetoin and diacetyl are given in Fig. 2 and for acetylpropionyl in Fig. 3. The distribution-coefficient/temperature relationship was linear for all three substances, and for all three there was a rise in distribution coefficient with rise in temperature. For acetoin the change between 85° and 200° F. was from 0·19 to 0·25

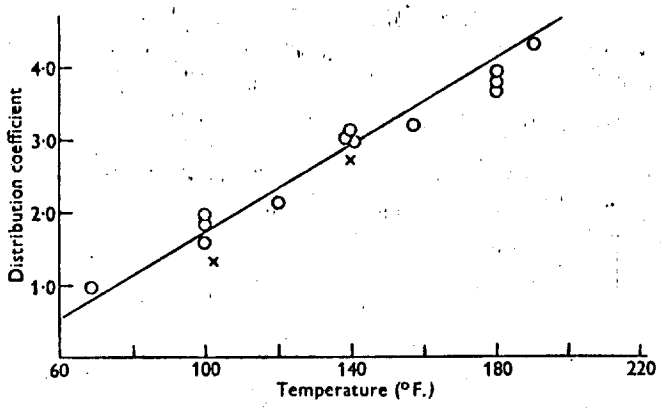

Fig. 2. Effect of temperature on coefficients of distribution of acetoin and diacetyl between butterfat and skim-milk. (Points marked × represent results obtained by direct method.)

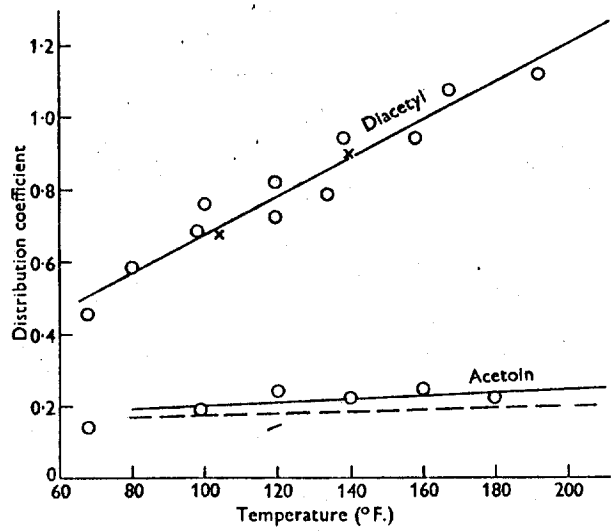

Fig. 3. Effect of temperature on coefficient of distribution of acetylpropionyl between butterfat and skim-milk. (Points marked × represent results obtained by direct method.)

and the extrapolated value at 212° F. was 0·255; for diacetyl the corresponding figures were 0·6–1·2, and 1·27; and for acetylpropionyl they were 2·3–5·6, and 5·9. The results obtained by the direct method for the coefficient of distribution of diacetyl and acetylpropionyl between butterfat and 4·5% lactose solution, shown as a cross in Figs. 2 and 3, were in reasonably good agreement with those obtained by the indirect method for the coefficients between butterfat and skim-milk.

*Distribution coefficients for acetyl-*iso*-butyryl, acetylvaleryl, acetylcaproyl and acetylbenzoyl at* 100° C.

In the continuous vaporization equilibrium still[9,10] the vapour/liquid equilibrium coefficients are measured at the prevailing atmospheric pressure, which at this Institute corresponds to a boiling-point for water of approximately 100° C. To permit computation of the expected vapour/liquid equilibrium relationship for a reference substance in cream, therefore, it was necessary to know the distribution coefficient at 100° C. For acetoin, diacetyl and acetylpropionyl this was read by extrapolation from the average lines in Figs. 2 and 3. For the higher diketones under investigation it was computed by extrapolation to this temperature from the experimental results obtained by the direct method at 40° and 60° C. (Table 1), on the assumption that the distribution-coefficient/temperature relationships were linear as was found for diacetyl and acetylpropionyl (Figs. 2, 3). The extrapolated values are shown in the last column of Table 1.

Effect of concentration of reference substance on the distribution coefficient

The results shown in Figs. 2 and 3 for diacetyl and acetylpropionyl were obtained with concentrations of 1 and 10 p.p.m. of reference substance in the original milk. From the close grouping of the points around the average line it is apparent that over this range of concentration there was no change in magnitude of the distribution coefficient with change in concentration. Results obtained by the indirect method over a wider range of concentrations are given in Table 2. At both temperatures the coefficients were constant within the range of accuracy of the method used.

Table 2. *Effect of concentrations of reference substance on butterfat/skim-milk distribution coefficients at* 112° F. *and* 180° F.

	Temperature of partition equilibrium			
	112° F.		180° F.	
Diketone	Concn. in skim-milk at time of partition	Distribution coefficient	Concn. in skim-milk at time of partition	Distribution coefficient
Diacetyl	0·9	0·67	0·8	1·15
	2·2	0·82	2·2	1·05
	4·2	0·75	4·0	1·05
	8·2	0·78	7·4	1·29
	18·4	0·61	15·0	1·14
	35·5	0·71	21·0	1·25
	41·0	0·83	28·5	1·29
	61·0	0·65	43·0	1·09
	76·0	0·68	54·0	1·40
	142·0	0·66	66·0	1·41
	210·0	0·77	120·0	1·31
	—	—	204·0	1·29
Acetylpropionyl	0·7	3·49	0·66	4·41
	1·4	3·15	1·26	4·71
	2·6	3·37	1·35	5·31
	4·5	3·55	4·2	4·85
	6·5	3·12	6·1	4·69
	9·8	3·50	8·8	4·45
	10·5	3·10	10·0	4·26
	25·5	3·21	21·0	5·02
	36·0	3·26	32·0	5·16
	47·0	3·85	41·0	5·34

SUMMARY

1. Coefficients of distribution for acetoin, diacetyl and acetylpropionyl between butterfat and skim-milk were measured over a range of temperatures up to 190° F. by passing preheated milk containing the reference substance through an hermetic cream separator and analysing the cream and skim-milk obtained.

2. The coefficients increased with rise in temperature, and the relationship between change in coefficient and change in temperature was linear for all three substances.

3. Coefficients of distribution for acetyl-*iso*-butyryl, acetylvaleryl, acetylcaproyl and acetylbenzoyl between butterfat and 4·5% lactose solution were measured at 40° and 60° C. by direct separation after equilibration, followed by estimation of the diketone in the lactose solution.

4. Coefficients of distribution at 100° C., obtained by linear extrapolation from these results, were: acetoin, 0·255; diacetyl, 1·27; acetylpropionyl, 5·9; acetyl-*iso*-butyryl, 20; acetylvaleryl, 23; acetylcaproyl, 152; acetylbenzoyl, 48.

5. The coefficients of distribution for diacetyl and acetylpropionyl between butterfat and 4·5% lactose solution were not affected by change in concentration of the diketone in the lactose solution.

The author is indebted to Mr B. Le Heron for assistance in the indirect measurement of the distribution coefficients at the Institute butter factory, and to Mr E. R. Elley for the analytical work.

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(*MS. received for publication* 19 *September* 1958)

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J. Food Sci. 34 (6): 493-96 (1969)

Wheat Flavor Components

SUMMARY—The volatile flavor components of a lightly milled, whole-grain soft wheat, Moro variety, were isolated and studied by gas-liquid chromatography, mass spectrometry, paper chromatography and/or chemical tests. The following 11 compounds were identified in the wheat flavor essence or in headspace vapors: acetaldehyde, isobutyraldehyde, butyraldehyde, hexanal, heptanal, octanal, crotonaldehyde, 3-methyl-2-butanone, 2,2-dimethyl-3-pentanone, diacetyl and ethyl acetate. Tentative identifications were made of seven additional compounds: butanone, valeraldehyde, isovaleraldehyde, cyclopentanone, phenylacetaldehyde, amyl alcohol and isoamyl alcohol.

INTRODUCTION

COMPONENTS of the flavor fractions of many foods have been the subject of intensive research in recent years. Yet the realm of cereal flavors has been practically untouched despite the fact that cereal grains constitute the basic food supply for many peoples of the world. In anticipation of food shortages throughout the world, new food products are being developed from previously unexploited sources. One well-known example of such a food formulated by man is Incaparina.

Palatability as well as nutritional value of prime importance in securing consumer acceptance of new foods. The use of well-accepted flavors such as those of cereal grains in new food products should contribute to their acceptance and utilization. When the significant components in the cereal volatiles are identified, synthesizing these important flavors will be possible.

In this study the volatile flavor components of whole grain wheat, Moro variety, were investigated. Eleven of the low-boiling compounds in the wheat volatiles were identified and seven were tentatively identified.

Two papers dealing directly with the flavor of cereal grains were found. In studies on rice flavor, Yasumatsu et al. (1966a) positively identified acetaldehyde and n-caproaldehyde, and tentatively identified methylethylketone, n-valeraldehyde and either propionaldehyde or acetone from the vapor of cooked rice. The stale flavor of stored rice was found to be due, at least in part, to the presence of n-caproaldehyde (Yasumatsu et al., 1966b).

Although bread doughs, pre-ferments and baked bread are more complex than the cereal grain alone, some of the important flavor components will arise from the cereal portion of the mix. Ng et al. (1960) identified acetaldehyde, ethyl pyruvate and furfural as constituents of fresh white bread volatiles, and isobutyraldehyde and n-valeraldehyde as compounds in the oven vapors. Acetaldehyde, acetone, 2-ethyl hexanal, 2-hexanone, 3-heptanone, crotonaldehyde, diacetyl, pyruvaldehyde and furfuraldehyde were identified in fresh bread by Wiseblatt et al. (1960).

Johnson et al. (1966) summarized studies conducted to that date in which 22 carbonyl compounds had been identified in pre-ferments, doughs, oven vapors, and bread. Ten esters had been detected in pre-ferment and bread, 24 organic acids in pre-ferments, doughs and bread, and seven alcohols in pre-ferments, oven vapors and bread.

EXPERIMENTAL

ANALYSES were carried out on a wheat flavor concentrate and on headspace vapor. The concentrated essence of the wheat flavor volatile components was prepared using a modification of the procedure of Herz et al. (1966). A sample of 1500 g wheat was mixed with 6.5 L of distilled water saturated with sodium chloride. Steam was directed through this slurry and the condensate was collected for 4 hr under vacuum. At the end of the distillation period the condensate in the traps was combined and extracted with ether. The ether was evaporated to yield 0.15 ml of a viscous yellow essence which had an intense, wheaty aroma.

Headspace vapor was obtained fresh for each analysis by bubbling steam through the wheat sample (200 g wheat and 600 ml distilled water) which was heated in a simple distillation apparatus under atmospheric pressure. A removable thermometer in the side arm of the flask allowed ready access to the exiting vapors.

Identification of components of the volatile flavor fraction of wheat was accomplished by means of functional group analysis, melting point determinations and paper chromatography of 2,4-dinitrophenylhydrazone derivatives of the carbonyl compounds present, retention times during gas-liquid chromatography, and mass spectrometry.

For functional group analysis a 5-ml sample of the vapors was injected into the chromatograph. As the various peaks emerged, the effluent stream was diverted into five vials containing selected reagents (Walsh et al., 1960). The reagents, chosen to indicate the presence of alcohols, carbonyls, esters, amines and mercaptans, were removed when a given peak (or peaks) had passed, and were replaced with a complete set of fresh reagents.

Collection times were 50–66 sec, 67–78, 79–100, 105–130, 133–160, and 160–180 sec total elapsed time from injection to elution. Each collection time corresponded to the elution of one or more components. The 6 ft × 1/4 in. I.D. column, packed with 6% diethylene glycol succinate supported on 90-100 mesh Anakrom ABS, was operated isothermally at 75°C under a nitrogen pressure of 5 psi.

Although a concentrate had been prepared by ether extraction of the vacuum condensate, enough of the volatile flavor components remained in the condensate to permit preparation of 2,4-dinitrophenylhydrazone derivatives (Shriner, et al., 1964). The crystals formed slowly, thus enabling partial separation of the various derivatives by filtration. Further separation was achieved by recrystallizing from ethyl alcohol, and by manual separation with the aid of a magnifying glass. Melting points of these derivatives were determined and compared with the melting points of known carbonyl derivatives prepared in the laboratory and also compared with values reported in the literature.

Column chromatography (Schwartz et al., 1962) and ascending paper chromatography, as outlined by Gaddis et al. (1959) and Ellis et al. (1958), were used to separate the aforementioned carbonyl derivatives into classes and to subsequently identify them as specific compounds.

Retention times of known compounds were determined by measuring the elapsed time from injection to the height of the peak, using a Barber-Colman gas-liquid chromatograph equipped with a flame ionization detector. Retention times were noted for three column packings, 6% Apiezon M, 6% diethylene glycol succinate and 3% Free Fatty Acid Phase; all supported on 90-100 mesh Anakrom ABS. Glass columns, 6 ft × 1/4 in. I.D. were used.

The columns were operated isothermally at 75°C with a nitrogen carrier pressure of 5 psi. Samples of wheat flavor essence, 0.2 μl

in size, and of wheat headspace vapor, 5 ml in size, were analyzed on the same columns under the same conditions. Retention times of the peaks in the chromatograms of compounds arising from the wheat flavor essence and headspace vapor were compared with those of known compounds.

For mass spectrometry, a 1 μl sample of the wheat flavor essence was injected into a Perkin-Elmer gas chromatograph which housed a 300 ft × 0.01 in. I.D. stainless steel capillary column coated with butanediol succinate. Operation was isothermal at 60°C for 15 min, followed by programmed heating at the rate of 4°C per min to a final hold temperature of 182°C. The effluent from the gas chromatograph was introduced directly into the Atlas-MAT CH-4 mass spectrometer (9-in., 60° sector, single-focusing instrument) with a rapid magnetic scan from m/e 25 to 200 requiring 2 sec. Spectra of the eluting peaks were recorded by a Honeywell Model 1508 Visicorder.

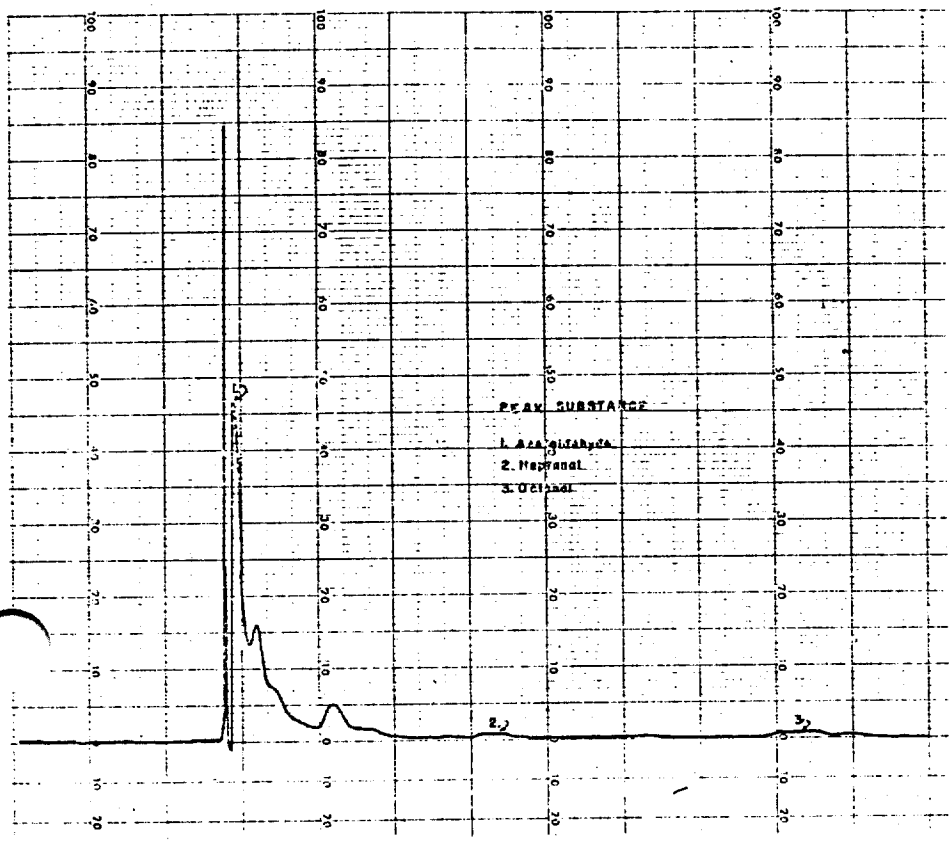

Fig. 1—Wheat headspace vapor, Apiezon M. 6%.

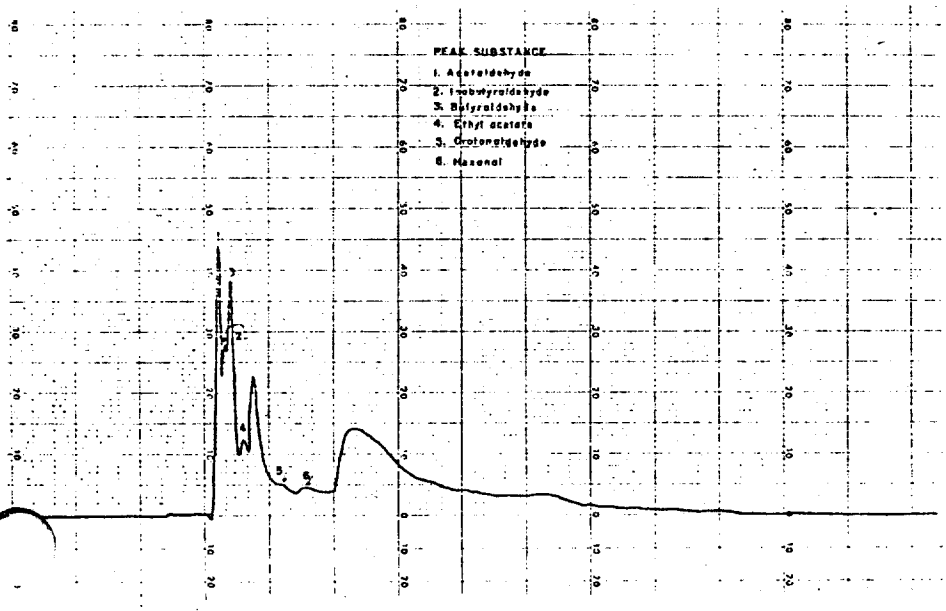

Fig. 2—Wheat headspace vapor, FFAP 3%.

RESULTS

CHROMATOGRAMS of headspace vapor and wheat flavor essence obtained by means of gas-liquid chromatography are shown in Figures 1 through 4. Retention times of wheat vapor components, Figures 1 through 3, indicated the presence of: 6% Apiezon column,—acetaldehyde, heptanal and octanal; 3% FFAP column, —acetaldehyde, isobutyraldehyde, butyraldehyde, 3-methyl-2-butanone, crotonaldehyde and hexanal; 6% DEGS column,—acetaldehyde, isobutyraldehyde butyraldehyde and crotonaldehyde.

The chromatogram of wheat flavor essence, Figure 4, indicates the presence of 3-methyl-2-butanone, crotonaldehyde, hexanal and heptanal. Tentative identification is indicated for cyclopentanone, isoamyl alcohol and amyl alcohol.

Functional group analysis of the effluent from the DEGS column during the periods mentioned under "Procedure" indicated the presence of carbonyl compounds through 180 sec. During each of the collection periods, precipitates formed in the vials containing 2,4-dinitrophenylhydrazine reagent, indicating the presence of the following compounds: 50–66 sec, acetaldehyde and/or isobutyraldehyde; 67–78 sec, butyraldehyde; 79–100 sec, 3-methyl-2-butanone, isovaleraldehyde and/or butanone; 105–130 sec, 2,2-dimethyl-3-pentanone, valeraldehyde and/or diacetyl; 133–160 sec, crotonaldehyde; 160–180 sec, hexanal.

Melting point determinations of the 2,4-dinitrophenylhydrazine derivatives of the unknowns in the flavor volatiles were compared with those obtained from known carbonyl derivatives prepared in the laboratory. Results indicated the presence of acetaldehyde, isobutyraldehyde, butyraldehyde, crotonaldehyde, 3-methyl-2-butanone, valeraldehyde, hexanal, 2,2-dimethyl-3-pentanone, heptanal, octanal, butanone and isovaleraldehyde. A comparison of observed melting points with values reported in the literature is presented in Table 1.

Additional confirmation of the presence of acetaldehyde, crotonaldehyde, and 3-methyl-2-butanone was secured by separating the 2,4-dinitrophenylhydrazone derivatives into classes by means of paper chromatography. The presence of 3-methyl-2-butanone was shown by eluting 2-alkanone region and subsequently chromatographing this sample with a derivative of the known compound. A similar procedure, using the alk-2-enal region, was used to identify acetaldehyde and crotonaldehyde. Ellis et al. (1958) had previously demonstrated that acetalde-

Table 1—Comparison of melting points of 2,4-dinitrophenylhydrazine derivatives of heat volatiles with those of known compounds reported in the literature.

Carbonyl	Melting point of 2,4-dinitrophenylhydrazone, °C	
	Laboratory	Literature[1]
Acetaldehyde	168	168
Isobutyraldehyde	182	182
Butyraldehyde	125	122
Crotonaldehyde	190	190
3-Methyl-2-butanone	119	117
Valeraldehyde	110	107
Hexanal	104	104
2,2-Dimethyl-3-pentanone	178	175
Heptanal	110	108
Octanal	109	106
Butanone	116	114
Isovaleraldehyde	125	122

Values as reported in Shriner et al. (1964) except those for valeraldehyde, 3-methyl-2-butanone, and 2,2-dimethyl-3-pentanone which were reported in Heilbron et al. (1953).

hyde was separated in the class separation step with the alk-2-enals rather than with the expected n-alkanals.

Mass spectral identifications for three compounds were made by comparing the spectra with those published in the ASTM tables (1963). The capillary column used in conjunction with the mass spectrometer provided good separation of volatile components of the wheat flavor essence.

In the first spectrum, the parent ion was at m/e 44 and the base peak was at [illegible]. This fragment suggests a cleavage [illegible] the first and second carbon atoms, and the relative abundance of the fragment ion at m/e 43 was consistent with the value reported in the ASTM tables for acetaldehyde. This spectrum was thus confirmed as being acetaldehyde.

Similarly, a second spectrum was identified as being ethyl acetate. The parent ion was at m/e 88 and the base peak was at m/e 43. The ions observed at m/e 43 and m/e 45 were the result of the cleavage of the ester linkage.

A third spectrum was analyzed, and the parent ion was at m/e 86. The base peak at m/e 43 was the result of a split in the middle of the symmetrical molecule, diacetyl. The intensities observed in both spectra 2 and 3 were consistent with the intensities for ethyl acetate and diacetyl, respectively, in the ASTM tables.

A fourth spectrum was examined and the parent ion was at m/e 120, and the base peak at m/e 91. Comparison of this spectrum with the values reported in the tables led to tentative identification of this spectrum as that of phenylacetaldehyde.

The identifications of components are summarized in Table 2.

DISCUSSION

ONE OF THE pitfalls of the flavor researcher is the possibility of creating or introducing experimental artifacts during the concentration process and ultimately endowing them with a natural existence. The wheat odor was carefully noted when the shipment arrived. The aroma of the heated sample for headspace vapor analysis was compared with the original aroma, as were the odors at the various stages of concentration during the processing of the wheat essence.

The aroma issuing from the headspace vapor apparatus when the thermometer was removed from the system was identified as strongly wheat-like. The odor of the wheat essence that resulted after extraction with anhydrous diethyl ether and distillation of the ether was intense and was easily recognized as that of wheat. The trapped effluent from the gas chro-

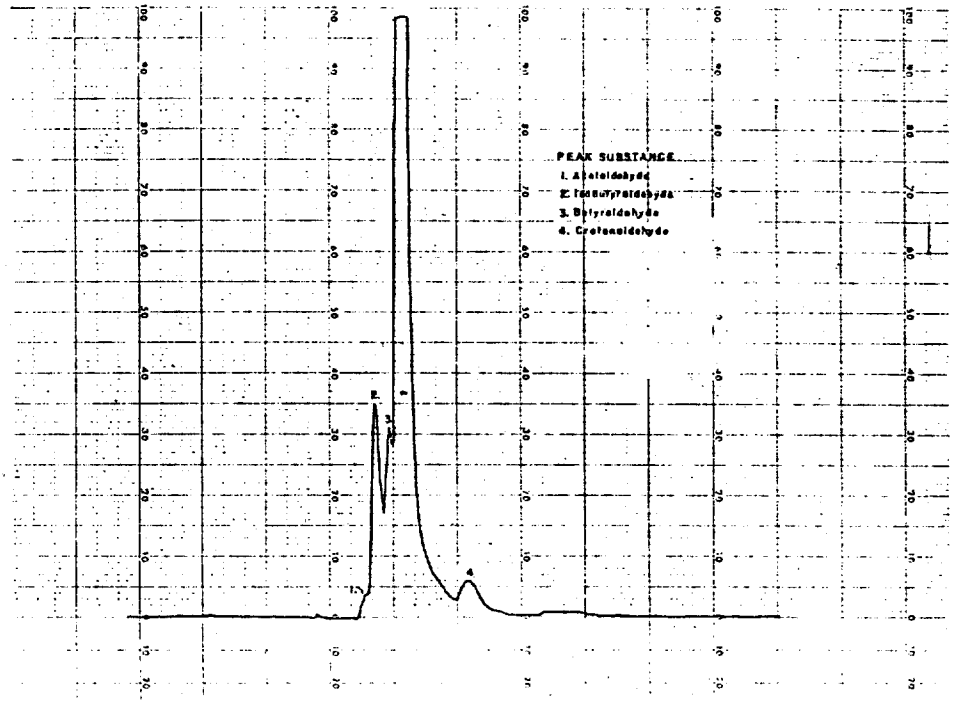

Fig. 3—Wheat headspace vapor, DEGS 6%.

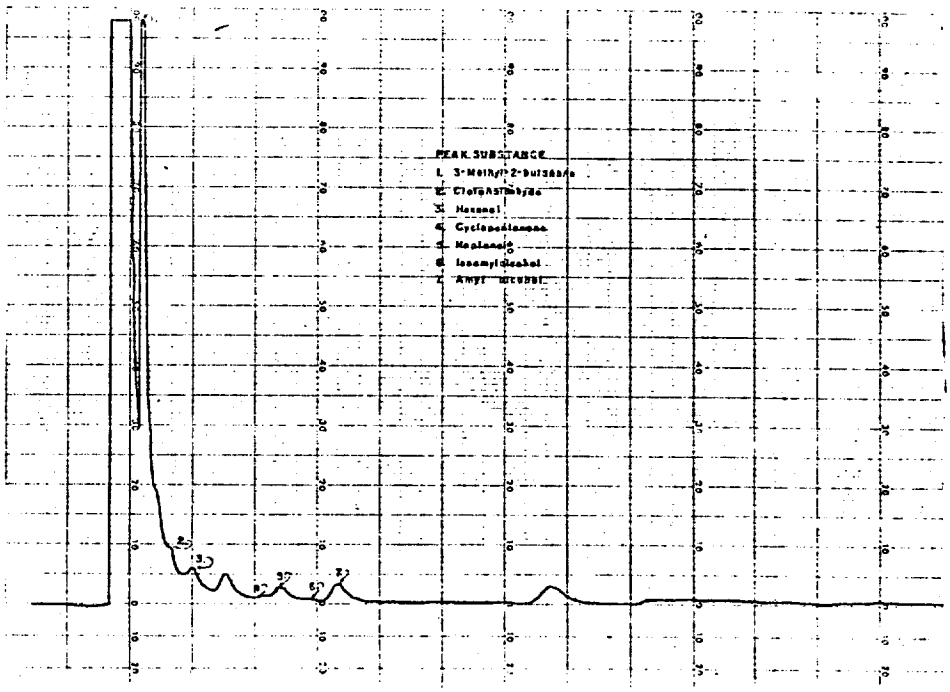

Fig. 4—Wheat flavor essence, FFAP 3%.

Table 2—Summary of identification of components in wheat headspace vapor or essence.

Compound	Effluent formed a precipitate in 2,4-DNPH reagent at retention times of known compound	Paper chromatography	Gas-liquid Chromatography						Mass Spectrometer	Melting point of 2,4-DNPH derivative
			Headspace Vapor			Essence				
			AM	FFAP	DEGS	AM	FFAP	DEGS		
Acetaldehyde	x	x	x	x	x				x	x
Isobutyraldehyde	x			x	x					x
Butyraldehyde	x			x	x					x
Hexanal	x			x			x			x
Heptanal			x	x			x			x
Octanal			x				x			x
Crotonaldehyde	x	x		x	x		x			x
3-Methyl-2-butanone	x	x		x			x			x
2,2-Dimethyl-3-pentanone	x								x	
Diacetyl	x								x	
Ethyl acetate									x	
Isoamyl alcohol							x			
Butanone	x									
Isovaleraldehyde	x									x
Valeraldehyde	x									x
Cyclopentanone										x
Phenylacetaldehyde							x		x	
Amyl alcohol							x			

matographic column was also readily identified as having the odor of wheat.

Experimental evidence from chemical, gas chromatographic and mass spectrometric techniques was used in identifying wheat flavor components. All results were brought to bear in establishing identification of 11 compounds and tentative identification of seven others.

Of the 18 compounds noted in this paper, all but 3-methyl-2-butanone and 2,2-dimethyl-3-pentanone have been reported as components of pre-ferments, doughs, oven vapors or bread in the summary by Johnson et al. (1966). It appears that much of the flavor of bread may arise from components present in the wheat.

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Ms. received 1/29/68; revised 10/31/68; accepted 10/17/69.

Financial support of the American Home Economics Association through granting of the AHEA Foundation–HEIB Section Fellowship to Mrs. McWilliams is gratefully acknowledged.

Fla. State Hort. Soc. 79: 312-21 (1966)

DIACETYL TEST AS A QUALITY CONTROL TOOL IN PROCESSING FROZEN CONCENTRATED ORANGE JUICE

D. L. MURDOCK[1]

Lactic acid bacteria are organisms of sanitary significance in processing frozen concentrated orange juice. They impart flavors and odors which have been characterized as similar to buttermilk (3) (7). The bacteria responsible are principally those organisms belonging to the genera *Lactobacillus* and *Leuconostoc*. They are present on and in the fruit when it arrives at the plant and grow well in single strength and partially concentrated orange juice. To prevent a buildup of these organisms during the processing operation, an efficient sanitation program must be maintained.

It soon became apparent among the early investigators that a rapid method was needed to detect the presence of lactic acid bacteria. The direct miscroscopic method was first used. It consisted of counting the number of cells present on a stained slide preparation. The difficulty in distinguishing between the bacteria and the debris present on the smear left much to be desired. Then in 1954, Hill et al. (4) and Byer (2) introduced to the citrus industry a rapid method for the detection of diacetyl and acetylmethylcarbinol (AMC), the metabolic

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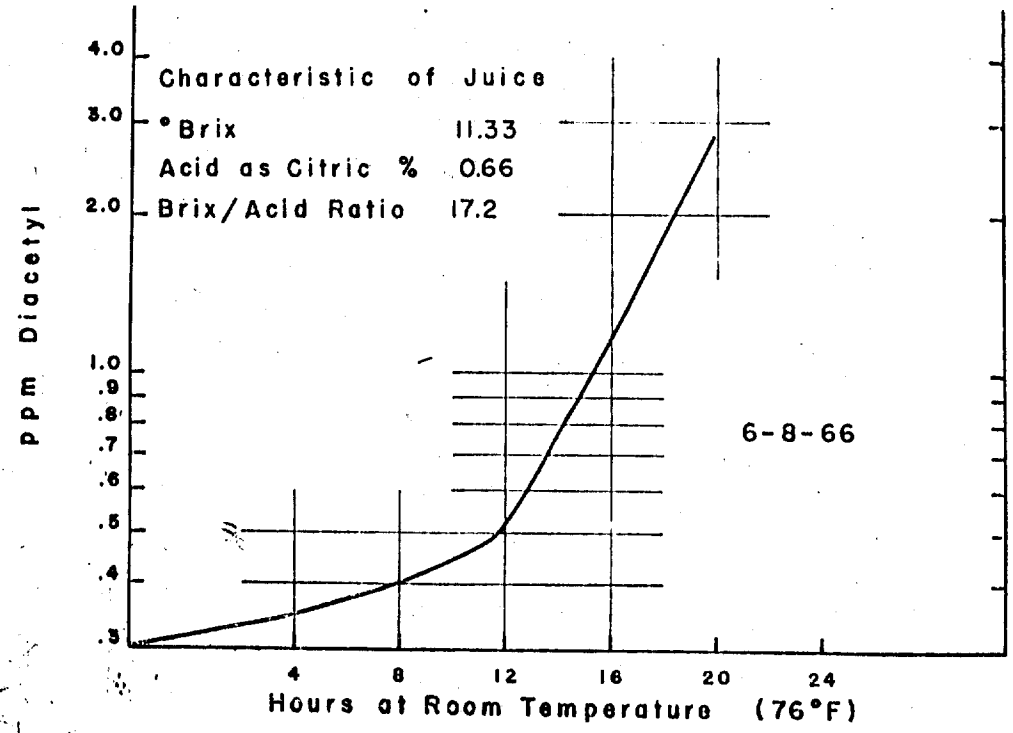
Fig. 1 DIACETYL VALUES OF ORANGE JUICE HELD AT ROOM TEMPERATURE

Sample Obtained From Finishers



products of the growth of lactic acid bacteria in orange juice. The test, a modification of the Voges-Proskauer (VP) reaction, is extremely sensitive to diacetyl and will detect concentrations of less than 1 ppm. Most processors of frozen concentrated orange juice, as indicated by a survey made by Murdock and Dennis in 1964 (8), use some form or modification of the VP test for the detection of diacetyl and AMC in orange juice. Variations in preparation and handling of VP reactants were reported by Murdock in 1965 (9).

The purpose of this paper is to show how the diacetyl test, which requires less than one-half hour to perform, can be used as a quality control tool to determine the microbial activity in processing frozen concentrate orange juice.

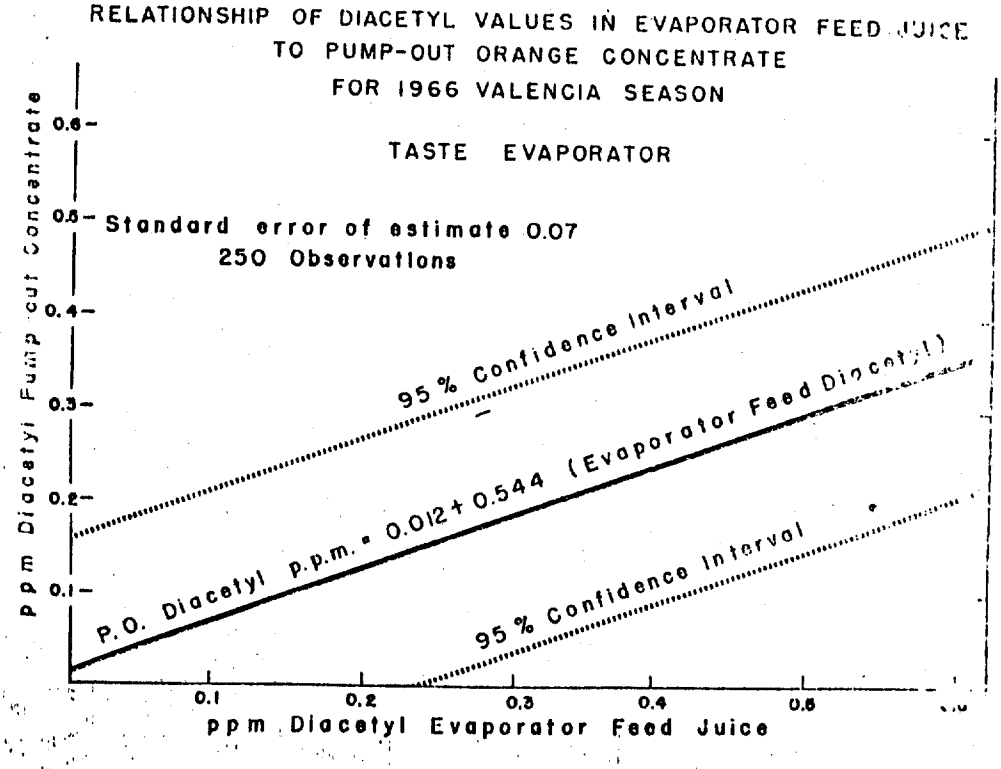
Procedure

The diacetyl test used in this investigation was described by Murdock (Fla. State Hort. Soc. Proc., Nov. 1965). Briefly, it consisted of adding 300 ml of single strength or reconstituted orange juice (12° Brix) to a large boiling flask connected to a water condenser, collecting 25 ml of distillate, adding the VP reagents, and reading the color reaction on a Lumetron colorimeter 60 seconds after addition of the reagents. The results are expressed as ppm of diacetyl in the distillate.

Results and Discussion

Relationship of fruit quality to diacetyl values.—A study was made to determine the relationship of fruit quality to diacetyl values. It consisted of collecting at the concentrate plant ½ box (approx. 45 lbs.) of sound acceptable fruit (oranges) and a similar amount of reject fruit, the latter consisting of splits, partially decomposed, soft spots, etc.. The fruit was brought to the laboratory where the juice was extracted from each lot by means of a hand extractor. The following diacetyl values were obtained:

Fig. 2



Fruit	° Brix	Ratio	Diacetyl
(Good)	12.16	14.17	0.15
(Bad)	12.98	14.42	0.80

et al. (1) also found that juice from fruit was higher in diacetyl than it sound fruit. Murdock et al (10) found was a direct correlation between fruit nd the microbial contamination of tracted juice. Therefore, it is quite at if unsound fruit entering the plant oved during the grading operation it ly "seed" the plant with microorgan- vill also result in an immediate surge during the processing operation.

l as an index of plant sanitation.—In essing plants, as in other food plants, occasionally remain stagnant in an eline; or a dead pocket may occur as a change in the piping arrangement. adhering to finisher chutes may not removed during a flush operation. It that pulp under optimum conditions equipment, such as finisher chutes, lop a sour odor in approximately 6 removed (11). When this occurred, sually had a total viable count of rganisms per gram and a diacetyl n of one or more ppm (6).

shows that diacetyl values of orange at room temperature (76° F) in- m 0.3 to 2.9 ppm in 20 hrs. The rease in diacetyl occurred after 12 these data, it is apparent that if is held in a dormant condition, such used pipeline and the equipment is a sanitary condition, an increase in occur.

values during evaporator operation. Wenzel in 1957 (5) reported on the ues obtained in processing orange n a low temperature pilot and com- orator. They noted an increase in rred during the concentrate process, the evaporator had been operated ore. The citrus industry within the two has replaced nearly all of their ure evaporators with high temper- olding time units, generally refer- TASTE evaporator (Thermal Ac- t Time Evaporator).

as made under commercial condi- ng a TASTE unit with a 6-stage low-temperature Buflovak evaporator in processing wash pulp concentrate. Samples for diacetyl analyses were drawn every 4 hrs. while the evaporators were in operation. Juice was collected before it entered the evaporator (evaporator feed) and when it left the units (pump-out concentrate). The Buflovak units were cleaned on a 48-72 hr. cycle and the TASTE evaporator every 10-12 hrs. A table of frequencies of diacetyl values (evaporator feed juice and pump-out concentrate) for the period the TASTE and Buflovak evaporators were in operation is presented in Table 1. It can be seen from the data that for the period April 6-13, 18 of the TASTE evaporator feed juice diacetyl values were in the range of 0.20 to 0.25 ppm. The distribution of the values leaving the evaporator were 14 in the range of 0.10 to 0.15 ppm, two in the range of 0.20 to 0.25, and two in the range of 0.30 to 0.35. The average diacetyl values for the test period and the percent change between evaporator feed juice and pump-out concentrate is shown in Table 2. Note that the average loss of diacetyl between these two points for the TASTE evaporator was 36%, which compares with a 15% increase in diaetyl for the Buflovak evaporator.

In another study, which was made at Plant A from April 29 through June 22, the orange juice entering the TASTE evaporator (evaporator feed) and pump-out concentrate 60° Brix were checked for diacetyl every 4 hrs. while the evaporator was in operation. A total of 250 analyses were made. The average loss of diacetyl for the season between the evaporator feed juice and the pump-out concentrate was 32% (Table 2). A table of frequencies of the diacetyl data for the test period is shown in Table 3. Also included are the diacetyl values obtained at Plant B while processing orange concentrate.

In analyzing the diacetyl data obtained from Plant A, the following regression equation was derived:

Pump-Out Diacetyl ppm = 0.012 + 0.544 x Evaporator Feed Diacetyl

This equation shows the functional relationship between the level of diacetyl in the evaporator feed juice entering the TASTE evaporator and the level of diacetyl in the 60° Brix concentrate pump-out. From the equation, it can be estimated that if the evaporator feed juice contains 0.4 ppm diacetyl there will be on the average 0.23 ppm diacetyl in the pump-out concentrate. The relationship for other diacetyl values in evaporator feed juice to pump-out concentrate is shown in Fig. 2. Using the standard error of the estimate of 0.07 ppm, a 95% confidence interval was calculated to show the variability associated with the estimate. For an evaporator feed juice diacetyl of 0.4 ppm, we would expect 95% of the estimates of pump-out diacetyl ppm to fall within a confidence interval of 0.09 to 0.37 ppm.

Although the strength of the regression equation for the other TASTE evaporators was weak due to the limited number of samples collected, it appears that the same functional relationship applies.

Table 1. Distribution of diacetyl data in processing wash concentrate during 1966 Valencia season

Evaporator Feed Juice — Diacetyl Range ppm	Total Freq.	Pump-Out Concentrate (60° Brix) — Diacetyl Range ppm: 0.00–0.05	0.10–0.15	0.20–0.25	0.30–0.35	0.40–0.45	0.50–0.55
		Frequencies					
TASTE EVAPORATOR — April 6-13							
0.40 - 0.45	1	1	0	0	0	0	0
0.30 - 0.35	14	1	2	9	2	0	0
0.20 - 0.25	18	0	14	2	2	0	0
0.10 - 0.15	2	1	1	0	0	0	0
	35						
BUFLOVAK EVAPORATOR — April 22- May 4							
0.40 - 0.45	5	0	0	1	3	1	0
0.30 - 0.35	19	0	0	7	7	4	1
0.20 - 0.25	16	0	2	4	4	5	1
0.10 - 0.15	4	1	1	2	0	0	0
	44						

Biological Control Program.—We have seen that diacetyl can be used as presumptive evidence of the quality of the incoming fruit and as an index of sanitation in a citrus concentrate plant. Since the diacetyl test will detect the end products of microbial growth in orange juice during processing operations, it can be used effectively as a quality control tool. In developing a biological control program, the following should be considered:

1. Sample stations
   Select definite check points to adequately cover the processing operation.

Average Diac alues for Test Period

	Evaporator	No. Tests	Avg. ppm of Diacetyl: Evaporator Feed Juice	Avg. ppm of Diacetyl: Pump-Out Concentrate	Diacetyl % Change
: (60° Brix)	TASTE	35	0.265	0.171	36% (-)
- (60° Brix)	Buflovak	44	0.282	0.323	15% (+)
nc. (65° Brix)	TASTE (1)	250	0.374	0.254	32% (-)
nc. (65° Brix)	TASTE (2)	36	0.260	0.160	38% (-)

(1) 40,000 lb. evaporator Plant A

(2) 50,000 lb. evaporator Plant B

ncy of tests

k product from each station every s. or more frequently if necessary.

ng of data

all diacetyl values. An example of in which the data can be plotted ically as it is accumulated is n in Fig. 3.

tation of data

ble to correctly interpret the data is obtained. In order to assist te Maid personnel in this regard, ide for Interpretation of Diacetyl ses has been developed (Fig. 4).

lues during normal and abnormal

3 is a copy of a log showing s obtained during a normal plant rted on May 10, 1966. Note the more or less constant within a range.

copy of a log showing a diacetyl ve the steady increase in diacetyl pulp discharge liquor starting at h 31 and the large increase in en 0900 on April 2 and 1300 on . During this time interval the sed from 0.4 ppm to 0.8 ppm in would indicate the contaminating in their optimum growth phase. easures are not immediately tak- occur in a short time. In this ice room was shut down and the ghly cleaned with a detergent solution. The source of contamination, when conditions exist as illustrated in Fig. 5, usually will be found in the juice room. It may occur from sour pulp in finisher chutes, stagnant juice in troughs or header lines, etc. If found, a flush down with chlorinated water is indicated and/or the equipment should be thoroughly cleaned with detergent solution.

Another abnormal condition is a sudden surge in diacetyl. This usually occurs when:

(1) A "slug" of sour juice is diverted into the system, ie. from an unused pipeline not cleaned prior to use,

(2) product remaining in an evaporator feed tank that has become stagnant, or

(3) a load of poor fruit being processed.

Fortunately, a sudden increase in diacetyl is usually followed by a corresponding decrease as the slug of contaminated product passes through the system. If diacetyl does not return to normal, a flush down or a clean up is indicated.

We have seen how the diacetyl test can be used as an effective quality control tool in the processing of frozen concentrated orange juice. It can also be used to a lesser degree as an indication of the quality of bulk concentrate which, at times, is purchased on the open market. If the product was produced in a TASTE evaporator, it must be remembered that approximately ⅓ of the diacetyl present in the evaporator feed juice will be lost during the concentrate operation. Therefore, if high diacetyl is found in bulk drummed product, the assumption can be made that the product was produced under insanitary conditions prior to evaporation.

Table 3. Distribution of diacetyl data in processing orange concentrate in TASTE evaporator during 1966 Valencia orange season.

PLANT A

April 29- June 22

Evaporator Feed Juice: Diacetyl Range ppm	Total Freq.	Pump-Out Concentrate (60° Brix): Diacetyl Range ppm 0.00 – 0.05	0.10 – 0.15	0.20 – 0.25	0.30 – 0.35	0.40 – 0.45	0.50 – 0.55
		Frequencies					
0.60 - 0.65	7	0	0	1	3	2	1
0.50 - 0.55	33	0	1	15	10	5	2
0.40 - 0.45	85	1	15	44	22	3	0
0.30 - 0.35	79	2	30	41	6	0	0
0.20 - 0.25	42	4	31	6	1	0	0
0.10 - 0.15	4	1	3	0	0	0	0
	250						

PLANT B

April 11 - May 10

0.40 - 0.45	3	0	3	0	0	0	0
0.30 - 0.35	12	1	10	0	1	0	0
0.20 - 0.25	13	4	9	0	0	0	0
0.10 - 0.15	8	1	6	1	0	0	0
	36						

Summary

Data are presented to show that the diacetyl test can be used as an index of juice quality and poor sanitation in processing frozen concentrated orange juice. Approximately ⅓ of the diacetyl present in the evaporator feed juice was lost in a TASTE evaporator during the concentrate operation. An increase in diacetyl usually oc red when a low temperature evaporator used.

1387

3 DIACETYL VALUES OBTAINED DURING NORMAL OPERATION

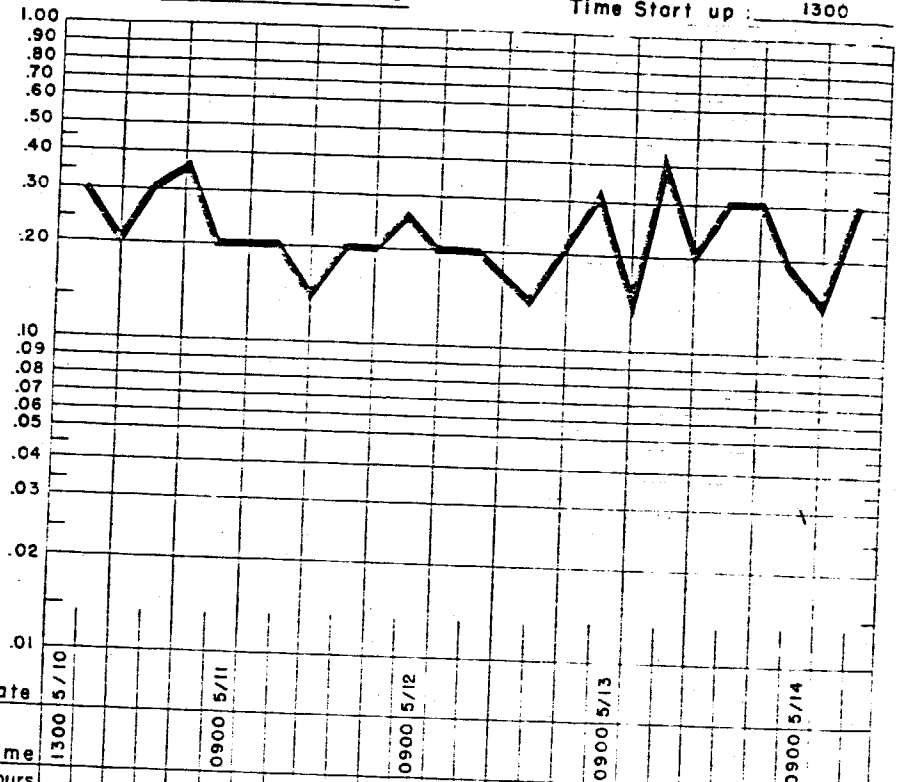
DIACETYL LOG

Plant B

Product Orange Concentrate

Date Start up: 5-10-66

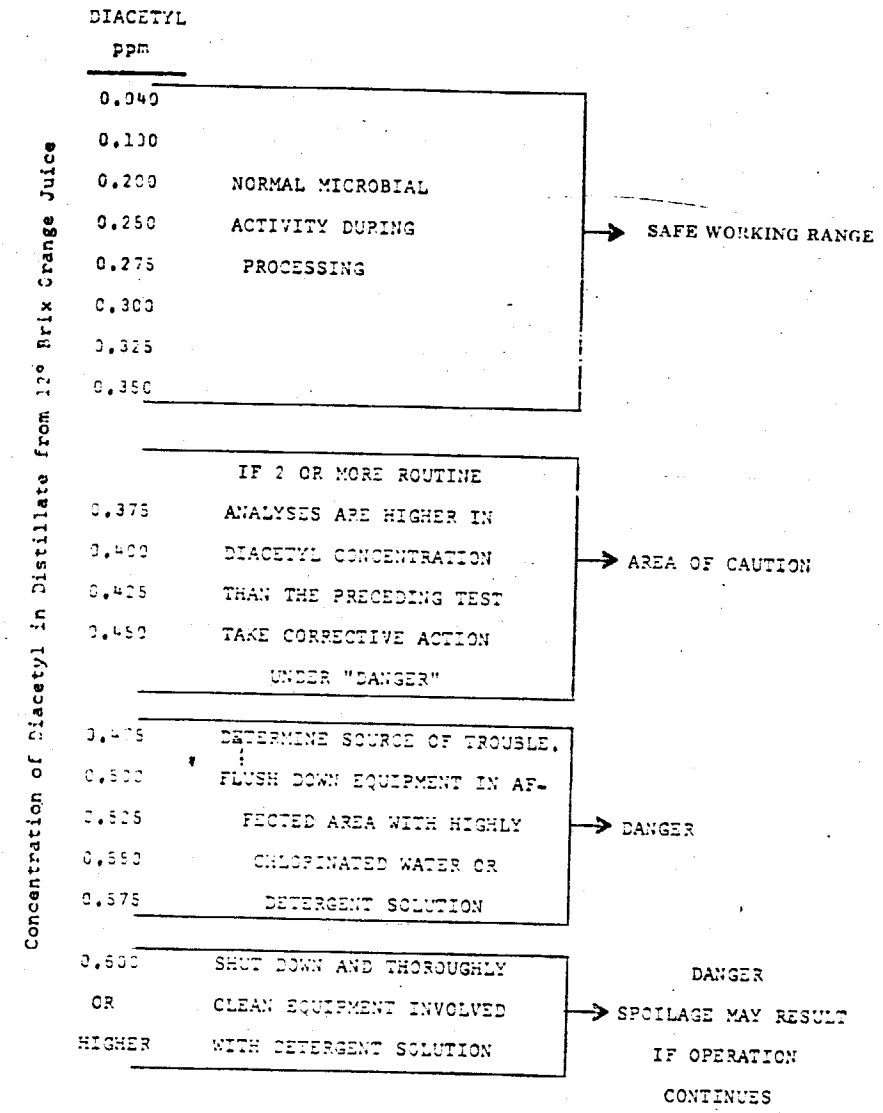
Time Start up: 1300



Date	5/10						5/11					5/12					5/13							5/14	
Time	1300						0900					0900					0900							0900	
Hours																									
Evap. Feed Juice: Bact.																									
Yeast																									
Total																									
ppm Diac.	.30	.20	.30	.35	.20	.20	.20	.15	.20	.20	.25	.20	.20	.18	.15	.20	.30	.15	.40	.20	.30	.30	.20	.15	.30
Tech.																									

Remarks: Samples analyzed every four hours.

Fig. 4 GUIDE FOR INTERPRETATION OF DIACETYL ANAL IN PROCESSING FROZEN CONCENTRATED ORANGE JUICE



Development of biological control program using the diacetyl test as a quality control tool is discussed. Interpretation of the diacetyl data and preventive measures used in a concentrate plant to prevent spoilage during periods of high microbial activity are mentioned.

ACKNOWLEDGMENT

The author gratefully acknowledges Mr. W. J. Hepburn for the statistical analyses of the data and Quality Control personnel for the diacetyl determinations.

1387

Fig. 5

LOG SHOWING DIACETYL BUILDUP

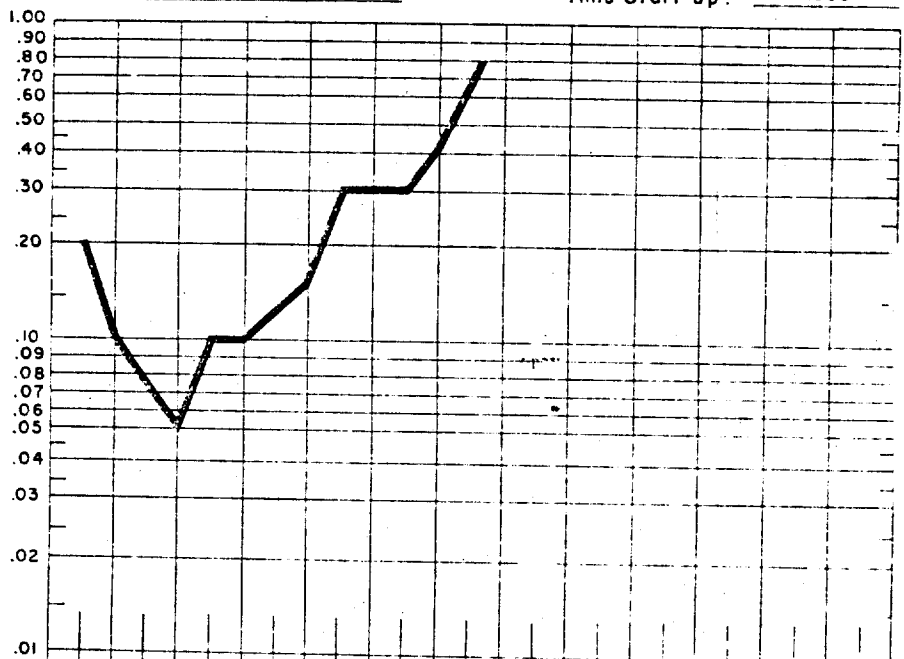
DIACETYL LOG

Plant A

Product Wash Pulp (W.P.)

Date Start up: 3-31-66

Time Start up: 1300



Date	3/31						4/1					4/2	
Time	1300			0100			1300			0100		0900	
Hours													
Bact.													
Yeast													
Total													
ppm Diac.	.20	.10		.05	.10	.10		.15	.30	.30	.30	.40	.80
Tech.													

SOURCE OF SAMPLE: Discharge Liquor

Remarks: Samples analyzed every four hours. Juice room equipment thoroughly clean at 1330 on 4-2-66

Fig. 5 LOG SHOWING DIACETYL BUILDUP

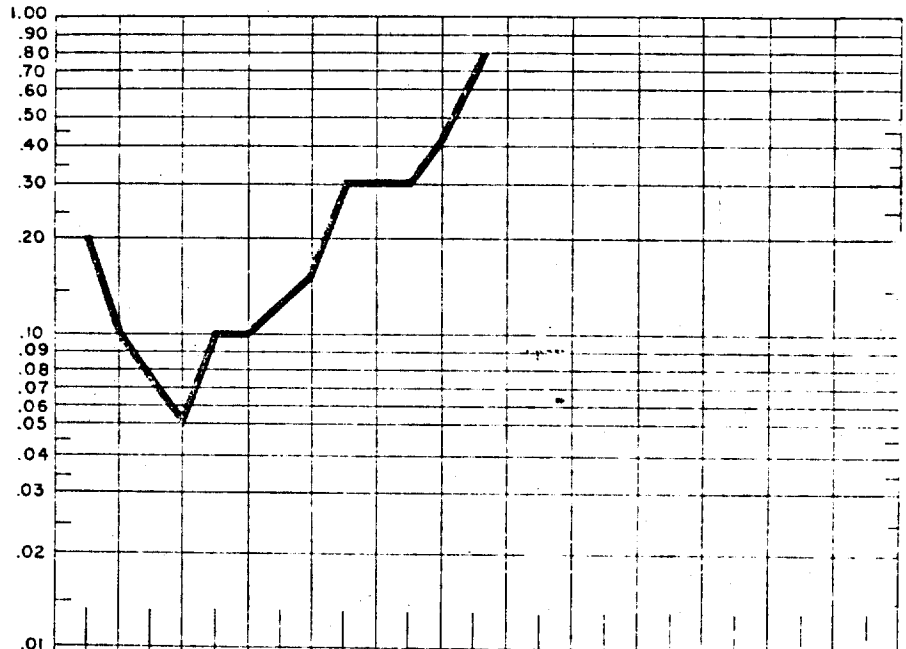
DIACETYL LOG

Plant A

Product Wash Pulp (W.P.)

Date Start up: 3-31-66

Time Start up: 1300



Date	3/31						4/1					4/2	
Time	1300			0100			1300			0100		0900	
Hours													
Bact.													
Yeast													
Total													
ppm Diac.	.20	.10		.05	.10	.10		.15	.30	.30	.30	.40	.80
Tech.													

SOURCE OF SAMPLE: Discharge Liquor

Remarks: Samples analyzed every four hours. Juice room equipment thoroughly clean at 1330 on 4-2-66

1387

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RESEARCH

PAGES 157–186—(Vol. 21, Pages 643–672)

Methods Employed by the Citrus Concentrate Industry for Detecting Diacetyl and Acetylmethylcarbinol

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SUMMARY

Variations in preparing and handling VP reagents were investigated. Alpha-naphthol reagent can be prepared from the technical grade of Eastman's compound, if the color of the powder is light pink. Otherwise, color produced by this reagent will interfere with VP reaction. Alpha-naphthol received in amber glass bottles from the manufacturer does not deteriorate when stored on the laboratory shelf. The reagent prepared from this compound is also stable at room temperature. The VP reaction for diacetyl and AMC showed color developed faster when the alpha-naphthol reagent was prepared with isopropyl alcohol than with ethyl alcohol.

KOH creatine reagent prepared from creatine from three different suppliers showed little or no difference in VP reaction. KOH solution containing 0.3% creatine developed color faster than it did with 5.0% at a given time interval. This reagent breaks down very rapidly at room temperature but is fairly stable at 0°F.

Diacetyl remaining on the laboratory shelf for an extended period should not be used for preparing a standard curve because it will give erroneous results. A method is described for detecting diacetyl in frozen concentrated orange juice.

INTRODUCTION

In producing frozen concentrated orange juice, there has been concern with off-flavors characterized as being similar to "buttermilk." Early investigators (Hays, 1951, and Murdock, *et al.*, 1951) were of the opinion that the bacteria responsible were principally those belonging to the genus *Lactobacillus*. It soon became evident that a rapid method was needed for detecting the metabolic end products of these organisms.

Byer (1954) and Hill *et al.* (1954) introduced to the citrus industry a rapid method for detecting diacetyl and acetylmethylcarbinol (AMC). The test, a modification of the Voges-Proskauer (VP) reaction, is used to determine end products of bacterial growth produced in orange juice, principally by organisms belonging to the genera *Lactobacillus* and *Leuconostoc*. Certain strains of yeast have also been reported to produce diacetyl, particularly *Saccharomyces cerevisiae* (Murdock, 1964).

The VP test is now almost universally employed in the citrus industry as part of their biological control program. However, most plants have modified it to meet their needs. Consequently, a considerable variation exists in the industry as to the procedure employed for detecting diacetyl or AMC, as a survey of 14 major Florida citrus concentrate plants indicated (Murdock and Dennis, 1964). Some plants read the color reaction 1 min after the addition of VP reagents, others at 8, 10 min, or intermediate periods. Even the distillation of 3 fractions is employed by some processors.

Confusion also exists as to the importance of AMC in the juice sample. Diacetyl content is generally used as an index of sanitation of the processing operation. AMC, on the other hand, is considered by most food technologists to have little or no sanitary significance. AMC, which is tasteless, is found naturally in citrus fruit and increases as the fruit matures (Hill, *et al.* 1954). According to Hill, *et al.* (1954), it may reach levels as high as 4 ppm in oranges. Although diacetyl has never been reported to exist in oranges, it is believed to be present in extremely low levels of 0.01 to 0.02 ppm, increasing as the fruit matures (Minute Maid Company, 1965).

The VP test is highly specific to diacetyl. Murdock and Dennis (1964) reported that after the addition of VP reagents 1 ppm of diacetyl reached its maximum color intensity between 1 and 2½ min, after which a slight decrease in color occurred. This was also reported by Hill and Wenzel (1957).

On the other hand, in comparison to diacetyl, the VP reaction for AMC is extremely slow. Low concentrations of less than 1 ppm show no color development at the end of 1 min (Minute Maid Company, 1965). With concentrations where color development does occur, more than 20 min are required to obtain maximum color intensity (Murdock and Dennis, 1964). Therefore, it would appear that those laboratories making colorimetric readings of their juice distillate samples at the end of 5, 8 and 10 min would show values due principally to AMC. This would especially be true when the color intensity was determined at the end of 10 min.

This paper concerns an investigation of the methods used by the Florida citrus industry to detect presence of diacetyl and AMC in processing frozen concentrated orange juice.

METHODS

Two stock solutions, of known concentration, were first prepared in distilled water—one using Eastman No. 1591 diacetyl and the other, Eastman No. P3788, acetylmethylcarbinol (AMC). Subsequent dilutions in distilled water were then made to obtain the desired concentration of each compound under investigation.

The VP test consisted of placing 25 ml of the test solution in a large test tube. To this was added 10 ml of a 5% solution of alpha-naphthol (Eastman No. 170) in 99% isopropyl alcohol and 4 ml of a 40% KOH solution containing 2.0% creatine (Eastman No. 951). The tube was then stoppered and shaken.

The intensity of color produced was determined by placing the tube in a Lumetron colorimeter, Model No. 401, equipped with a 530mμ filter. Readings were recorded as optical density (O.D.) at various time intervals under investigation. To compensate for color development in the reagent blank, optical density values were recorded at the various time intervals. These values were then subtracted from those

1389

Table 1. Effect of reagent mixing time on color development of diacetyl reaction. Data avg. of 4 replicate tests.

Diac. ppm	Sec reagents mixed: 5	15	30	45
	O. D. at 60 sec			
0.2	0.22	0.24	0.22	0.20
0.5	0.51	0.53	0.51	0.51
0.8	0.85	0.86	0.83	0.80
1.0	1.11	1.08	1.01	1.00

Table 2. Comparison of swirling and shaking of reagents on color development. All O.D. values avg. of 2 determinations.

Diac. ppm	Reagents mixed 20 sec: O. D. values at 60 sec: Swirled	Shaken
0.2	0.19	[illegible]
0.5	0.50	[illegible]
0.8	0.79	[illegible]
1.0	0.99	[illegible]

obtained at the same time interval for the test sample. The results obtained correspond to the concentration of diacetyl or AMC present in the distillate after a sample of juice has been distilled.

In preparing standard solutions of 1 ppm or less, using volumetric technique, slight variations occurred between solutions of the same concentration.

RESULTS AND DISCUSSION

Effect of mixing time of reagents on color development. Since there is no unanimity in the industry as to the time required to mix VP reagents, an investigation was made of this variable. Four solutions of diacetyl were prepared ranging from 0.2 to 1.0 ppm. Each solution was then divided into 4 equal parts. VP reagents were added. One set of samples was mixed for 5 sec, another for 15, 30, and 45 sec, respectively. Color development was determined at 60 sec.

The results show no significant difference in color intensity, whether or not the VP reagents were mixed 5, 15, 30 or 45 sec (Table 1). However, if the sample was mixed 5 to 15 sec, more time was available to transfer the tube to the Lumetron when making the 60-sec colorimetric reading.

Method of mixing reagents. Brown (1964) reported that he preferred to pour the 25 ml juice distillate sample into a 125 ml Erlenmeyer flask, add the VP reagents and mix by swirling, instead of shaking in a stoppered test tube (Fig. 1). This, he stated, resulted in less interference from air bubbles when the samples were swirled. Also, the need for rubber stoppers was eliminated. They can be a source of error if they are not washed properly between determinations. A comparison was made between shaking and swirling the reagents. Data (Table 2) show no difference between methods of mixing.

Alpha-naphthol reagent grade versus technical. In preparing the alpha-naphthol reagent, some investigators use the technical grade—Eastman T-170 (Fields, 1964; Hill and Fields, 1966). Others use the reagent grade—Eastman 170 (Fields, 1962; Hill *et al.*, 1954; Hill and Wenzel, 1957). There was little or no difference between certain lots of T-170 and 170 (Table 3), while other lots of T-170 produced a color of greater intensity than was developed by the test sample, making it impossible to get accurate O.D. readings. Therefore, the technical grade of this compound is not recommended for preparing this reagent.

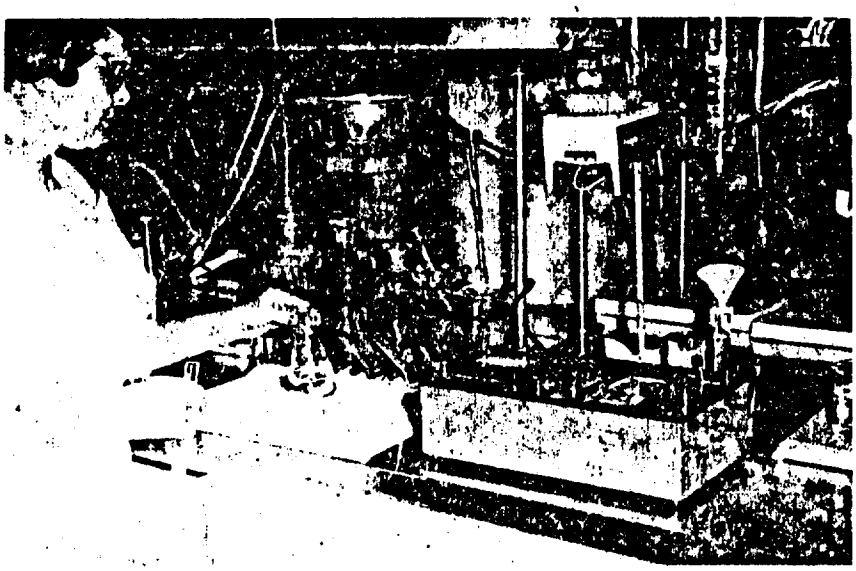

Fig. 1. Mixing of distillate in Erlenmeyer flask. Note Lumetron in foreground and portion of distillation apparatus in background.

Effect of temperature and length of storage of alpha-naphthol used for preparing VP reagents. It is the opinion of some citrus laboratory personnel that alpha-naphthol received from the manufacturer in amber colored bottles will deteriorate on the laboratory shelf. Consequently, many plants store it in the freezer at 0 to −10°F. To test this assumption, a fresh shipment of alpha-naphthol, Eastman 170, was placed in three brown screw-cap bottles, one at 0°F, 40°F, and room temperature (70–75°F), respectively for 12 months.

The VP activity of the test material was checked against 1 ppm of diacetyl, using freshly prepared KOH, and 2% creatine as the other reagent. The results (Table 4) show alpha-naphthol does not break down at room temperature. For sake of space, O.D. values for only the start and end of the test period are given. The other values were very similar to those presented.

Effect of temperature and length of storage of alpha-naphthol reagent on VP reaction. The stability of alpha-naphthol reagent, after it has been prepared, has been one of conjecture. To investigate this variable, alpha-naphthol reagent prepared from Eastman 170 was placed in three brown screw-cap bottles. One was stored at 0°F, 40°F, and room temperature, respectively, for 50 days. The VP activity of the test material was determined as described in the previous experiment. Temperature had little effect on the stability of alpha-naphthol reagent (Table 5). In terms of percent light transmission (L.T.), the 50-day readings were 82, 81, and 79 for 0°F, 40°F, and room temperature, respectively.

Comparison of ethyl and isopropyl alcohols for preparing alpha-naphthol reagent. Isopropyl alcohol (99%), because of its accessibility, is used by most plants instead of ethyl as a solvent for alpha-naphthol. In this study, two alpha-naphthol reagents were prepared, one from each alcohol. VP activity of diacetyl and AMC was then determined using KOH containing 2% creatine as the other reagent. Al-

1389

though there was only a slight difference in the rate of color development between the two alcohols, isopropyl consistently developed color faster than ethyl at 0.5 and 1.0 ppm diacetyl levels (Table 6).

The rate of color development for 0.2 ppm diacetyl was about the same for each alcohol. Similar results, not shown in data, were obtained with AMC in all concentrations up to 4 ppm, the highest level tested; i.e., color developed faster with isopropyl alcohol.

KOH creatine reagent prepared from creatine from different suppliers. There has been some question, in the frozen citrus concentrate industry, that KOH creatine reagent prepared from creatine from one supplier varies from that of another. To check this assumption, four different KOH (40%) creatin (2%) solutions were prepared using creatine from three suppliers—Eastman, Pfanstiehl, and Matheson. Two different grades of Eastman creatine were also compared, Eastman Grade (951) and 90% Practical (P951). Freshly prepared 5% alpha-naphthol (Eastman 170) in 99% isopropyl alcohol was used as the other reagent.

All readings were made after 60 sec, using a solution containing 1 ppm diacetyl as the standard. It would appear from the data in Table 7 that either grade of Eastman creatine can be used. Also, there was little or no difference in the VP reaction when creatine from three different suppliers was used.

The effect of concentration of creatine and KOH solution on VP reaction for diacetyl and AMC. A survey of the Florida citrus concentrate industry (Murdock and Dennis, 1964) showed that the percentage of creatine employed in preparing the KOH creatine reagent varied from 0.3% to 2.0%, with the majority of plants using 0.3%. To study the effect of creatine in the KOH reagent, 4 different solutions were prepared containing 0.3, 0.5, 2.0, and 5.0 g per 100 ml of KOH, respectively. Each solution was used as one of the reagents in determining the VP reaction against known concentrations of diacetyl and AMC.

Data in Tables 8 and 9 show the fastest color reaction, at a given time interval, occurred with 0.3% creatine, and the slowest with 5%. For diacetyl, maximum color development was obtained between 110 and 140 sec, regardless of the concentration of creatine employed. For AMC, the color reaction was not complete at the end of the 10-min test period. Hill and Wenzel (1954) found that 25 min were required for 5 ppm of AMC to reach maximum color intensity.

Table 3. Comparison of reagent and technical grades of Eastman alpha-naphthol for VP activity. Data from 10 replicate tests.

ppm Diac.	Reagent avg. O.D.	Reagent range	Technical avg. O.D.	Technical range
	Reaction time 60 sec			
0.5	0.41	0.35–0.45	0.46	0.42–0.50
1.0	1.05	0.98–1.10	0.99	0.95–1.00

Table 4. Effect of temperature and length of storage of alpha-naphthol on VP activity. Data avg. of 3 replicate tests.

Reaction time min	O.D. values for 1 ppm diacetyl at start and end of 12-mo. test period: Control	Temperature of storage: 0°F 12 mo.	40°F 12 mo.	RT 12 mo.
1.0	0.94	0.91	0.98	0.95
2.5	1.10	1.25	1.28	1.26
5.0	1.10	1.23	1.25	1.19
7.5	1.07	1.20	1.20	1.15
10.0	1.03	1.13	1.19	1.12

Table 5. Effect of temperature and length of storage of alpha-naphthol reagent on VP activity. Data avg. of 3 replicate tests.

Days stored	O.D. values for 1 ppm diacetyl at 60 sec: Temperature of storage 0°F	40°F	RT
0	0.85	0.85	0.85
2	0.78	0.82	0.93
3	0.75	0.80	1.13
4	0.78	0.98	1.10
5	0.88	0.92	1.03
8	0.88	0.90	1.00
11	0.88	0.90	1.00
15	0.87	0.90	1.00
22	0.88	0.90	1.13
43	0.87	0.90	1.00
50	0.87	0.90	1.02

Table 6. Comparison of ethyl and isopropyl alcohols for preparation of alpha-naphthol reagent. Data avg. of 4 replicate tests.

ppm Diac.	Reaction time in sec O.D. values: 60	100	140
	Ethyl alcohol		
0.2	0.20	0.28	0.30
0.5	0.48	0.58	0.61
1.0	0.94	1.10	1.14
	Isopropyl alcohol		
0.2	0.22	0.26	0.27
0.5	0.55	0.65	0.69
1.0	1.05	1.21	1.25

Table 7. Comparison of creatine from different suppliers. Data avg. of 10 replicate tests.

Creatine	Reagent prepared from each compound	O.D. values for 1 ppm diac. at 60 sec avg.	range
Eastman (Eastman Grade)	951	0.92	0.90–1.0
Eastman (Practical)	P951	0.91	0.90–1.0
Pfanstiehl Lab.	C.P.	0.93	0.90–1.0
Matheson	Monohydrate	0.92	0.90–0.93

Effect of temperature and length of storage of KOH creatine reagent. To study this variable, a 40% KOH solution containing 2% creatine C.P. (Pfanstiehl Laboratories) was prepared. Equal portions were stored at 0°F, 40°F, and room temperature for 127 days. The test reagents were used to determine the VP activity, as previously discussed. As indicated in Table 10, KOH creatine solution breaks down rapidly at room temperature. A 22% reduction occurred in 24 hours and 38% in 48 hours. This compares with 11% and 7%, respectively, during a similar period at 40°F. At 0°F, storage up to 3 weeks appears possible without any appreciable change.

Effect of order of adding reagents on VP reaction. In the original VP test described by Byer (1954), alpha-naphthol solution was added first to the distillate followed by the KOH creatine reagent. Byer stated that adding the solutions in reverse order would cause erroneous results.

In our survey of the concentrate industry, it was found that all plants, except one, added the reagents in the order Byer specified. To study this variable, two sets of samples contain-

1389

Table 8. Effect of concentration of creatine in KOH solution on VP reaction for diacetyl. Data avg. of 4 replicate tests.

% Creatine	Reaction time in sec O.D. values 60	90	120	150
	0.2 ppm diac.			
0.0[a]	0.05	0.06	0.06	0.05
0.3	0.25	0.31	0.33[b]	0.33
0.5	0.24	0.29	0.30[b]	
2.0	0.22	0.27	0.28[b]	0.28
5.0	0.17	0.22	0.24	0.25
	1.0 ppm diac.			
0.0[a]	0.10	0.12	0.12	0.11
0.3	1.12	1.38	1.50[b]	1.54
0.5	1.13	1.35	1.42[b]	1.45
2.0	1.05	1.19	1.25[b]	1.26
5.0	0.85	0.98	1.02[b]	1.04

[a] Light brown instead of pink color obtained when creatine was omitted from the reagent.
[b] Maximum color intensity obtained between 110 and 140 sec.

Table 9. Effect of concentration of creatine in KOH solution of VP reaction for AMC. Data avg. of 4 replicate tests.

% Creatine	Reaction time in min O.D. values 4	6	8	10
	1.0 ppm AMC			
0.3	0.86	1.09	1.28	1.40
2.0	0.73	0.89	1.03	1.14
5.0	.....	0.52	0.61	0.68
	2.0 ppm AMC			
0.3	1.46	1.94	2.29	2.51
2.0	1.16	1.53	1.78	1.95
5.0	0.76	0.99	1.22	1.37
	4.0 ppm AMC			
0.3	2.56	3.48	4.14	4.61
2.0	2.17	2.88	3.39	3.77
	1.27	1.76	2.11	2.40

Table 10. Effect of temperature and length of storage of KOH creatine reagent on VP activity. Data avg. of 3 replicate tests.

Days stored	O.D. values for 1 ppm diac. Reaction time 60 sec. Temperature of storage 0°F	40°F	RT
0	0.90	0.90	0.90
1	0.83	0.89	0.70
2	0.83	0.84	0.56
3	0.83	0.86	0.40
4	0.85	0.78	0.35
7			0.35
9	0.85	0.70	0.32
11	0.86	0.82	0.30
21	0.80	0.55	0.20
66	0.70	0.30	0.05
127	0.65	0.23	0.01

ing 1 ppm diacetyl were prepared. To one, alpha-naphthol reagent was added first; to the other it was added last. Reaction time was recorded after the addition of the second reagent. Data in Table 11 indicate that the order of adding the reagents has no effect on the VP reaction.

Effect of amount of distillate used for VP reaction. The usual practice in the citrus concentrate industry is to collect the first 25 ml of distillate. Some plants use the full amount for their VP reaction, while others use a 10 ml portion, adding 5 ml of alpha-naphthol and 2 ml KOH creatine. Twice the amount of each reagent is used for the 25 ml sample. The data in Table 12 show that either 10 or 25 ml of the distillate can be used without affecting the VP reaction, if the correct amount of each reagent is added.

Table 11. Effect of order of adding reagents on VP reaction. Data avg. of 3 replicate tests.

Order of adding reagents	O.D. values for 1.0 ppm diac. Reaction time in min 1.0	2.5	5.0	7.5	10.0
Alpha-naphthol — 1st	0.97	1.25	1.23	1.17	[illegible]
KOH creatine — 1st	0.96	1.20	1.17	1.12	[illegible]

Table 12. Effect of amount of distillate used for VP reaction. Data avg. of [illegible] replicate tests. All values are O.D. Reagents added to 10 ml distillate—5 ml of alpha-nap[illegible] and 2 ml of KOH creatine; for 25 ml twice these amounts were used.

ppm diac.	Tests based on 25 ml of distillate recovered — Reaction time 1.0 min: ml of distillate used for VP reaction 10	25	Reaction time 10.0 min: 10	[illegible]
0.2	0.15	0.17	0.23	[illegible]
0.5	0.43	0.47	0.58	0.58
1.0	0.90	0.93	1.11	[illegible]

Table 13. Effect of age of diac. on VP reaction.

Age of diacetyl	O.D. values for 1 ppm diac. Reaction time in min 1.0	2.5	5.0	10.0
New[a]	1.00	1.13	1.11	1.06
2 years on lab. shelf (previously opened)	0.61	0.72	0.75	0.67

[a] Sealed bottle received from Eastman. Opened at time of experiment.

Effect of age of diacetyl on VP reaction. It is customary to prepare a calibration curve by applying the VP test to samples of water to which known amounts of pure diacetyl have been added. This compound is usually received from the supplier in small glass sealed bottles, and when not in use is stored on the laboratory shelf. Since diacetyl is volatile, the effect of storage at room temperature on the strength of the compound was determined. The standard VP test, as previously discussed, was performed using 2 bottles of pure diacetyl (Eastman 1591) as the test material. One bottle represented a fresh shipment and the other had remained on the laboratory shelf for 2 years.

Data (Table 13) show diacetyl held for a prolonged period at room temperature should not be used for preparing a standard curve because it will give erroneous results. However, subsequent investigation has shown that if a bottle is tightly stoppered and stored in the refrigerator (40°F), it can be held for an extended period without any reduction in strength.

Detection of diacetyl in frozen concentrated orange juice. The following is a suggested procedure for the detection of diacetyl in frozen concentrated orange juice. Interference from AMC is negligible. It follows closely the original procedure described by Byer (1954).

Potassium hydroxide (40%) containing creatine. Dissolve 40 g of potassium hydroxide pellets in sufficient quantity of distilled water to make 100 ml of solution. Then add 0.3 g of creatine (reagent or technical grade). Stir mixture until dissolved. *Caution:* Reagent breaks down very rapidly at room temperature. Store not over 3 days at 40°F, and 21 days at 0°F.

Alpha-naphthol reagent. Dissolve 5 g of alpha-naphthol (Eastman 170 or C.P.) in 100 ml of 99% isopropyl alcohol. Temperature of storage not critical.

1. Add 300 ml of reconstituted juice (12° Brix) to a large boiling flask connected to a water condenser to allow for rapid distillation.

2. Add 1 or 2 drops of antifoam solution to each 300 ml sample. Solution made by mixing 1 g of Dow-Corning Antifoam, AF emulsion, in 10 ml distilled water.

3. Distill off sufficient distillate to recover 25 ml, when filtered through a No. 1 Whatman filter paper (used to eliminate cloud or oil interference).

4. Pour into a 125 ml Erlenmeyer flask. Add 10 ml of alpha-naphthol solution. Reagents may be added to a

10 ml portion of the 25 ml distillate. To this amount, add 5 ml of alpha-naphthol and 2 ml of KOH creatine.

5. Add 4 ml of KOH solution containing creatine. Order of adding reagents is not critical.

6. Mix by swirling gently for 20 sec, and immediately transfer to a colorimetric tube. Record reading 60 sec after addition of reagents as % light transmission (L.T.), or as optical density (O.D.). Use a Lumetron colorimeter with a 530mμ filter.

Null the colorimeter by holding a blank containing distilled water and the two reagents for the same period as the test sample.

7. Prepare a calibration curve by applying this method to samples of distilled water to which known amounts of diacetyl have been added. Use a sealed, unopened bottle of diacetyl, recently purchased if possible.

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Ms. rec'd 8/12/66.

The author acknowledges the assistance of Messrs. T. H. Shang, A. J. Mercer and R. E. Dennis who performed the analyses.

1391

(1391)

Diacetyl Test as a Quality-Control Tool in Processing Frozen Concentrated Orange Juice

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Food Tech. 22 (1), 90-94, (Jan. 1968)

SUMMARY

Data are presented to show that the diacetyl test can be used as an index of juice quality and poor sanitation in processing frozen concentrated orange juice. Approximately 1/3 of the diacetyl present in the evaporator feed juice was lost in a TASTE evaporator during the concentrate operation. An increase in diacetyl usually occurred when a low temperature evaporator was used.

Selection of sample stations, frequency of tests, recording and interpretation of data are all integral parts of a biological control program. During normal operation diacetyl values fluctuate within a narrow range. When a buildup occurs, insanitary conditions usually exist in the juice extraction room. Poor fruit being processed may also be a factor.

INTRODUCTION

Lactic acid bacteria are organisms of sanitary significance in processing frozen concentrated orange juice. They impart flavors and odors which have been characterized as similar to "buttermilk" (Hays *et al.*, 1952; Murdock *et al.*, 1952). The bacteria responsible are principally those organisms belonging to the genera *Lactobacillus* and *Leuconostoc*. They are present on and in the fruit when it arrives at the plant and grow well in single strength and partially concentrated orange juice. To prevent a buildup of these organisms during the processing operation, an efficient sanitation program must be maintained.

It soon became apparent among the early investigators that a rapid method was needed to detect the presence of lactic acid bacteria. The direct microscopic method was first used. It consisted of counting the number of cells present on a stained slide preparation. The difficulty in distinguishing between the bacteria and the debris present on the smear left much to be desired. Then in 1954, Hill *et al.*, and Byer introduced to the citrus industry a rapid method for detecting diacetyl and acetylmethylcarbinol (AMC), the metabolic products of the growth of lactic acid bacteria in orange juice. The test, a modification of the Voges-Proskauer (VP) reaction, is extremely sensitive to diacetyl and will detect concentrations of less than 1 ppm. Most processors of frozen concentrated orange juice, as indicated by a survey made by Murdock *et al.* in 1961, use some form or modification of the VP test for detecting diacetyl and AMC in orange juice. Variations in preparing and handling VP reactants were reported by Murdock in 1967.

The purpose of this paper is to show how the diacetyl test, which requires less than one-half hour to perform, can be used as a quality control tool to determine the microbial activity in processing frozen concentrated orange juice.

PROCEDURE

The diacetyl test used in this investigation was described by Murdock (1967). Briefly, it consisted of adding 300 ml of single strength or reconstituted orange juice (12° Brix) to a large boiling flask connected to a water condenser, collecting 25 ml of distillate, adding the VP reagents, and reading the color reaction on a Lametron colorimeter 60 sec after addition of the reagents. The results are expressed as ppm of diacetyl in the distillate.

RESULTS AND DISCUSSION

Relationship of fruit quality to diacetyl values. A study was made to determine the relationship of fruit quality to diacetyl values. It consisted of collecting at the concentrate plant ½ box (approximately 45 lb) of sound acceptable fruit (oranges) and a similar amount of reject fruit; the latter consisting of splits, partially decomposed, soft spots, etc. Juice from each lot of fruit was extracted in the laboratory by means of a hand extractor. The following diacetyl values were obtained:

Type of fruit	° Brix	Brix/acid ratio	ppm Diacetyl
Sound (good)	12.16	11.47	0.15
Reject (bad)	12.98	11.42	0.80

Beisel, *et al.* (1954) also found that juice from defective fruit was higher in diacetyl than that from sound fruit. Murdock *et al.* (1958) found that there was a direct correlation between fruit quality and the microbial contamination of freshly extracted juice. Therefore, if unsound fruit entering the plant is not removed during the grading operation, it will not only "seed" the plant with microorganisms but will also result in an immediate surge in diacetyl during the processing operation.

Diacetyl as an index of plant sanitation. In citrus processing plants, as in other food plants, juice may occasionally remain stagnant in an unused pipeline; or a dead pocket may occur as a result of a change in the piping arrangement. When juice is held under these conditions for any extended period, or if the equipment is not kept in a sanitary condition, an increase in diacetyl will occur. This is shown in Fig. 1 where diacetyl values obtained from orange juice held at room temperature (76°F) increased from 0.3 to 2.9 ppm in 20 hr with the greatest increase occurring after 12 hr. Other data not presented herein also showed that juice held under similar conditions increased in diacetyl.

An increase in diacetyl may also occur when pulp adhering to equipment is not removed during a flush operation. Pulp adhering to equipment, such as finisher chutes, developed a sour odor in approximately 6 hr (Murdock, 1966). When this occurred, the pulp usually had a total viable count of 1,000,000 organisms per g and a diacetyl concentration of 1 or more ppm (Minute Maid Co., 1965).

Diacetyl values during evaporator operation. Hill *et al.* (1957) reported on the diacetyl values obtained in processing orange concentrate in a low temperature pilot and commercial evaporator. An increase in diacetyl occurred during the concentrate process, usually after the evaporator had been operated 24 hr or more. The citrus industry within the last year or two has replaced nearly all of their low temperature evaporators with high temperature short holding time units, generally referred to as a TASTE evaporator (Thermal Accelerated Short Time Evaporator, Fig. 2).

A study was made under commercial conditions comparing a TASTE unit with a 6-stage low temperature Buflovak evaporator in processing wash pulp concentrate. Samples for diacetyl analyses were drawn every

Table 1. Distribution of diacetyl data in processing wash pulp concentrate during 1966 Valencia season.

Evaporator feed juice		TASTE evaporator April 6–13					
		Pump-out concentrate (60° Brix)					
Diacetyl range ppm		Diacetyl range ppm Reconstituted juice basis					
		0.00–0.05	0.10–0.15	0.20–0.25	0.30–0.35	0.40–0.45	0.50–0.55
	Total freq.						
0.40–0.45	1	1	0	0	0	0	0
0.30–0.35	11	1	2	0	2	0	0
0.20–0.25	18	0	14	2	2	0	0
0.10–0.15	2	1	1	0	0	0	0
	35						
		Buflovak evaporator April 22–May 4					
0.40–0.45	5	0	0	1	3	1	0
0.30–0.35	19	0	0	7	7	4	1
0.20–0.25	16	0	2	4	4	5	1
0.10–0.15	4	1	1	2	0	0	0
	44						

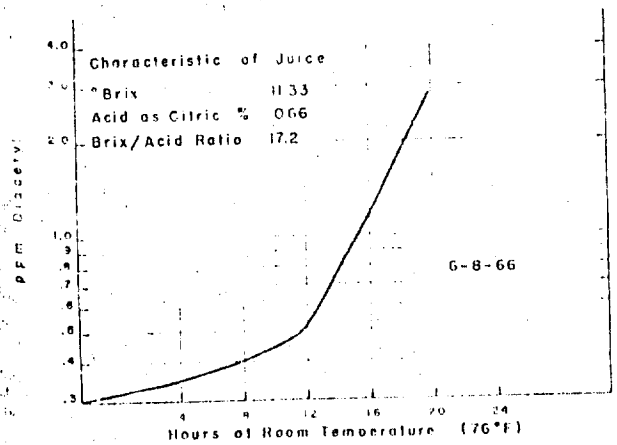

Fig. 1. Diacetyl values of orange juice held at room temperature. Sample obtained from finishers.

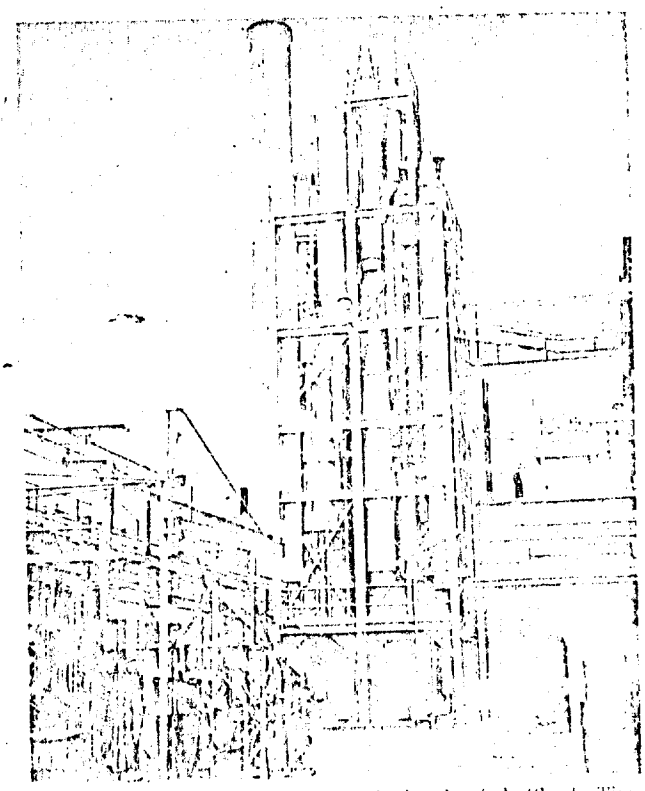

Fig. 2. The TASTE (Thermal Accelerated Short Time Evaporator) is now in general use by the citrus industry.

4 hr while the evaporators were in operation. Juice was collected before it entered the evaporator (evaporator feed) and when it left the units (pump-out concentrate). The Buflovak units were cleaned on a 48–72 hr cycle, and the TASTE evaporator every 8–12 hr; the usual practice in the industry. TASTE evaporators require more frequent cleaning to remove hesperidin which plates out in this unit.

A table of frequencies of diacetyl values (evaporator feed juice and pump-out concentrate), for the period the TASTE and Buflovak evaporators were in operation at Plant A, is presented in Table 1. Table 2 shows a frequency distribution of the diacetyl values obtained in processing orange concentrate in a TASTE evaporator at Plants A and B. An increase in diacetyl usually occurred when concentrating juice in a low temperature evaporator (Buflovak); in a TASTE unit the opposite was true (Tables 1 and 2).

This is also shown in Table 3, where the average diacetyl values for the test period and the percent change between evaporator feed juice and pump-out concentrate are presented. Note when these two evaporators were processing wash pulp, the average loss of diacetyl for the TASTE evaporator was 36%, which compares with a 15% increase for the Buflovak evaporator. A similar loss occurred when the TASTE evaporator processed orange concentrate at Plants A and B.

Plate counts made on products entering and leaving the TASTE evaporator have also shown it is not a source of contamination. Similar data from the Buflovak evaporator, on the other hand, show a microbial buildup in the low Brix stages, even after a short period of operation (Minute Maid Co., 1965).

Table 2. Distribution of diacetyl data in processing orange concentrate in TASTE evaporator during 1966 Valencia orange season.

Evaporator feed juice: Diacetyl range ppm	Total freq.	Pump out concentrate (60° Brix): Diacetyl range ppm, Reconstituted juice basis 0.00–0.05	0.10–0.15	0.20–0.25	0.30–0.35	0.40–0.45	0.50–0.55
		Frequencies					
Plant A, April 29–June 22							
0.60–0.65	7	0	0	1	3	2	1
0.50–0.55	33	0	1	15	10	5	2
0.40–0.45	85	1	15	41	22	3	0
0.30–0.35	79	2	30	41	6	0	0
0.20–0.25	42	4	31	6	1	0	0
0.10–0.15	4	1	3	0	0	0	0
	250						
Plant B, April 11–May 10							
0.10–0.15	3	0	3	0	0	0	0
0.30–0.35	12	1	10	0	1	0	0
0.20–0.25	13	4	9	0	0	0	0
0.10–0.15	8	1	6	1	0	0	0
	36						

Table 3. Average diacetyl values for test period.

Product	Evaporator	No. tests	Avg. ppm of diacetyl, Reconstituted juice basis: Evaporator feed juice	Pump-out concentrate	Diacetyl % change
Wash pulp (60° Brix)	TASTE	35	0.265	0.171	35% (−)
Wash pulp (60° Brix)	Buflovak	44	0.282	0.323	15% (+)
Orange conc. (65° Brix)	TASTE[1]	250	0.374	0.251	32% (−)
Orange conc. (65° Brix)	TASTE[2]	36	0.260	0.160	38% (−)

[1] 40,000 lb. evaporator Plant A.
[2] 50,000 lb. evaporator Plant B.

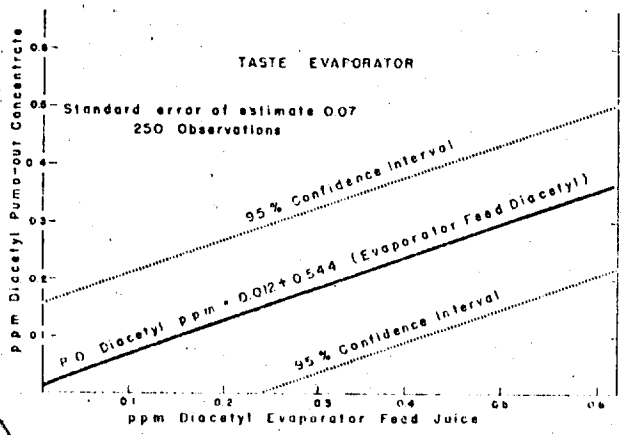

Fig. 3. Relationship of diacetyl values in evaporator feed juice to pump-out orange concentrate for 1966 Valencia season.

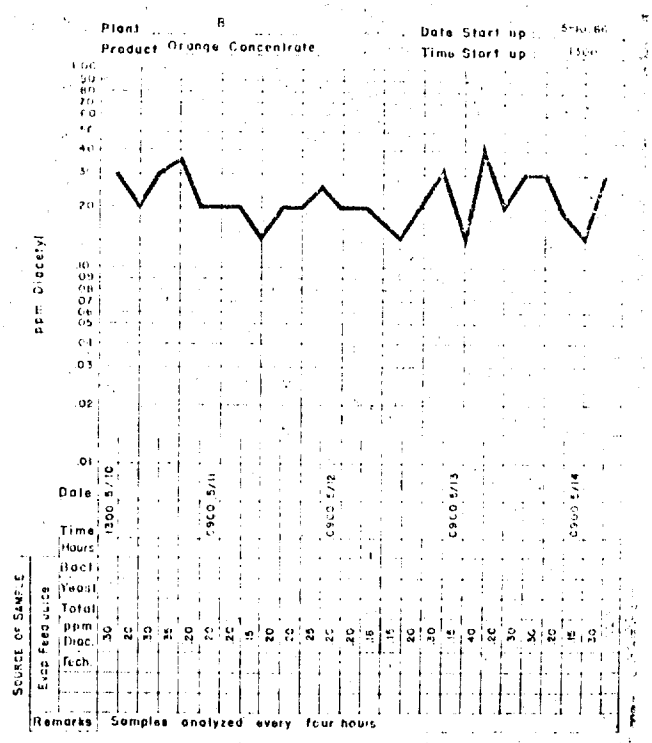

Fig. 4. Diacetyl values obtained during normal operation.

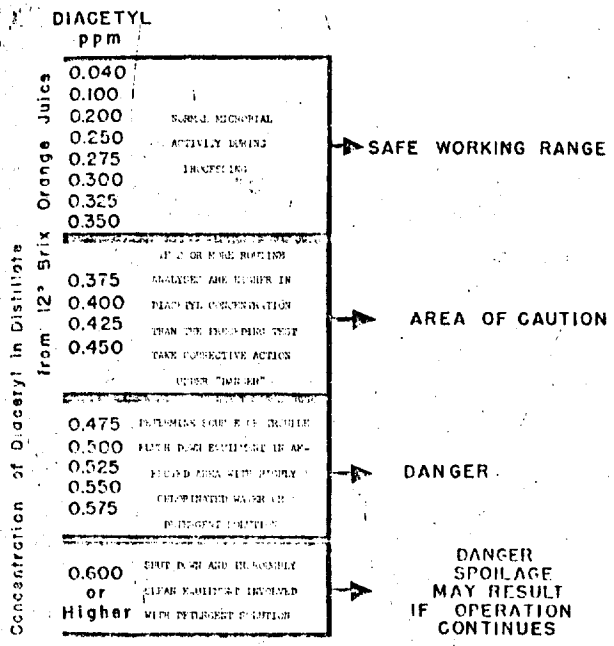

Fig. 5. Guide for interpretation of diacetyl analysis in processing frozen concentrated orange juice.

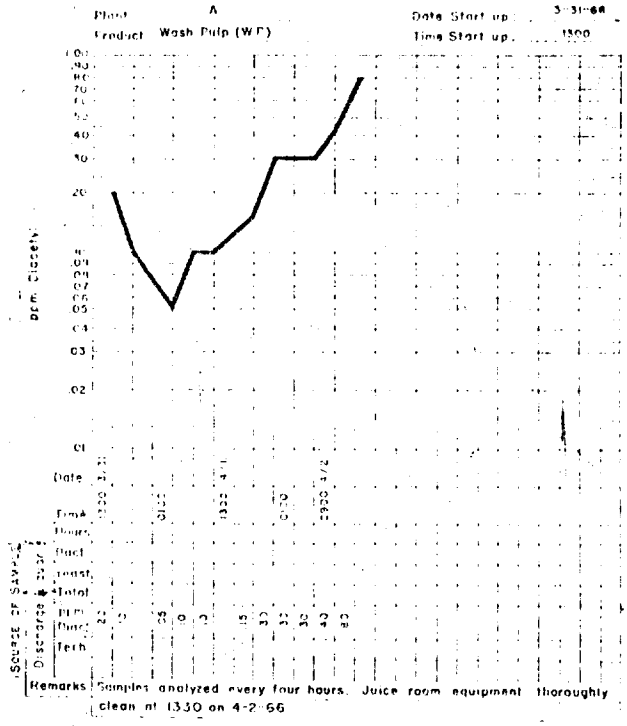

Fig. 6. Log showing diacetyl buildup.

In analyzing the diacetyl data obtained from Plant A, the following regression equation was derived:

$$\text{Pump-out diac. ppm} = 0.012 + 0.544 \times \text{evap. feed diac.}$$

This equation shows the functional relationship between the level of diacetyl in the evaporator feed juice entering the TASTE evaporator and in the 65° Brix concentrate pump-out. From the equation, it can be estimated that if the evaporator feed juice contains 0.4 ppm diacetyl there will be, on the average, 0.23 ppm diacetyl in the pump-out concentrate.

The relationship for other diacetyl values in evaporator feed juice to pump-out concentrate is shown in Fig. 3. Using the standard error of the estimate of 0.07 ppm, a 95% confidence interval was calculated to show the variability associated with the estimate. For an evaporator feed juice diacetyl of 0.4 ppm, we would expect 95% of the estimates of pump-out diacetyl ppm to fall within a confidence interval of 0.09 to 0.37 ppm.

Although the strength of the functional relationships for the other TASTE evaporators studied was weak due to the limited number of samples collected, it appears that similar regression equations apply.

Biological control program. We have seen that diacetyl can be used as presumptive evidence of the quality of the incoming fruit, and as an index of sanitation in a citrus concentrate plant. Since the diacetyl test will detect the end products of microbial growth in orange juice during processing operations, it can be used effectively as a quality control tool. In developing a biological control program, the following should be considered:

1. *Sample stations*—select definite check points to adequately cover the processing operation.
2. *Frequency of tests*—check product from each station every 4 hr, or more frequently if necessary.
3. *Recording of data*—log all diacetyl values. An example of a log in which the data can be plotted graphically as it is accumulated is shown in Fig. 1.
4. *Interpretation of data*—be able to correctly interpret the data as it is obtained. To assist Minute Maid personnel in this regard, a Guide for Interpretation of Diacetyl Analyses has been developed (Fig. 5).

Diacetyl values during normal and abnormal periods. Fig. 4 is a copy of a log showing diacetyl values obtained during a normal plant run which started on May 10, 1966. Note that the values remain more or less constant within a very narrow range.

Fig. 6 is a copy of a log showing a diacetyl buildup. Observe the steady increase in diacetyl in the wash pulp discharge liquor starting at 0100 on April 1, and the large increase between 0900 on April 2, and 1300 on that same day. During this time interval the diacetyl increased from 0.4 ppm to 0.8 ppm in 1 hr. This indicates that the contaminating organisms were in their optimum growth phase.

If preventive measures are not taken, spoilage will occur in a short time. In this instance, the juice room was shut down and the equipment thoroughly cleaned with a detergent solution. The source of contamination, when conditions exist as illustrated in Fig. 6, usually will be found in the juice room. It may occur from sour pulp in finisher chutes, stagnant juice in troughs or header lines, etc. If found, a flush down with chlorinated water is indicated and/or the equipment should be thoroughly cleaned with detergent solution.

Another abnormal condition is a sudden surge in diacetyl. This usually occurs when:

1. A "slug" of sour juice is diverted into the system, i.e., from an unused pipeline not cleaned prior to use;
2. product remaining in an evaporator feed tank that has become stagnant; or
3. a load of poor fruit being processed.

Fortunately, a sudden increase in diacetyl is usually followed by a corresponding decrease as the slug of contaminated product passes through the system. If diacetyl does not return to normal, a flush down or a clean-up is indicated.

We have seen how the diacetyl test can be used as an effective quality control tool in the processing of frozen concentrated orange juice. It can also be used to a lesser degree as an indication of the quality of bulk concentrate which, at times, is purchased on the open market. If the product was produced in a TASTE evaporator, it must be remembered that approximately $\frac{1}{3}$ of the diacetyl present in the evaporator feed juice will be lost during the concentrate operation. Therefore, if high diacetyl is found in bulk drummed product, the assumption can be made that the product was produced under insanitary conditions prior to evaporation.

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Presented at the 79th meeting of the Florida State Horticultural Society, Miami Beach, Florida, Oct. 27, 1966.

The author gratefully acknowledges Mr. W. J. Hepburn for the statistical analyses of the data and Quality Control personnel for the diacetyl determinations.

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SANITATION PROBLEMS IN THE PRODUCTION OF FROZEN CITRUS CONCENTRATE[1]

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J. of Milk and Food Technology
31(8): 245-250

Abstract

Extensive repairs are made during the off season in food plants operating on a seasonal basis. Precautions taken to protect equipment during down periods are discussed.

Microorganisms causing food poisoning do not grow, but die readily in citrus juices and concentrates. Coliform bacteria are of no apparent public health significance in frozen citrus concentrates, partially concentrated or single strength citrus juices. Lactic acid bacteria, which constitute the most important index of processing sanitation, are held in check by constant sanitation efforts.

Soundness of incoming fruit and efficiency of grading are important factors in final product quality. Chlorinated water sprays are used to sanitize fruit handling equipment. A chlorine concentration of 15 ppm was found to be effective in controlling conveyor belt contamination at one plant.

The TASTE evaporator (thermal accelerated short time evaporator), now used almost universally in the citrus industry, is not a source of microbial contamination. Data indicate that a loss of diacetyl occurs when it is used for processing.

In distributing finished product from processor to consumer, an occasional can may infrequently be exposed to conditions conducive to the development of spoilage. Tests results indicate that for a 6 oz can of frozen concentrated orange juice to swell or burst, 3 or more days are required at room temperature, and 5 or more wk at 40 F.

Microbial contamination is controlled in the citrus industry by strict adherence to fruit quality and by maintaining an efficient sanitation program. The diacetyl test is used as a quality control tool to detect microbial activity in processing orange and grapefruit concentrates.

Good housekeeping is an important adjunct to sanitation. One company employs a good housekeeping/safety contest as a method of maintaining a safe and neat-appearing plant. The contest has resulted in improved morale of employees, along with better over-all plant sanitation and efficiency.

Webster defines sanitation as the application of measures to make environmental conditions available to health. Modern usage does not restrict the term to conditions affecting health. According to the Food and Drug Administration, the term must be interpreted broadly encompassing the field of food plant sanitation including conditions that not only lead to contamination of food with microorganisms or other elements dangerous to health, but also filth and other extraneous matter that have no place in food. Sanitation, an integral part of plant operation, is of utmost importance in the production of high quality frozen food products with wide consumer acceptance. It is for this reason that the citrus industry emphasizes sanitation so strongly in its plants. The industry's policy is to give the consumer not just a good product, but the best and finest attainable. At no time does it want the consumer to open a can of citrus concentrate which is "off-flavored" or contains foreign matter. The citrus industry has one of the highest sanitary codes in the frozen food field.

The Florida citrus industry is big business. Last season (1965-66) the on-tree-dollar value was $227,952,000, with 72% of the oranges in the United States produced in Florida, 26% in California, and the remainder in Texas, Arizona and other states *(3)*. This season (1966-67) an estimated 147,000,000 boxes of oranges will be produced in the state of Florida. Over 50% of the orange crop annually is used for the production of frozen concentrated orange juice. Processing this tremendous volume has led to the development of the most modern equipment and processing technique.

Processing Frozen Orange Concentrate

The processing of frozen orange concentrate is briefly reviewed below. At most plants fruit is received by truck, dumped onto a conveyor system, graded for maturity and soundness, stored in bins, washed, regraded, and sanitized prior to entering the extractors. Juice leaving the extractors passes through a series of finishers which remove the seeds and pulp. Product, prior to entering the evaporators, is pumped to large storage tanks which are either held under atmospheric pressure or vacuum. In the evaporators juice is concentrated to the desired solids content measured as degrees Brix. It is flash-heated to reduce enzyme activity, and thus retard separation and gelation. The concentrate then enters the blending tanks where it is mixed with fresh juice to replace some of the volatile constituents which were lost during evaporation. In some plants volatile essence is recovered and restored to the product along with cutback juice. The blended (45 ° Brix) product is either pumped through votators where it is slush-frozen or to cold wall tanks prior to filling into cans. The finished canned concentrate is quick-frozen in a blast or alcohol freezer, cased, and stored in a cold

[1]Presented at the 54th Annual Meeting of the International Association of Milk, Food, and Environmental Sanitarians, Miami Beach, Florida, August 14-17, 1967.

storage warehouse at 0 to −10 F until shipped through commercial channels to the market.

The frozen citrus concentrate season in Florida usually starts in December and extends into June of the following year. Operation during this period is usually 7 days a wk and 24 hr a day. At the end of the season's operation all equipment is given a thorough renovation. Most plants, during the summer months, make necessary equipment changes to eliminate processing "bottlenecks" which have shown up the previous season. Occasionally certain phases of the processing operation are completely modified to improve manufacturing efficiency. An extensive painting program is usually carried out. Buildings are painted both inside and out where needed. Equipment is given a thorough overhaul and defective parts replaced. Floors, both concrete and tile, are inspected and repaired as required. Since the citrus industry in Florida is located in a semitropical area, problems associated with high humidity are of concern. Consequently, steps are taken to minimize rust formation by use of rust inhibitors, grease, and by use of paint on equipment where needed.

Prior to the start of seasonal operation all equipment is reassembled and checked to make sure it is in running order. Each plant is inspected to insure that: (*a*) motor guards removed during the summer have been replaced, (*b*) windows are installed where broken or absent, (*c*) doors or windows that require rescreening have been rescreened, and (*d*) plant appearance and housekeeping are satisfactory.

SANITATION PROBLEMS ENCOUNTERED DURING PROCESSING SEASON

Up to this point, the operations that are generally followed by the citrus industry during their off-season have been briefly discussed. Now, some of the sanitary problems encountered during the processing season will be mentioned. However, before proceeding, mention should be made of the microflora encountered in processing frozen concentrated orange juice.

Significance of pH

The pH of citrus concentrate, excluding lemon and lime, which averages from 3.4 to 4.0, makes this product unique in comparison with other food products. Citrus juices, therefore, act similar to a differential plating medium in that they restrict the growth to only those organisms capable of tolorating this acid environment. Consequently, enteric organisms such as *Salmonella* and *Shigella* types cannot survive for long periods in citrus juices or concentrates *(1, 5)*. Furthermore, the spores of *Clostridium botulinum*, Types A and B, apparently cannot germinate even though they may be present *(18)*. In other words, microorganisms causing food poisoning do not grow, and often die readily in citrus juices and concentrates.

Coliform bacteria have been reported to be present from time to time in frozen citrus concentrates by various public health agencies. In this regard, there are no data to indicate that these organisms actually grow in citrus juices. On the other hand, there is considerable evidence to show that coliform bacteria can retain their viability for extended periods in frozen orange concentrate, but die off rapidly in fresh or reconstituted juice *(2, 5, 19)*. It is the opinion of Dack *(2)*, and others *(7, 8)* that the coliform index of frozen citrus products is of no public health significance. Dack *(2)* also states, "Millions of cans of frozen concentrated orange juice have been consumed without causing any known cases of enteric infections."

Microorganisms capable of growing in citrus products

Up to this point, microorganisms which fail to grow in citrus products have been discussed. Now those capable of tolerating this acid medium will be considered. Organisms known to grow in single strength juices of these acid foods (not including lemon or lime juice) are lactic and acetic acid bacteria, yeasts and molds. Of this group, organisms belonging to the genera *Lactobacillus* and *Leuconostoc* are of prime concern. Lactic acid bacteria are organisms of sanitary significance in processing frozen concentrated orange juice. They impart flavors and odors which have been characterized as similar to "buttermilk" *(6, 7, 15)*.

Fruit handling

One of the most important factors in the production of high grade product is the quality of the incoming fruit. It must be carefully graded prior to juice extraction. If defective fruit entering the extractors is not removed, it will affect the microbial load of the expressed juice (Table 1) and may produce "stale" and "old" fruit flavors in the finished product.

In some plants, fruit is washed prior to bin storage and again before juice extraction. The effect of pre-bin washing was investigated *(16)* and results indicate a decrease in fruit surface contamination occurred immediately after the initial fruit wash. The overall effect of pre-washing was not statistically significant. However, fruit handling equipment (particularly the storage bins) remained in a more sanitary condition, and the washed fruit was cleaner from the standpoint of surface soil up to the point of final brush washing. Graders preferred handling washed fruit and culled more oranges entering bins when they were unwashed. Pre-bin washing did not affect the performance of the final graders (Table 2).

One of the most effective means of removing soil

TABLE 1. RELATIONSHIP BETWEEN QUALITY OF FRUIT ENTERING EXTRACTORS AND CONTAMINATION OF EXTRACTED JUICE[a]

Defects[b]	Frequency of occurrence	Juice sampled before finishers Microorganisms per ml		
(%)		(Min.)	(Max.)	(Avg.)
0	10	1,000	21,000	8,835
4	1	37,000	98,000	67,000
6	1	90,000	120,000	105,000
8	2	47,000	320,000	176,000

[a]Period covered—3 days. Total samples examined—14.
[b]Per cent defects based on examination of 50 oranges collected at random from belt feeding one line of extractors.

from fruit surfaces is the brush washer. It consists of a detergent applicator and a set of rotating brushes. The fruit is thoroughly rinsed prior to leaving the unit. The brush washer is an essential piece of equipment used in all citrus plants, and is generally placed in the line prior to the final graders.

Fruit from the bins to the extractors is transported by a series of conveyor belts and elevators. It is important that this equipment not only be cleaned at periodic intervals, but also kept properly sanitized. This is usually accomplished by placing chlorinated water sprays at strategic locations on the conveyor belts and elevators. The sprays must completely cover the entire width of the belt. Otherwise, dark areas at the edges of the belt consisting of mold and bacterial slime will accumulate (Figure 1). When sprays are positioned to adequately cover the entire belt surface, top as well as the underside, microbial contamination will be kept to a minimum. Likewise, the elevators must be effectively sprayed.

The relationship of chlorine concentration on a conveyor belt feeding a line of extractors and the bacterial counts of expressed juice is shown in Table 3. Data show a 15 ppm chlorine residual held expressed juice microbial population to an acceptable level. Although this concentration of chlorine proved to be effective in the present study *(4)*, it could vary from one installation to another. Chlorine concentration is dependent upon the degree of contamination; length and speed of the conveyor belt; and the number, location and volume of the sprays.

Preparation of juice for concentrate

Up to this point the need of proper grading to insure that only sound fruit enters the extractors, and the importance of maintaining the fruit handling system in a sanitary condition have been discussed. An efficient sanitation program is also needed in processing the juice from the extractors to the finished product.

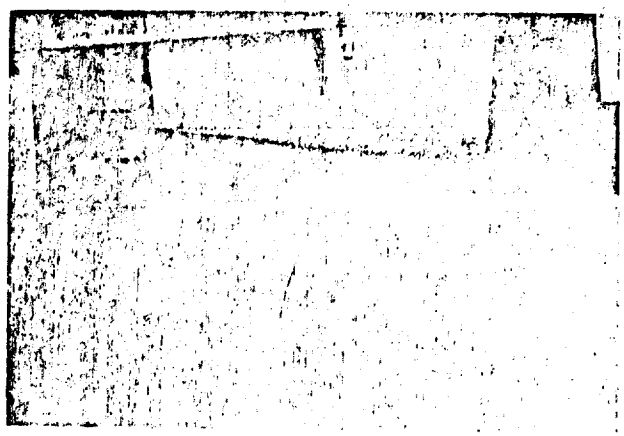

Figure 1. Inadequate coverage of conveyor belt with chlorinated water spray. Dark edges of belt indicate presence of mold and bacterial slime.

The juice extractors, of which there are 2 principal types, Brown (Automatic Machinery Corp.) and the FMC In-line (FMC Corp.), must be flushed with chlorinated water and cleaned with detergent solution at regular intervals. This also applies to the finishers which are used to remove seeds and pulp from the juice. The usual practice at Minute Maid is to flush all juice handling equipment, prior to the evaporators, at 4 hr intervals with water containing between 50 and 100 ppm chlorine. This is a very effective method of controlling microbial buildup.

Evaporators and finished product

Generally speaking, there are two types of evaporators used in the citrus industry, low temperature (approximately 65 to 80 F) and high temperature

TABLE 2. FRUIT CULLED BY GRADERS (WASHED VERSUS UNWASHED FRUIT)

	Unwashed			Washed		
	Total			Total		
	Boxes received	Boxes culled	Per cent culled	Boxes received	Boxes culled	Per cent culled
Fruit to bins	199,031	982	0.493	201,142	749	0.372
Fruit to process	198,049	956	0.483	200,393	1,028	0.513

TABLE 3. GEOMETRIC MEAN BACTERIAL COUNTS OF EXPRESSED JUICE DURING CHLORINATION OF BELTS

Chlorine dosage (ppm)	No. tests	Count per ml
5	10	114,000
10	11	105,000
15	14	19,000
20	4	16,000

TABLE 4. AVERAGE DIACETYL VALUES OBTAINED WHEN PROCESSING WASH PULP CONCENTRATE (60° BRIX)

Evaporator	No. tests	Avg. ppm of diacetyl: Evaporator feed juice	Pump-out concentrate	Diacetyl % change
Low Temp.	44	0.282	0.323	15 (+)
TASTE	35	0.265	0.171	36 (−)

short holding time units (195 to 200 F). The latter are generally referred to as TASTE evaporators (thermal accelerated short time evaporator). Within the last year or two, the citrus industry has replaced nearly all of their low temperature units with the TASTE evaporator. Product leaving the TASTE evaporator is, for all practical purposes, free from microorganisms *(11)*. On the other hand, an increase in contamination usually occurs in a low temperature evaporator. Table 4 shows a comparison of diacetyl values between juice entering and product leaving a TASTE evaporator in comparison with a low temperature unit when wash pulp concentrate was being processed. Note a 36% decrease in diacetyl occurred in the TASTE evaporator which compares with a 15% increase in the low temperature unit. A similar reduction in diacetyl occurred when the TASTE evaporator was used to process orange concentrate.

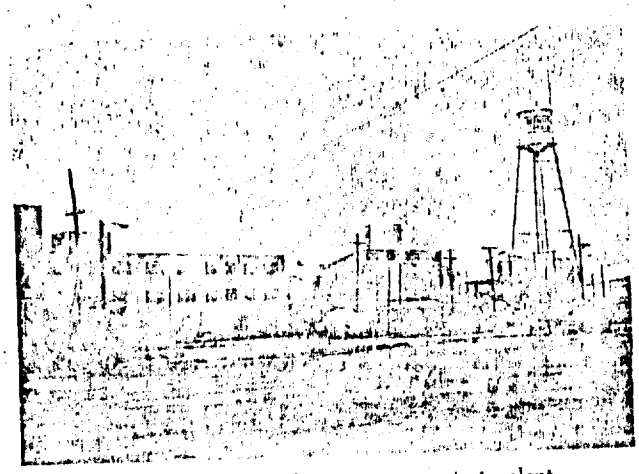

Figure 2. Partial view of a citrus concentrate plant.

Subsequent processing equipment consisting of blending tanks, votators and filler bowls are not a source of contamination during operating periods since concentrate in these units is usually in the temperature range of 25 to 30 F.

Up to this point, sanitation problems encountered from the time the fruit enters the plant to the point where concentrate is placed into the can have been discussed. One would think that there are no more problems after the product has been canned, frozen, cased and placed in primary storage at 0 to −10 F. This is not true. It is entirely possible along the distribution route, between the processor and consuming public, that an occasional can of frozen concentrated citrus juice may be exposed to conditions conducive to the development of spoilage. For example, the consumer may place it in a refrigerator (40 F) or even on the kitchen shelf or in the pantry where the can may explode. Fortunately, these complaints have been exceedingly low, averaging less than 1.5 per 10,000,000 units of 6 and 12 oz cans of frozen concentrated orange juice sold *(10)*.

Lorant *(9)*, in his study on distribution and handling of frozen fruits, vegetables, and juices, states that the major sources of adverse exposure for frozen concentrated orange juice are: (*a*) transport, (*b*) break-up operation, (*c*) retail store, and (*d*) home storage. Of the 4 categories mentioned, the greatest abuse occurs during home storage.

The question is frequently asked, "How long does it take for a 6 oz can of frozen concentrated orange juice to swell or burst when abused?" An investigation *(17)* showed for the cans to swell or burst, 3 days or more were required at room temperature (70-74 F) and 5 wk or more at 40 F. Yeasts were the predominant organisms which grew rapidly in the product stored 5 days at room temperature and were the only ones found capable of growing at 40 F in 42° Brix concentrate (Tables 5 and 6).

Methods used to control microbial contamination

As previously stated, the frozen citrus concentrate industry strives to produce a product that has the appearance and all of the flavor characteristics of freshly squeezed orange juice. How is this possible with large scale production with its inherent sanitation problems? This is resolved by strict adherence to fruit quality and maintenance of an efficient sanitation program. The prerequisites may be briefly stated as follows:

1. Only clean sound fruit must be permitted to enter the extractors.

2. Fruit must be carefully graded before it enters the bins, properly washed, regraded and sanitized prior to juice extraction. If unsound fruit composed of a large percentage of drops, soft deteriorated spots, splits, etc. is permitted to

TABLE 5. SPOILAGE DEVELOPING AT ROOM TEMPERATURE (70-74 F) IN 6 OZ CANS OF ORANGE CONCENTRATE. PRODUCT REPRESENTATIVE OF 2 CITRUS SEASONS

Season	No. cans tested	No. days all cans flat	Days for 1 or more cans: To swell, Days	To swell, %	To burst, Days	To burst, %
1961 Midseason[a]	150	2	3	21	3	2
1961 Valencia	100	2	3	5	3	1
1962 Midseason	100	2	3	1	4	20
1962 Valencia	50	3	4	62	4	16

[a]Samples from 2 plants.

TABLE 6. SPOILAGE DEVELOPING AT 40 F IN 6 OZ CANS OF ORANGE CONCENTRATE. PRODUCT REPRESENTATIVE OF 2 CITRUS SEASONS

Season	No. cans tested	No. weeks all cans flat	Weeks for 1 or more cans: To swell, Wk	To swell, %	To burst, Wk	To burst, %
1961 Midseason[a]	150	4	5	10	[b]	[b]
1961 Valencia	100	4	5	3	7	1
1962 Midseason	100	4	5	3	16	2
1962 Valencia	50	5	6	2	8	16

[a]Samples from 2 plants.
[b]Not checked to determine time required to burst.

enter the extractors, it not only contaminates juice room equipment and evaporators, but may also result in stale, old fruit flavors in the finished product.

3. Fruit handling equipment must be kept in a sanitary condition to prevent recontamination of fruit surfaces. Recontamination may result from slimy belts, sizers, elevators, and from the extractors just before the fruit is extracted if they are not cleaned properly. This problem is resolved through the use of chlorinated water which is used as a germicidal rinse to control fruit surface contamination, and as a spray on conveyor belts, elevators, etc. to minimize slime formation.

4. An effective cleaning and sanitizing program must be maintained to prevent contamination by microorganisms during the processing operation. Otherwise microbial growth, if not controlled, may result in yeasty, fermented, and buttermilk-like flavors in the finished product.

DETECTION OF CONTAMINATION BY LABORATORY METHODS

The citrus industry is very fortunate that there is a rapid method, generally referred to as the diacetyl test *(12)*, for the detection of microbial activity in orange and grapefruit concentrates. It is a colorimetric procedure requiring about 30 min for the detection of diacetyl and acetylmethylcarbinol, end products of microbial growth in orange juice principally by those organisms belonging to the genera *Lactobacillus* and *Leuconostoc*. The diacetyl test is used very effectively as a quality control tool during processing operations *(13)*.

Plate counts are also employed by the citrus industry to determine the total number of viable microorganisms in a product. Generally, they are restricted to finished product. However, in some plants juice from various processing operations are plated. These tests are usually referred to by the citrus industry as line checks.

Minute Maid has been able to detect microbial buildup in their processing operations, source of contamination, and determine the efficiency of each clean-up through their biological control program. This is accomplished by making routine diacetyl determinations and line checks. These procedures have also been highly successful in preventing product spoilage.

HOUSEKEEPING—AN ADJUNCT TO SANITATION

The citrus industry has made great strides in improving plant appearance and maintaining good

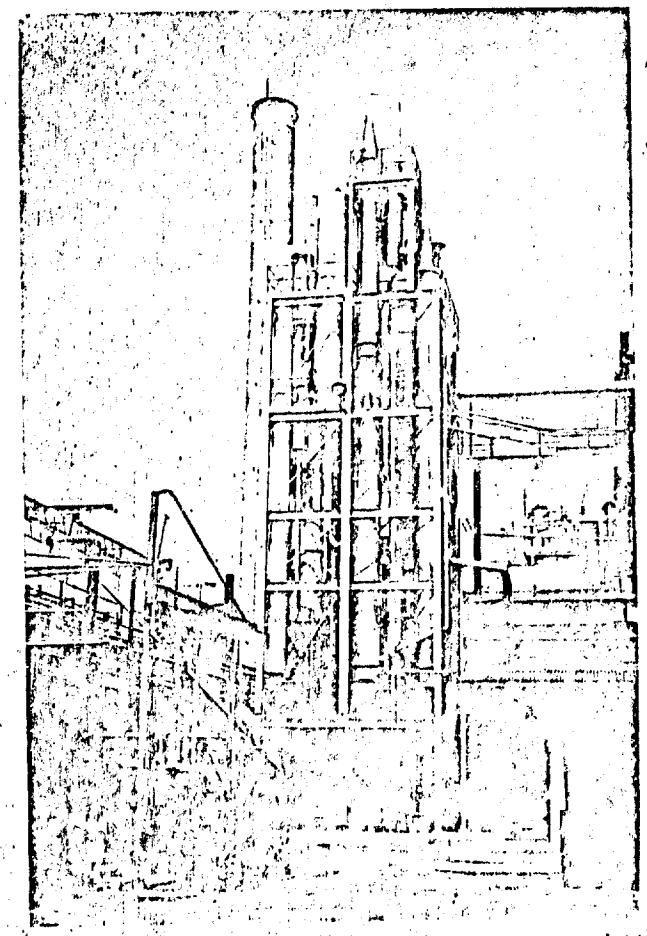

Figure 3. TASTE evaporator typical of the type used in the citrus industry.

housekeeping throughout the processing season. In plants of the Minute Maid Company it was decided this could best be accomplished through a good housekeeping contest *(14)*. Since a clean plant is generally a safe one, a combination was made of both safety and good housekeeping. Posters using a "catchy" slogan announcing the contest are placed in each plant prior to the start of the program. Some examples are: "WANTED – Cleaner and Safer Plants for 1965"; "GET ON TARGET for '–66, Play it Safe, Keep it Clean", and "LET'S ALL PULL TOGETHER for a CLEANER – SAFER 1967." Prizes are awarded as an incentive to keep up interest in the housekeeping program. This year "Safety Sam" was introduced to make employees more safety conscious. A different safety slogan is placed in a plant twice each mo. Employees' names are drawn from a hat, and the first employee to repeat the slogan word for word receives a gift certificate. Since the contest has been in force, a substantial reduction in accident frequency rate has been obtained. For example, during a recent season 1 of our plants received national recognition by working 1,000,000 man-hr without a "lost-time" accident. Morale of employees, over-all sanitation, and plant efficiency have been improved.

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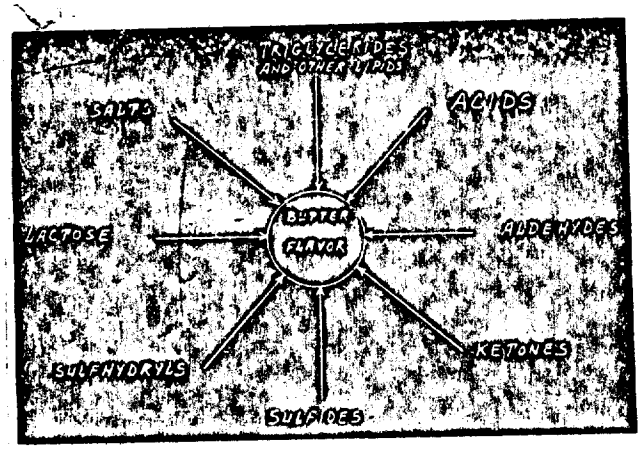


FIGURE 1

PLANT CLINIC

By V. H. NIELSEN

Components in more or less than sufficient quantities determine the quality of the product

Making Butter Flavor Better

Amer. Dairy Rev. 28(11) 50, 62-3 (1966)

IN TERMS OF OLD KNOWLEDGE AND OF RECENT FINDings the flavor of normal, high quality butter is a rich conglomerate of compounds which may be grouped and illustrated as in the diagram in Figure 1.

The complex can also be described as in Figure 2 in terms of the derivation of the known and demonstrated flavor components from certain major constituents of butter or of the cream from which it is made.

Neither of these can, however, be definitions of normal butter flavor because they lack the quantitative relationships between the flavor substances. They may, however, serve to illustrate the complex nature of a flavor concept generally described with simple but inadequate words.

A scanning of the array of compounds contributing to the flavor of normal butter reveals that many of them also are the causes of highly objectionable flavor defects. Thus much of the distinction between normal and defective butter flavor stems from a difference in concentration of certain components.

Free Fatty Acids

Bills et al (2) found that the concentrations of the low molecular weight, volatile fatty acids in rancid cream were 2-4 times higher in rancid cream than in fresh cream from the same source or in fresh butter (Table 1). The same was true of the higher molecular weight fatty acids although these probably contribute less to the normal flavor of butter or to the rancid defect. We can assume, therefore, that a blend of volatile fatty acids in extremely low concentrations is part of the natural butter flavor as it is part of the typical flavor of coconut and chocolate. Higher concentrations will give rancid or bitter flavor.

ACID	FRESH CREAM	FRESH BUTTER	RANCID CREAM
	Mg/Kg Fat		
Butyric	43.4	36.0	77.8
Caproic	13.6	17.8	34.8
Caprylic	12.8	22.8	75.8
Capric	40.4	38.8	139.0
Lauric	68.8	115.4	191.0
Myristic	164.6	302.2	489.2
Palmitic	583.4	620.2	1293.7
Stearic	265.2	329.2	594.0
Oleic	851.4	1050.8	1842.8
Linoleic	85.4	127.4	205.2
Linolenic	53.2	73.6	136.6

[1]From data of Bills et al (2)

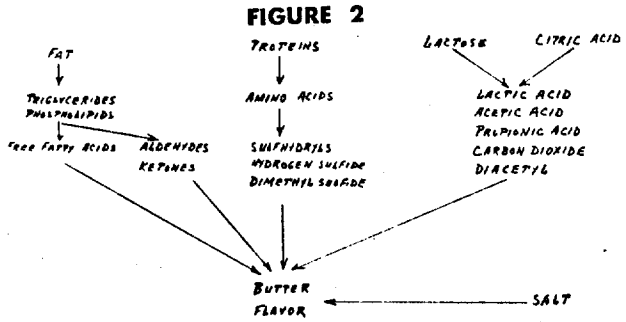
The lipolytic reactions which bring this about are caused by the following abuses:

1. Excessive agitation and foaming in raw milk in pumps and pipelines.

2. Repeated alternate warming (60-80° F) and cooling of raw milk or cream.

(Please turn to Page 62)

FIGURE 2



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Making Butter Better

(Continued from Page 50)

3. Blending homogenized milk or cream with raw milk or cream.

4. Growth of lipolytic bacteria in milk or cream.

Ketones

Diacetyl, in concentrations of 2-4 p.p.m. has by many investigators been identified as one of the important flavor components of highly aromatic butter. Diacetyl alone produces a coarse flavor and there is little doubt that other products of the fermentations by which it is produced in butter cultures, such as lactic, and propionic acids, also must be present to yield the typical, desired flavor. The classic method for producing this flavor is to prepare a culture grown in milk and adding the ripened culture to the cream prior to churning or to the butter granules after churning. A widely used method is to add a synthetic diacetyl-acid or "starter distillate" preparation directly to the butter before the working process.

Aldehydes

The presence of aldehydes in butter or in milk fat is generally regarded as the cause of oxidized, tallowy, oily, metallic or other flavor in the group of off flavors which is produced by oxidation and cleavage of the unsaturated fatty acids in milk fat. This reaction will produce a series of aldehydes of varying molecular weight and chemical structure and many of these have been shown to be associated with oxidized and similar flavors.

Here, again, the defect as a function of these compounds is a matter of degree.Some of them have been shown to be present in normal milk fat in extremely small but measurable concentrations and in oxidized milk fat in concentrations several times greater.

Recent work by Badings (1) demonstrated that the presence of one part per billion (p.p.b.) of 4-cis-heptenal in tasteless butterfat (produced by high vacuum degassing) gave a pleasant cream-like flavor. In concentrations of 10 p.p.b. it produced a cold-storage grainy flavor.

The reactions producing these aldehydes are caused by oxygen dissolved in milk and cream and they are catalyzed by the presence of cupric ions. The exact critical level of cupric ion is not known and susceptibility to oxidation is also influenced by other factors such as pH and the presence of natural antioxidants. In a survey of Canadian butter the samples which turned oxidized upon storage had copper contents ranging from 0.07 to 0.8 p.p.m. compared with 0.09 to 0.16 p.p.m in those which did not become oxidized.

The best defense against this defect is therefore to avoid copper contamination at all stages of milk and cream handling and butter manufacturing, to adjust the pH of the butter to 6.6-7.0 and to avoid excessive agitation and exposure to light.

Sulfhydryls and Sulfides

Several compounds containing sulphur are known to contribute to the flavor of butter as to the flavor of other dairy products. Upon heating milk or cream some of the amino acids in the serum proteins become reactive. One of these, cysteine, contains in its molecule a sulfhydryl (-SH) group. Upon hydrolysis of this group, hydrogen sulfide (H_2S) is produced. Hydrogen sulfide is believed to be partly responsible for the cooked flavor in milk, cream, butter and other milk products. In low concentrations it is an almost desirable, or at least acceptable, flavor constituent in butter. It is specifically included in the definition of U.S. Grade AA butter.

Recently Day et al (3) demonstrated that dimethyl sulfide was a desirable component of butter flavor. In concentrations of 10-40 parts per billion (p.p.b.) it smoothed the harsh flavor of diacetyl and the acids associated with culture flavor. Concentrations of 100 p.p.b. produced a slight feed flavor and of 200 p.p.b. a definite feed flavor.

The origin of dimethyl sulfide is obscure but much of the experimental evidence suggests that it enters the milk directly from feed by way of the rumen.

This discussion is not an exhaustive review of the components of normal butter flavor. It merely points out that the concept is much more complex than the official specification of the product leads us to believe. It also sugguests the possibility that some day it may be feasible to define by reliable and reproducible physical-chemical methods (gas-liquid chromatography, infrared spectrophotometry and mass spectrometry) exactly how good butter should taste.

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The Journal of
Pathology and Bacteriology
Vol. XXXIV., No. 4

51053 576.809.8

A SIMPLE DELICATE AND RAPID METHOD OF DETECTING THE FORMATION OF ACETYLMETHYLCARBINOL BY BACTERIA FERMENTING CARBOHYDRATE.

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DURING the fermentation of glucose and certain other substances by yeast and many different varieties of bacteria a butylene glycol is produced, of the formula $CH_3.CHOH.CHOH.CH_3$. The immediate precursor of this glycol is acetylmethylcarbinol or acetoin $CH_3CO.CHOH.CH_3$ which is reduced by the organism to give the glycol. Acetoin is produced by condensation of two molecules of acetaldehyde, an intermediate product in the breakdown of the sugar, under the influence of an enzyme "carboligase," which is present in some bacteria, *e.g.*, *Ærobacter aërogenes* [*B. lactis aërogenes*], but thought to be absent from others, *e.g.*, *Escherichia coli*. The formation of acetoin during carbohydrate fermentation is therefore a biochemical characteristic of certain organisms, just as the formation of indol from protein is a property of many species. While however the available tests for indol production are delicate and convenient in their application and are consequently much used, there is no method of testing for acetoin formation comparable with them in these essentials. In consequence, the characteristic property of acetoin production has not received the recognition to which its importance would entitle it.

The usual routine method of detecting the formation of acetoin is by the Voges-Proskauer test, which has been used extensively (in a somewhat empirical fashion, it is true) in connection with water analysis, more especially in the United States. The common method of performing the test is that advocated in *Standard methods of water*

analysis published by the American Public Health Association; it involves incubation of cultures at 37° C. for 4 days, followed, in most cases, by 24 hours standing at room temperature after the addition of caustic soda to the cultures, before the results can be read. Even with this technique acetoin cannot be detected in every case in which it is formed since the method is essentially crude.

The principal alternative to the Voges-Proskauer test at the present time is the method employed by Lemoigne. This method, which is not suited to routine work, consists in oxidising both the 2·3 butylene glycol and acetoin formed in the cultures to diacetyl $CH_3.CO.CO.CH_3$, which is then distilled. The diacetyl in the distillate is detected by precipitation in the form of the red nickel dimethyl glyoxime.

Attempts too numerous to mention have been made to hasten and increase the delicacy of the Voges-Proskauer reaction. They have, in general, been based upon the concept of the mechanism of the reaction put forward by Harden and further elaborated by Harden and Norris, and have usually taken the form of adding oxidising agents such as hydrogen peroxide, sodium peroxide or ferric chloride to the cultures, in addition to caustic soda, at the time of doing the test. Although success has been claimed for some of these methods their very multiplicity is in itself proof of their failure. Having tried the oxidising agents mentioned and many others as well, I was satisfied that they were unreliable since not only did they fail to hasten the detection of acetoin with fair regularity but actually prevented its detection in certain instances when its presence could be demonstrated by the ordinary Voges-Proskauer technique.

Owing to the failure of oxidising agents to achieve their purpose when they might, with reason, have been expected to give satisfactory results, a reinvestigation of the mechanism of the Voges-Proskauer reaction was undertaken and it was established that the accepted views are incorrect in a number of respects. It is not proposed to discuss this matter further in the present paper, though it may be mentioned that, as a result of the investigation, it became apparent that hastening of the reaction would have to be achieved by some other method than the addition of oxidising agents. It was noticed during some experimental work in connection with the mechanism of the reaction that creatine gives with acetoin in the presence of air and strong alkali a red colour which develops very rapidly and is very intense. It was decided to employ this colour reaction as a method of testing for acetoin formation. The choice of a method of applying the test was guided by certain theoretical and practical considerations.

In the first place it is desirable, in carrying out delicate colour tests, that one should be freed from the disadvantage of having to work with solutions already coloured or with solutions capable of interfering with the proper development of the colour sought. It was decided therefore to adopt a colourless synthetic medium for the

1472

purpose of growing the organism to be studied, bearing in mind that its most probable application would be to the detection of acetoin production among the gram-negative bacilli of soil water and the intestinal canal. Secondly, in accordance with the law of mass action, the velocity of a chemical reaction is proportional to the product of the concentrations of the reactants. The concentration of creatine was capable of alteration at will, but the concentration of acetoin could be varied only within comparatively narrow limits depending upon the conditions of its production.

With these principles in mind, a trial was made of Koser's synthetic citrate medium, with 0·5 per cent. of added glucose as the culture fluid. The organism to be tested was grown for 24 hours at 37° C. in this medium and a knife point of creatine was then added to the tube of growth followed by a volume of 40 per cent. caustic soda equal to that of the culture. On agitating the tube acetoin formation by the organism could be detected by the development of a well-marked red colour in two minutes or less. In a series of 36 organisms of the *coli, lactis aërogenes* and Friedländer types, kindly given to me by Dr A. H. Thompson, acetoin was found to be formed by 23 in the time specified. Of the 23, 18 were definitely Voges-Proskauer positive and 1 was doubtful. Of the other 4, a faint positive reaction was given by 3 when saccharose was used instead of glucose in testing for acetoin formation by the Voges-Proskauer technique. The remaining organism was negative with both glucose and saccharose. It has been well established that certain organisms give a Voges-Proskauer reaction when the glucose in the culture medium is replaced by saccharose, although they fail to do so when glucose is used. This, however, does not mean, as will be seen from the above results, that they fail to produce acetoin from glucose. It merely signifies that the method in common use for detecting the production of this substance fails to show its formation even though some is actually produced in the course of fermentation. It will be remembered that acetoin, after its formation by condensation of two molecules of acetaldehyde, is reduced with varying degrees of rapidity to 2·3 butylene glycol. The glycol does not give the colour reaction and if the rate of reduction is high the test may in consequence be negative. Anything which increases the reduction potential of the medium tends both to lessen the production of acetoin and to accelerate the formation of the glycol, and consequently tends to convert a reaction which should be positive into one which is negative. Glucose is such a reducing substance whereas saccharose is not and, as a result, the Voges-Proskauer technique may fail to show acetoin when glucose is the test sugar but may show it when saccharose is used instead of glucose, provided that the organism studied is capable of utilising saccharose.

It was found, bearing upon this point, that acetoin tends to disappear rather rapidly from cultures made in the above modification

of Koser's medium, leading to somewhat irregular results. The colour reaction was much less intense after 48 hours incubation than after 24 hours. This was found to be due to reduction of acetoin to the glycol, for if to the 48 hour culture a few drops of 10 volume hydrogen peroxide were added and the culture re-incubated for a further 24 hours the intensity of the reaction was restored to even greater proportions than in the 24 hour culture. It was, furthermore, found that adding more glucose to the medium so far failed to increase the intensity of the reaction that when 1 per cent. of glucose was used the reaction was much less marked than with 0·5 per cent. To increase the formation of acetoin and counteract the reduction of acetoin to butylene glycol an alternative hydrogen acceptor was added to the medium.

After a number of trials with potassium nitrate, sodium fumarate, sodium aspartate and methylene blue, it was found that the fumarate gave very constant and reliable results when present in a concentration of 1 per cent. Potassium nitrate was also quite effective but less regular in the results which it yielded. Sodium aspartate and methylene blue were much less satisfactory. The sodium fumarate probably acts in two ways in both by virtue of being a hydrogen acceptor. In the first place it increases the formation of acetoin by lessening the amount of acetaldehyde reduced during fermentation, leaving more of that substance available for conversion into acetoin, and secondly it tends to lessen the rate of reduction of acetoin to butylene glycol so that the amount of acetoin available at the time of doing the test is increased.

The composition of the culture medium arrived at in the above manner is therefore:—

Sodium chloride	5·0 grams
Magnesium sulphate	0·2 ,,
Ammonium phosphate $(NH_4)H_2PO_4$	1·0 ,,
Potassium phosphate K_2HPO_4	1·0 ,,
Sodium citrate (crystalline)	2·77 ,,
Glucose	5·0 ,,
Sodium fumarate	10·0 ,,
Distilled water to	1000 c.c.

The medium requires no adjustment of reaction, is put up in wide tubes ($\frac{3}{4}$ in. internal diameter) 5 c.c. being allowed to each tube, and may be sterilised in the autoclave. The object of putting a comparatively small amount of culture medium in so wide a tube is to ensure as liberal a supply of oxygen as possible, since increased aeration favours the production of acetoin. Organisms of the colon aërogenes type grow freely in the medium and may be tested for acetoin production as follows. A tube of the medium is inoculated with a loopful of a recent growth of the organism on solid medium. The culture is incubated at 37° C. for 24 hours and is then tested for acetoin by adding a knife point of solid creatine (about 25 milligrams)

1472

followed by 5 cc. of concentrated caustic soda. The caustic soda solution used should never be less than 40 per cent. After the addition of these reagents the tube is well agitated when, if acetoin is present, a red colour appears in a couple of minutes, and owing to the fact that the medium is colourless, may be appreciated very readily. Once the colour has appeared it deepens rapidly on further agitation. The amount of acetoin capable of detection by this procedure is very minute. The presence of 1 part in 20,000 can be detected with great ease and 1 part in 50,000 is just capable of recognition. Instead of creatine, dicyandiamide may be used in the above test, and has the advantage of being cheaper. As the cost of the creatine used, however, is only about one-tenth of a penny per test, this advantage is outweighed by the fact that dicyandiamide is not so delicate and is much slower, the colour requiring up to 15 minutes or more to appear.

On testing the series of organisms mentioned above by the modified method, it was found that the reaction was positive with the same organisms as previously but was now much more intense and was uniform in its behaviour so that it could be relied upon to give constant results. Those organisms which had previously given only faint reactions were now strongly positive, and those which gave strong reactions before continued to do so. None of the organisms which were consistently negative in the trial method were found to give a positive result with the modified technique.

The above method of detecting acetoin may be applied as a qualitative test to the classification of organisms, to bacterial analysis of water and to the study of fermentations. To deal briefly with these applications, it is to be presumed in the first place, when dealing with closely related organisms that, other things being equal, a closer relationship to one another exists between those which possess a particular biochemical property than to others which do not possess it. In the present case the biochemical property is 2·3 butylene glycol fermentation which is believed to rely upon the presence of the enzyme carboligase in the bacterial cell. It has been shown by Harden and confirmed by other workers that *Escherichia coli* does not carry out this type of fermentation whereas *Ærobacter aërogenes* does. This is further confirmed by the present test with which a number of strains of *E. coli* have been found negative and *A. aërogenes* strongly positive. Between these two extremes, however, there exist many bacterial types whose exact classification remains in doubt, since in some respects they resemble one of these organisms and in some the other. *Escherichia communior* (Durham) is a case in point. This organism is Voges-Proskauer negative, but in other respects resembles members of the *Ærobacter* rather than the *Escherichia* genus. It can now be shown to produce acetoin freely from glucose by the test described above and should consequently be numbered among those

organisms which carry out the butylene glycol fermentation. This, in conjunction with its property of utilising citrate and fermenting a wide range of sugars, including saccharose, with active gas production, should suffice to place it in *Ærobacter*.

In bacterial analysis of water reliance is placed upon acetoin formation, using the Voges-Proskauer reaction, in conjunction with other tests, to distinguish lactose-fermenting bacilli of intestinal origin from those which may have gained access to the water from unpolluted sources. The new test provides a more rapid delicate and certain method of detecting acetoin formation by these organisms than the Voges-Proskauer reaction. This aspect of the subject, however, requires further study. Suffice it to say here that in the above series of gram-negative bacilli some 25 to 30 per cent. more were shown to be acetoin producers than the Voges-Proskauer reaction indicated.

It has been shown by Lemoigne that many varieties of bacteria are capable of carrying out the butylene glycol fermentation. Up to the present there has been no ready method of demonstrating this property. That the technique described above provides such a method will be appreciated from the fact that with its aid acetoin formation can be readily shown to occur with such organisms as *Vibrio finkler-prior*, *Corynebacterium diphtheriæ* and *Staphylococcus aureus*. Owing to the slow rate of growth of the two last mentioned organisms in the synthetic medium, it was found advisable to employ a heavy inoculation, two or three loopfuls of organisms being used and to incubate them for 48 hours before testing for acetoin.

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1472

1483

Volatile Chemical Compounds in Dry-Cured Hams[a,b]

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(Manuscript received March 11, 1963)

J. Food Sci. 29(2): 123-9 (1964)

18

SUMMARY

Volatile compounds isolated from dry-cured hams were tentatively identified by gas chromatography retention times and further verification of the compounds made by infrared spectroscopy. These compounds were as follows: formaldehyde, acetaldehyde, propionaldehyde, isobutyraldehyde, *n*-valeraldehyde, isovaleraldehyde, acetone, diacetyl, methyl ethyl ketone, formic acid, acetic acid, propionic acid, butyric acid, and isocaproic acid. R_F values and selective indicators were used to identify ammonia and methylamine. Selective trapping was used to identify hydrogen sulfide and trace amounts of disulfides and/or monosulfides.

INTRODUCTION

The commercial economic importance of dry-cured country-style hams (hereinafter referred to as CS hams) is increasing in the Southeastern United States. This type of ham was described by Dunker and Hankins (1951).

The acceptability of CS hams is determined by odor and flavor, among other quality factors. A few studies have attempted to relate odor and/or flavor to specific chemical constituents (Besley and Carroll, 1942; Blumer, 1954, 1958; Brady et al., 1949; Cecil and Woodroof, 1954; Cook and Barbella, 1937; Hunt *et al.*, 1939; Wilson *et al.*, 1957, 1961).

Other studies of CS hams related to aroma and flavor have been conducted primarily by subjective methods. These methods may indicate the range of acceptability, but they are not precise enough to measure accurately the effect of treatments on the development of aroma and flavor. The aroma from CS hams is distinctive and resembles the flavor; therefore, it seemed reasonable to assume that the determination of volatile constituents is important for objective evaluation of these quality criteria. This study was initiated to determine methods satisfactory for the isolation, separation, and identification of volatile compounds in dry-cured CS hams under prescribed experimental conditions.

MATERIALS AND METHODS

Curing and aging of hams. Sixty-four regular short-cut skinned hams, weighing 14–16 lb, were cured for 2 days per lb at 4°C with 1 oz of curing mixture (8 lb NaCl, 2 lb white sucrose, and 3 oz potassium nitrate) per pound of ham. One-third of the total quantity required for each ham was applied to the surface on each of the 1st, 3rd, and 10th days. After curing, the hams were soaked 2 hr in cold water, dried, and then smoked at 21°C by burning hardwood sawdust. They were aged at 23±1°C, relative humidity 60±3%, and air flow 35 ft/min. Hams were sampled after they were cured and also after they were smoked. They were sampled during aging at monthly intervals for 6 months, and after 9, 12, 15, and 24 months. One ham was randomly selected for analysis at each of the sampling periods.

The NaCl and moisture content of hams will be quite uniform after curing 2 days/lb and storing for an additional 30 days.

Vacuum distillation. A 400-g portion of a 1-in.-thick cross-sectional center-cut ham slice was freed of subcutaneous fat, ground in a Waring blender and placed in a sample flask, and the volatile compounds were separated by vacuum dis-

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Contribution from the Food Science Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with approval of the Director of Research as Paper 1581 of the Journal Series.

This work is cooperative with the U. S. Department of Agriculture.

From a portion of a Ph.D. thesis, North Carolina State College, 1962.

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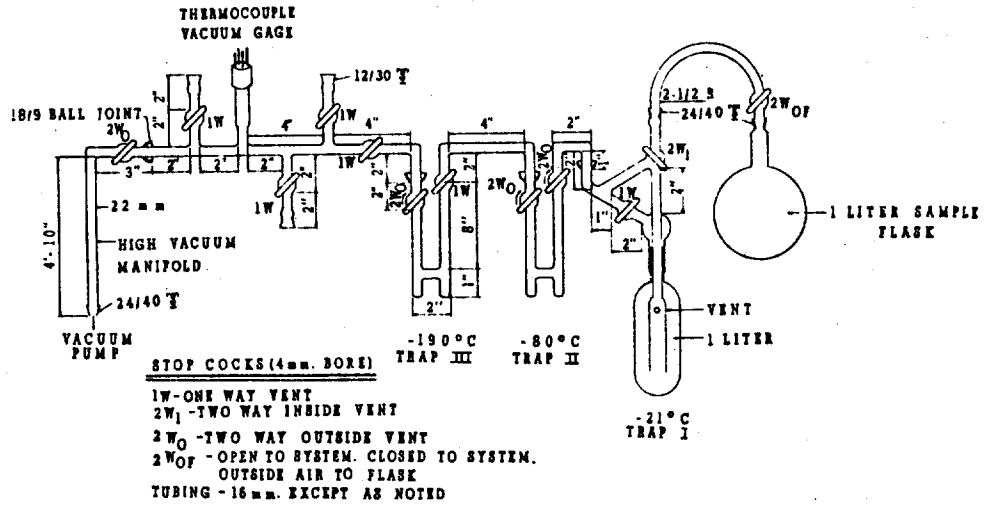

Fig. 1. Vacuum distillation apparatus (modification of Niegisch and Stahl, 1956).

tillation (Fig. 1). The sample was distilled 24 hr at 60°C under a vacuum of 200 μ. Typical aroma and flavor were developed under these conditions. Traps, arranged in order of decreasing temperature, were cooled with ice-salt, dry ice-methanol, and liquid nitrogen. After distillation, the contents of the traps were poured or bubbled into appropriate trapping solutions as described below. The odor after distillation was considered nearly neutral when the odor of the residue was compared with the odor of cooked portions of similar hams. Thus, it appeared that the distillation procedure for removal of volatile constituents was satisfactory. However, it is conceivable that higher distillation temperatures would produce compounds different from those produced here.

Trapping the volatile carbonyl compounds. Volatile carbonyl compounds were trapped in a solution containing 2 g 2,4-dinitrophenylhydrazine (DNP) per liter of 2*N* hydrochloric acid. The resulting 2,4-dinitrophenylhydrazones were collected by filtration and dried, first in air and then in a vacuum desiccator over phosphorus pentoxide.

Micro quantities of other carbonyl compounds, if present, may not have been detectable by the above technique.

Volatile carbonyl compounds from hardwood smoke were also trapped in DNP solution by pulling the smoke with a vacuum through a gas washing bottle containing the solution.

Gas chromatography of volatile carbonyl compounds. The carbonyl derivatives were analyzed by gas chromatography using a modification of the method of Ralls (1960a). An approximate 10-mg portion of a mixture of the hydrazones and alpha-ketoglutaric acid (1:3 by wt) was introduced into a Pyrex capillary tube sealed at one end, and prepared for injection into a gas chromatography (GC) apparatus (Fig. 2). The parent carbonyl compounds were regenerated and swept into the GC apparatus (Model 154-B Vapor Fractometer, Perkin-Elmer Corp.) by heating the capillary tube to 250°C in 15 sec and maintaining this temperature for an additional 15 sec.

The column (2 m × 6.35 mm) used to separate the carbonyl compounds was packed with Celite coated with diisodecyl phthalate, and maintained at 82°C. The carrier gas (helium) pressure in the column was regulated at 10 lb/sq in. and gave a flow rate of 32 ml/min (Woods, 1960).

To recover the separated carbonyl compounds, the tip of a glass tube attached to the exit port of the gas chromatograph was immersed in 5 ml of DNP solution whenever the recorder response indicated that a carbonyl compound was being eluted from the column. Several runs were made to obtain a sufficient quantity of hydrazone for subsequent identification by infrared spectroscopy. Each hydrazone was centrifuged from solution washed with 2*N* HCl and then with distilled water, and dried over phosphorus pentoxide.

Infrared analysis of 2,4-dinitrophenylhydra-

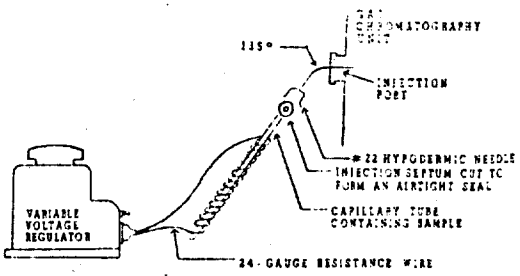

Fig. 2. Flash exchange and injection apparatus.

...nes. The dried hydrazone was mixed with potassium bromide (0.5:99.5) and placed in an ...mable potassium bromide die (Research and ...strial Instrument Co.). Vacuum was applied ...the die for 15 min, and the die, still under ...uum, was placed in a press (17,000 lb/sq in.) ...3 additional minutes. The pellet thus formed ...s placed in the sample beam of a double beam ...del 137 Infracord Spectrophotometer (Perkin-...mer Corp.) and the spectrum recorded. The ...ctrum was compared with 2,4-dinitrophenyl-...drazones of known carbonyl compounds (East-...an Chemical Co.) purified by gas chromatography ...described above. Thus, known compounds from ...ferent homologous series could be differentiated ...well as compounds having different chemical ...oups.

Trapping the volatile acids. Short-chain volatile ...ids were trapped in 1*N* potassium hydroxide ...lution. The potassium salts thus formed were ...ected over a steam bath and dried in a vacuum ...n at 100°C.

Gas chromatography analysis of volatile acids. ...e method of Ralls (1960b) was used in the ...analysis of the volatile acids. The potassium ...ts of the acids were heated with potassium ethyl ...ate to form the ethyl esters (Evans and ...ertson, 1917). The esters were separated by ...procedure similar to that described above for ...carbonyl compounds, except that the injection ...illary temperature was raised to 300°C and the ...umn temperature to 120°C.

...s the individual ethyl esters emerged from the ...apparatus, they were trapped in a U-tube sub-...ged in liquid nitrogen. The esters were then ...med to room temperature and dissolved in car-...tetrachloride (CCl_4).

Infrared analysis of esters. An aliquot of a ...tion of each ester in CCl_4 (5:95) was placed ...a sodium chloride cell, and the infrared spectrum ...s recorded with CCl_4 the blank in the reference ...am.

Trapping of volatile basic compounds. The ...n samples were steam distilled at atmospheric ...ssure, and the volatile basic compounds were ...ssed into a cold trap (ice and salt). The non-...ndensable gas was then bubbled through 1*N* ...Cl. The trapped components were combined and ...ied on a steam bath, and the drying was com-...ted in a vacuum oven at 100°C.

Paper chromatography of volatile basic compounds. The hydrochlorides of the volatile basic compounds were separated by ascending paper chromatography by the method proposed by Davies *et al.* (1953), as modified by Hornstein *et al.* (1960) and Hornstein (1960). Whatman No. 1 paper was used as the stationary phase. The chromatogram was developed in a test tube (25 × 250 mm) for 90 min.

Selective trapping of volatile sulfur compounds. A slurry was made by blending 600 g of ham with 300 ml of distilled water. A sulfur absorption train as described by Dateo *et al.* (1957) and Hasselstrom (1957) was used to trap the volatile sulfur components. Refluxing and sweeping with nitrogen (30 ml/min) was continued for 5½ hr.

RESULTS AND DISCUSSION

Carbonyl compounds in hams. Fig. 3 is a representative chromatogram of the carbonyl compounds from one ham. The retention times for known carbonyl compounds are given in Table 1. From these values, the peaks of the chromatogram in Fig. 3 were tentatively identified.

For a more positive identification, the compounds labeled in Fig. 3 as acetaldehyde, propionaldehyde, isobutyraldehyde, diacetyl, 2-butanone (methyl ethyl ketone),

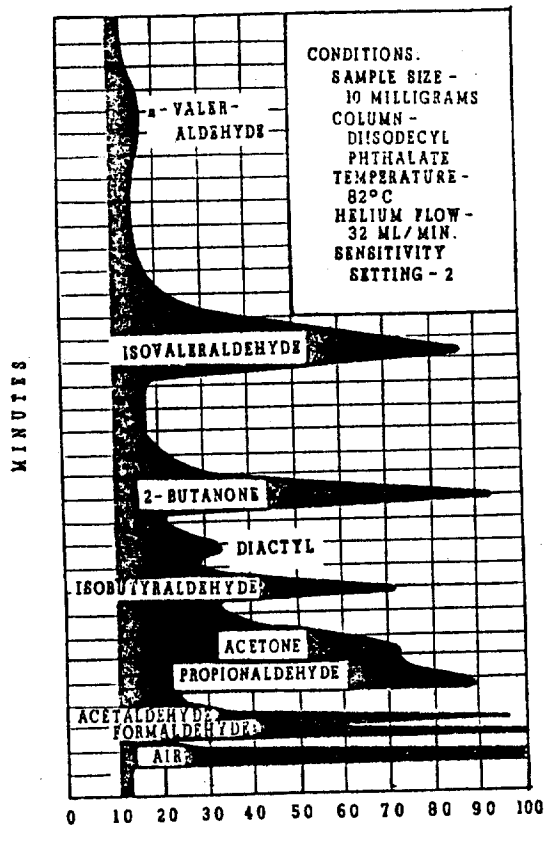

Fig. 3. Gas chromatogram of volatile carbonyl compounds isolated from a ham aged six months.

Table 1. Retention times of carbonyl compounds regenerated from 2,4-dinitrophenylhydrazones by exchange with alpha-ketoglutaric acid.

Parent carbonyl	Retention time (min) [a]	Retention volume (ml) [b]
Formaldehyde	1.4	45
Acetaldehyde	2.2	70
Propionaldehyde	4.0	128
Acrolein	4.2	134
Acetone	4.7	150
Isobutyraldehyde	7.7	246
n-Butyraldehyde	11.4	365
Diacetyl	11.5	368
Methyl ethyl ketone	11.8	378
Methyl isopropyl ketone	16.8	538
Isovaleraldehyde	17.8	570
Crotonaldehyde	20.5	656
Aldol	21.0	672
Methyl *n*-propyl ketone	21.5	688
Diethyl ketone	22.2	710
n-Valeraldehyde	25.7	822

[a] Conditions: 2-meter column of diisodecyl phthalate, temperature of 82°C, and a helium flow of 32 ml/min.
[b] Retention volume = retention time × flow rate (32 ml/min.).

and isovaleraldehyde were trapped in the DNP solution as they emerged from the gas chromatograph, after which they were subjected to infrared spectroscopy. Excellent agreement was found between different classes of the known and sample compounds. However, the spectrum of a known sample of diacetyl was similar to the spectrum produced by a 75:25 mixture of diacetyl and isobutyraldehyde. It is therefore probable that the "diacetyl" peak of the sample contained some isobutyraldehyde. Other investigators have reported the following volatile carbonyls in meat: acetaldehyde, Pippen *et al.* (1958), Gaddis *et al.* (1959), Gaddis and Ellis (1959), Landmann (1960), Hornstein (1960), and Kramlich and Pearson (1960); propionaldehyde, Pippen *et al.* (1958), Gaddis *et al.* (1959), and Gaddis and Ellis (1959); diacetyl, Pettet and Lane (1940) and Pippen *et al.* (1958); and 2-butanone, Gaddis *et al.* (1959) and Pippen *et al.* (1958).

Attempts failed to trap the gas corresponding to formaldehyde (Fig. 3) and produce the hydrazone derivative. This result may have been due to the highly volatile nature of the gas.

Other carbonyl compounds listed in Fig. were found in trace amounts only; therefore, no further identification was made. However, acetone has been reported in pork fat by Gaddis *et al.* (1959), in chicken by Pippen *et al.* (1958), and in beef and pork by Landmann (1960) and Hornstein (1960). Callow (1927) identified formaldehyde in smoked meats, and Hornstein (1960) listed it as a constituent of beef.

Wood smoke was analyzed, and peaks were recorded with retention times characteristic of formaldehyde, acetaldehyde, acetone, methyl ethyl ketone, isovaleraldehyde, and *n*-valeraldehyde. Pettet and Lane (1940) also isolated formaldehyde, acetaldehyde, and acetone; however, they also found diacetyl, which was not identified with the procedure used here. Callow (1927) also found formaldehyde to be present in wood smoke.

The ratios among the carbonyl compounds as determined by gas chromatography remained quite constant during the entire aging period, except for 2-butanone. For this compound, the proportion appeared to increase during the latter part of the aging period. Although the ratios remained constant, the total quantity of carbonyl derivatives obtained from the hams increased with aging time. This agrees with the results of Pippen *et al.* (1958), Gaddis and Ellis (1957), Gaddis *et al.* (1959), Hornstein (1960), and Mendelsohn and Steinberg (1962), who found that conditions favorable to oxidation gave an increase in carbonyl compounds.

Whether or not a quantitative relationship exists between flavor and carbonyl compounds is not reported in this paper.

Volatile acids in hams. Fig. 4 is a representative chromatogram indicating the volatile acids (esters) obtained from a ham aged 9 months. These acids were identified by comparison with chromatograms of known acids, retention times for which are shown in Table 2. The acids found in the hams were tentatively identified as formic, acetic, propionic, butyric, and isocaproic.

Sufficient quantities of acetic and butyric acids were trapped to allow comparison of their infrared spectra with those of known

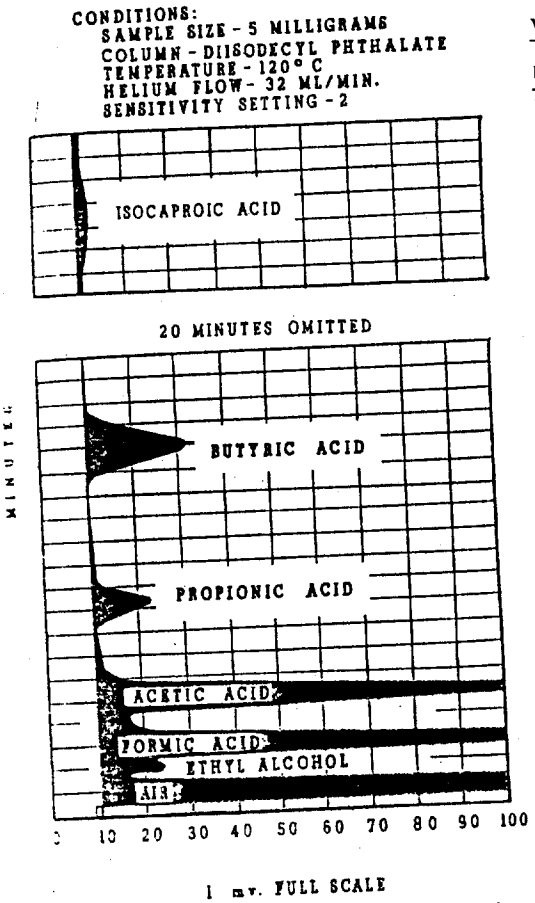

Fig. 4. Gas chromatogram of volatile acids isolated from a ham aged nine months.

acids. Excellent agreement was found; thus, verification of the same chemical grouping was shown. Acetic acid was isolated from U.S. hams by Blumer (1954). The other acids mentioned above could not be isolated in quantities sufficient to form derivatives. Only one peak was obtained from the smoke analysis having a retention time the same as that of the formic ester.

The number and quantity of acids isolated were found to increase with the length of the aging period, except for the quantity of formic acid. The percentage of this acid increased with storage time, but the relative decrease was probably due to an increase of longer-chain acids rather than to a decrease in amount of formic acid. It should be mentioned at this point, however, that formic acid is a constituent of wood smoke, and some decrease of the acid from this source

Table 2. Retention times for ethyl esters of volatile acids.

Parent acid	Retention time (min) [a]	Retention volume (ml) [b]
Ethyl alcohol [c]	1.9	61
Formic	2.4	77
Acetic	4.3	138
Acrylic	7.7	246
Propionic	7.9	253
Isobutyric	10.3	330
Trimethylacetic (pivalic)	11.8	378
Methacrylic	12.5	400
Butyric	14.2	454
Isovaleric	20.4	653
Crotonic	21.5	688
Valeric	27.5	880
Dimethylacrylic	36.7	1174
Isocaproic	39.7	1270
Caproic	53.3	1706

[a] Conditions: 2-meter column of diisodecyl phthalate, temperature of 120°C, and a helium flow of 32 ml/min.

[b] Retention volume = retention time × flow rate (ml/min).

[c] Ethyl alcohol is sometimes a disintegration product of the ester formation, thus forming a peak on the gas chromatography tracing.

may have occurred, especially since the residual of wood smoke is deposited primarily upon the meat surface.

Volatile bases in hams. Table 3 shows the paper chromatography results for known amine hydrochlorides. Comparison of these values with those obtained from the spots on the chromatogram from the ham samples indicates that the major portion of the volatile bases was ammonia and a very small amount was methylamine. This is in agreement with the results reported by Hornstein *et al.* (1960) and Hornstein (1960) for beef, and with the basic components of meat flavor as listed by Landmann (1960); however, Landmann also noted the presence of ethylamine, which was not detected in this study. Pippen and Eyring (1957) found only ammonia in chicken broth.

Volatile sulfur compounds in hams. Almost immediately upon refluxing and sweeping with nitrogen, lead sulfide precipitate was noted in the solid lead acetate trap, indicating that hydrogen sulfide had been liberated from the ham under these conditions. Several research workers have reported the presence of hydrogen sulfide in extracts of

Table 3. Paper chromatography separation of known amine hydrochlorides.

Compound	Rf	Ninhydrin test	Methyl orange test
NH_4Cl	0.50	Negative	Positive
$CH_3NH_2 \cdot HCl$	0.45	Positive	Positive
$CH_3CH_2NH_2 \cdot HCl$	0.53	Positive	Positive
$CH_3(CH_2)_2NH_2 \cdot HCl$	0.60	Positive	Positive
$CH_3(CH_2)_3NH_2 \cdot HCl$	0.68	Positive	Positive
$(CH_3CH_2)_2NH \cdot HCl$	0.78	Negative	Positive

meats (Crocker, 1948; Bouthilet, 1951a,b; Pippen and Eyring, 1957; Hornstein, 1960). A 3½-hr period of continuous refluxing followed before a trace of other precipitates was noted in the aqueous mercuric chloride traps. Thus, with a longer refluxing time, a trace of disulfides and/or monosulfides was obtained. Under the conditions of this experiment no mercaptans were detected.

The methods of analysis used in this study appear to be, in general, adequate for determining the volatile compounds in CS hams. Since these hams were aged for long periods, some differences in the amount and kind of these compounds were noted at the several sampling periods. Some association may exist between aged flavor and the development of certain of these compounds. In future quantitative work dealing with flavor of CS hams, the possibility should be taken into consideration that carbonyl compounds do make some contribution to the flavor complex.

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Analyses Simplified:

I. Chlorides, II. Pasteurization, III. Diacetyl[1]

Am. Brewer 94:23-5, (1961)

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The rapid development of new methods for analyzing beer components has created a need for simplifying established procedures wherever feasible. It is the intent of this paper to present simple but precise and accurate methods for performing three beer analyses.

1. CHLORIDES

The several classical direct methods for determining chlorides—namely the Mohr and the Volhard titrations—either are not applicable or are applicable only with difficulty to beer. The procedure in the A. O. A. C. Official Methods of Analysis for chlorides in beer (2) involves ashing and is, therefore, time-consuming. There has been no dearth of methods for determining chlorides in beer. In 1958 Hansen and Mølgaard (7) adapted the method of Dubsky and Trtilek (5) to beer; however, in an apparent attempt to gain some sensitivity the published method evolved into a somewhat complicated procedure. In 1959 a rather elegant method was presented for determining chlorides (12) but, alas, it requires special apparatus and takes over an hour of elapsed time.

The method given here may be applied to beers directly without any pretreatment whatsoever. It is titrimetric and requires no special apparatus. It involves titration with a standard solution of mercuric nitrate, using diphenyl carbazone as the indicator. The mercuric ions first react with chloride to form undissociated mercuric chloride; an excess of mercury then reacts with the diphenyl carbazone to give a colored complex. The end point is the appearance of a violet-blue color, which is quite easy to see.

TABLE I

CHLORIDES, as Cl, P.P.M.

This Procedure	Mohr	Volhard	A.O.A.C.
300	320		
323	323		
274	288		
240	252		
227	227		
259	235		
315	310		
360	355		
244	250		
317	330		
454	445		
349	350		
635	630	610	545
452	460	390	421
500	500	420	450

TABLE II

RECOVERY OF ADDED CHLORIDES

Chlorides, As Cl, P. P. M.

Beer	Added	Total	Recovery, % Added
A.	—	133	—
	—	133	—
	90	219	95
	100	237	104
	150	291	105
	170	313	106
	200	335	101
	230	372	104
B.	—	139	—
	—	139	—
	75	213	99
	125	263	99
	150	297	105
	175	312	99
	200	341	101
	250	394	102

This procedure has been in constant use in our laboratories for several years now. In addition, the method is widely used in clinical laboratories to determine chlorides in biological fluids (1). Since these liquids have compositions quite similar to beer, the method should be, simply by extension, applicable to beer. Comparison with other methods and recoveries of added chlorides indeed show this to be the case.

Table I shows a comparison of the results obtained by this method and those gotten from the A. O. A. C. and the Mohr titration. A calculation of Student's "t" for this method and for the Mohr titration gave $t=0.61$, indicating that the difference between the methods is not significant.

Table II shows recoveries of chlorides added to a commercial beer.

The details of the method follow:

Method:

Reagents: 1. *Mercuric nitrate solution*: Place a few hundred ml. of water in a 1000-ml. volumetric flask, add 20 ml. of 2*N* nitric acid and 2.9-3.0 g. of mercuric nitrate (c. p.), dissolve, and fill to 1000 ml. with water. This solution is stable indefinitely and need not be protected from light.

2. *Indicator solution*: Dissolve 100 ml. of s-diphenyl carbazone (Eastman No. 4459) in 100 ml. 95% alcohol, and store in the cold in an amber bottle. The orange-red solution must be protected from light, as it turns yellow when exposed to day-

1. Paper presented at the Annual Meeting of The American Society of Brewing Chemists, May 1960, and published in the 1960 ASBC Proceedings.
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light for a few days and cannot be used. Even in the refrigerator a slow change takes place, and the solution does not give a sharp end-point after about 2 months of storage. Consequently, it is necessary to prepare a fresh solution each month.

3. *Chloride standard*: Dry some sodium chloride c. p. at 120°C., dissolve 584.5 mg. in water, and make up to 1000 ml. The solution contains 10 meq. of chloride per liter and is used for the standardization of each new batch of mercuric nitrate solution.

4. *Standardization of mercuric nitrate solution*: Titrate in a 125-ml. Erlenmeyer flask a 5-ml. portion of the standard sodium chloride solution, using 4 drops of indicator. The end-point is the first appearance of an intense violet-blue color. From the titration calculate the strength of the solution, expressing it as mg. chloride per ml. of mercuric nitrate solution. Example: if 0.5906 g. NaCl were dissolved in 1000 ml. then a 5 ml. aliquot contains 1.79 mg. Cl. If titration was 1.68 ml., then strength of $HgNO_3$ soln. is:

$$\frac{1.79}{1.68} = 1.07 \text{ mg. Cl per ml. } HgNO_3 \text{ soln.}$$

Procedure: A 5 ml. sample is pipetted into a 125-ml. Erlenmeyer flask, diluted with about 5 ml. water, and 0.1 ml. indicator is added. It is then titrated with the standard $HgNO_3$ solution until a violet-blue color appears, which does not change with additional titrant.

Calculation: $\frac{a \times c}{v} \times 1000$ = ppm chloride, where

a = ml. $HgNO_3$ used in titration,
c = strength of $HgNO_3$ as mg. Cl per ml.
v = volume of sample used, in ml. (usually 5 ml.)
Example: 5 ml. beer used 1.63 ml. $HgNO_3$
a = 1.63
c = 1.07 (from standardization of $HgNO_3$)
v = 5

$$\frac{1.63 \times 1.07}{5} \times 1000 = 349 \text{ ppm. Cl}$$

II. Test For Pasteurization

The present trend toward reduced time and/or temperature of pasteurization has made necessary a rapid test to determine whether or not beer is pasteurized.

Although pasteurization should ideally be judged by the absence of microbial life, such tests are not rapid or definitive enough for routine purposes. Chemical tests based on the fact that the enzyme invertase is present in fermented beer and is destroyed at approximately the same rate as are bacteria have been in use for some time (3, 9). Since invertase hydrolyzes the non-reducing sugar sucrose to glucose and fructose, both of which are reducing, most previous methods are based on the appearance, or the increase, of reducing substances. The change in optical rotation which occurs when sucrose is inverted has also been used as a test for invertase. It was found by Haas and Fleischmann (6) in a very thorough study, that invertase is completely destroyed after exposure to five pasteurization units. The parallel between the destruction of this enzyme and the destruction of microbes leaves little to be desired.

The method we are presenting uses the destruction of invertase as the measure of pasteurization but simplifies the enzyme determination.

The development of sensitized paper strips which can specifically discriminate glucose (4) has made it possible to detect the presence of invertase with these.

The test proposed here simply involves the addition of sucrose to the beer being tested, allowing time for enzyme action—if any— to occur, and then detecting its action by a change in color of the sensitive paper strip. No laboratory reagents or facilities at all are necessary.

The specificity of the paper strips has been adequately demonstrated (4) and was not further investigated. The color change depends on the reaction of glucose (in the beer) with glucose oxidase (on the paper strip) to give gluconic acid and hydrogen peroxide. The hydrogen peroxide, in a reaction catalyzed by peroxidase (on the strip), gives a blue color with o-tolidine (also on the paper strip).

The reactions are shown below:

$$\text{Glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \textit{O}\text{-tolidine} \xrightarrow{\text{peroxidase}} \text{blue color}$$

Paper strips sensitized in the described manner are commercially available (in most drug stores). One produced by Eli Lilly, called "Tes-tape," has an added yellow dye, which makes the final positive test a green color. The strips produced by Ames, called "Clinistix," are not dyed and are less sensitive than "Tes-tape."

For those beers which are primed or otherwise contain glucose, the test requires some judgment. Since such beers will contain at most only a small amount of glucose, and since the procedure as presented below produces a large amount of glucose, the test may still be applied, but needs some care.

The sensitized strips—since they test for glucose—may be used also to determine the presence of most priming syrups in beer.

Method:

Reagents: 1. Sucrose, preferably powdered
2. "Tes-tape"—a glucose oxidase sensitized paper strip, produced by Eli Lilly & Company, and available in drug stores; or other similar strip.

Procedure: One gram of powdered sucrose is dissolved in 5 ml. of beer being tested. After standing for one-half hour at room temperature, a short strip of "Tes-tape" (1 to 2 in.) is partially immersed in the beer, withdrawn, and observed after one or two minutes. A distinct green color is a positive test for glucose and, consequently, for invertase, and indicates unpasteurized beer. Beers which have been exposed to some pasteurization (but not quite five units) will turn the strip light-green—more noticeable when observed two minutes after withdrawing the strip from the beer. Pasteurized beers will not change the color of the strip at all, even after two minutes. Any discoloration which occurs after two minutes should be ignored.

III. Diacetyl, or Vicinal Diketones (VDK)

Among the still few flavor components in beer which can be detected quantitatively, the compound diacetyl is of known and sometimes serious importance. In 1952 a method was developed for beer which, while admirably precise and accurate, is somewhat involved and time-consuming (11). In addition, it has been occasionally found that a beer which by odor and taste seems to contain diacetyl gives rather low values by the above procedure.

It is for these two reasons that a systematic search was made for a simpler and perhaps more meaningful diacetyl determination. A method originally used by

Diacetyl, P. P. M.

TABLE III
COMPARISON OF VDK PROCEDURE WITH WEST et al.

Beer	VDK	West et al.
1	0.14	0.17
2	0.17	0.15
3	0.18	0.18
4	0.20	0.18
5	0.23	0.22
6	0.27	0.21
7	0.35	0.33
8	0.54	0.55
9	0.63	0.54
10	0.72	0.68
11	0.63	0.34
12	0.65	0.24
13	0.88	0.42
14	1.05	0.70

Voges and Proskauer (10) for other purposes was subsequently modified by Westerfeld for biological fluids (13). It is a variation of this procedure which we have found to be appreciably simpler and also more meaningful. The method determines diacetyl and related compounds which contain vicinal diketone groupings. It is for this purpose that we intend to refer to this method as the determination of VDK compounds. Since it is fairly safe to assume that compounds of structure related to diacetyl have similar flavor characteristics, we feel that this method, while not so specific as the one based on the color salt of dimethylglyoxime (11,), actually shows better correlation with flavor.

In many cases the results given by both methods are practically identical, but in some there is a marked difference. The results in Table III illustrate this. In the last four beers, where the VDK results are appreciably higher than the diacetyl figures, the taste of the beer belied the lower results.

A list of compounds which were found to give negative results with this procedure is given in Table IV. The only compounds which were found to give positive tests were 2,3-pentanedione and 1-phenyl-1,2-propanedione. The mechanism which has been suggested for the creation of diacetyl in beer (8), could well lead to the production of 2,3-pentanedione as a by-product of the synthesis of amino acid isoleucine.

A typical standard curve for this method is given in Figure 1.

Recoveries of added diacetyl from several beers are shown in Table V.

The details of the procedure follow:

Method:

Reagents: 1. **Alpha-naphthol solution.** Four g. of alpha-naphthol are dissolved in 100 ml. of isopropanol, 99.6%. About ½ g. vegetable carbon is added, the mixture is shaken for about ½-hour and then filtered. The filtrate should be stored in the dark in an amber bottle.

2. **KOH—Creatine solution.** Dissolve 0.3 g. creatine in 80 ml. of 40% KOH soln. (aqueous), and filter. Store in polyethylene container under refrigeration.

3. **Diacetyl, stock solution.** Prepare an aqueous soln. containing 500 mcgm/ml. Stock soln. should be stored in an amber bottle in the refrigerator.

4. **Diacetyl, working solution.** Prepare immediately before use by diluting one ml. of stock soln. to 100 ml. with water. One ml. equals 5.0 mcgm diacetyl.

TABLE IV
VDK — NEGATIVE COMPOUNDS

ACETONE
GLYOXAL
ACETYLACETONE
ACETALDEHYDE
BUTYRALDEHYDE
1,2—CYCLOHEXANEDIONE
METHYL HYDROXY BUTANONE

TABLE V
RECOVERY OF ADDED DIACETYL

Diacetyl, P. P. M.

Added	Total VDK	Recovery, % Added
0.0	0.25	—
0.5	0.77	104
1.0	1.22	97
0.0	0.18	—
0.2	0.37	95
0.5	0.65	94
0.7	0.90	103
1.0	1.22	104

Apparatus: 1. Photoelectric colorimeter or spectrophotometer.

2. Distillation setup, preferably all glass.

Procedure: 1. Prepare a standard curve from 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 ml. of the working diacetyl soln. in 10 ml. volumetric flasks. Add water to bring volume in each to approximately 5 ml. Use 5 ml. water for reagent blank. Develop color as in 3.

2. One hundred ml. of decarbonated beer is distilled into a 50 ml. graduated cylinder containing 5 ml. of water. Collect about 15 ml. of distillate and make up to 25 ml. A 5 ml. aliquot is pipetted into a 10 ml. volumetric flask.

3. Color development: Add 1 ml. of the alpha-naphthol soln. to each flask and swirl. Add 0.5 ml. of the KOH-creatine soln. to not more than 4 or 5 flasks at a time. Make to the mark and shake vigorously for exactly one minute. Let stand, and read absorbance at 530 mmu against a reagent blank, between 5 and 6 minutes after shaking. Repeat this procedure until all samples have been read.

4. Plot values for standards, expressed in mcgm. diacetyl. Read the unknowns from the graph and calculate diacetyl content of the beer.

(*Continued on page* 42)

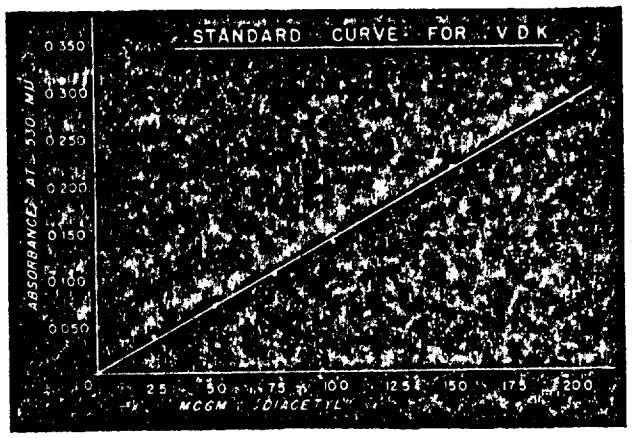

Figure 1

Analysis

(*Continued from Page* 25)

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Nitrogen Metabolism during Fermentation in the Brewing Process. II. Mechanism of Diacetyl Formation

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Pro. Amer. Soc. Brewing Chem 22-26 (1959)

THE RELATIONSHIP OF nitrogen metabolism (14), specifically the behavior of the amino acid valine, to a seemingly unrelated problem — diacetyl formation — is the subject of this paper.

Considerable effort has been devoted to diacetyl in beer in recent years (2,3,8,9,28). It is now well known that not only brewing bacteria, particularly *Pediococcus cerevisiae* (6,8,9,18,28), but also pure-culture yeast can produce diacetyl. This has been demonstrated by Czarnecki and Van Engel (3) and also in our laboratories. It is the production of diacetyl by yeast which concerns us here.

The purpose of this work is to demonstrate that yeast produces diacetyl as a by-product of its synthesis of valine. The proposed mechanism for the biosynthesis of both valine and diacetyl is shown in Fig. 1.

The biosynthesis of valine in microorganisms has been the focus of considerable investigation (6,10,11,12,19,20,21,22,23,24,25,26,27). In particular, it has recently been found that acetolactate is precursor to both valine (11) and acetoin in *Saccharomyces cerevisiae* (4) as well as in other microorganisms (24).

Krampitz (10) first showed that acetoin formation in *Staphylococcus aureus* involved condensation of pyruvic acid and acetaldehyde to form acetolactate. Juni (6) demonstrated this with *Aerobacter aerogenes*, but said that yeast and mammalian tissue did not accomplish the conversion of acetolactate to acetoin. Strassmann, Thomas, and Weinhouse (20) were first to show that acetolactate is the precursor of valine in *Torulopsis utilis*. McManus (12) and Wang (27) confirmed this for *S. cerevisiae*.

In 1958 Umbarger and Brown (23) found that a valine-requiring mutant of *Escherichia coli* accumulates acetolactate (and acetoin) when grown in valine-deficient media. These intermediates have also been found in sulfited apple juice fermentations, and were attributed to the restricted nitrogen supply (29). Wagner, Berquist, and Forest (26) found similar results with a valine-requiring mutant of *Neurospora crassa*. These findings provide strong evidence for the scheme presented above.

Lewis and Weinhouse (11) demonstrated unequivocally that bakers' yeast converts pyruvate to acetolactate. *S. carlsbergensis* has not yet been implicated in these schemes.

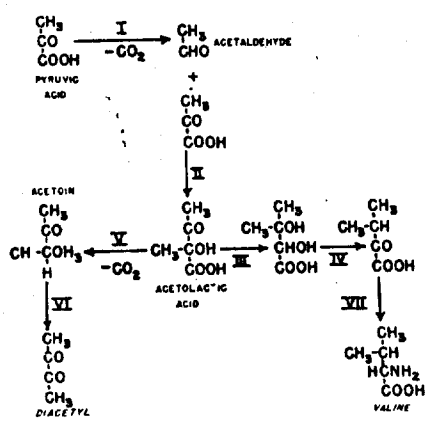

Fig. 1. Mechanism of diacetyl formation.

Experimental

If diacetyl is produced by brewers' yeast as a by-product of its synthesis of valine, then the addition of excess valine to wort should reduce the quantity of

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TABLE I
Effect of Valine on Diacetyl Formation

Fermentation Medium	Diacetyl: At End of Fermentation	Diacetyl: As End of Storage
	ppm	*ppm*
1. Wort	0.43	0.32
Wort + 5 ppm. valine	0.55	0.35
Wort + 50 ppm. valine	0.33	0.30
Wort + 200 ppm. valine	0.17	0.12
Wort + 500 ppm. valine	0.20	0.18
2. Wort	1.1	0.25
Wort + 500 ppm. valine	0.27	0.17
3. Wort	3.1	...
Wort + 50 ppm. valine	0.20	...
4. Wort	1.9	...
Wort + 50 ppm. valine	1.8	...
Wort + 500 ppm. valine	0.46	...
5. Wort	1.5	...
Wort + 500 ppm. valine	0.30	...

diacetyl produced by the yeast. This was actually found to be the case, as shown in Table I. While the addition of 50 ppm. or less of valine had little influence on the diacetyl level, an increment of 200 ppm. or more does have a marked depressive effect on diacetyl formation.

The possibility that any amino acid, or nitrogen source, supplied to wort could be responsible for diacetyl suppression, and not valine *per se*, was tested. Several amino acids, mixtures of amino acids, and ammonium sulfate were added to wort and then fermented. In no case did the additive depress diacetyl formation as well as did valine. The results are shown in Table II.

TABLE II
Effect of Other Nitrogen Compounds on Diacetyl Formation

Additive	Diacetyl, After Fermentation
	ppm
1. Control wort	0.95
2. Wort + alanine, 500 ppm.	0.65
3. Wort + ammonium sulfate, 500 ppm.	0.75
4. Wort + arginine, 500 ppm.	0.66
5. Wort + aspartic acid, 500 ppm.	0.96
6. Wort + glutamic acid, 500 ppm.	0.75
7. Wort + glycine, 500 ppm.	0.68
8. Wort + leucine, 500 ppm.	1.09
9. Wort + methionine, 500 ppm.	0.97
10. Wort + lysine, 500 ppm.	0.76
11. Wort + threonine, 500 ppm.	2.90
12. Wort + tryptophane, 500 ppm.	0.61
13. Wort + yeast extract, 10,000 ppm.	0.83
14. Wort + casein hydrolysate, 10,000 ppm.	0.82

Furthermore, if diacetyl production is related to valine synthesis by yeast, then diacetyl should be formed during the early stage of fermentation, when yeast is anabolizing, or building up yeast substance. The curves shown in Fig. 2 demonstrate that this actually occurs.

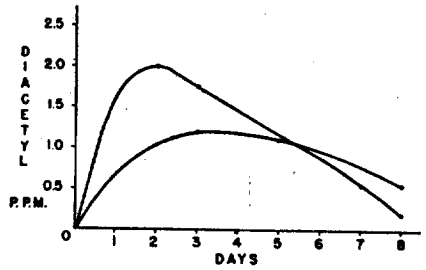

Fig. 2. Diacetyl levels during fermentation.

The addition of acetolactate to wort also was found to suppress the formation of diacetyl by yeast (Table V), as could be predicted from a consideration of the feed-back principle (30). According to this, a product inhibits its own formation by inhibiting the enzyme which is essential to its synthesis. This leads to a piling-up of the immediate precursor, which in turn binds the enzyme necessary to its formation, and so on. In this experiment, the addition of acetolactate inhibited the enzyme which decarboxylates it (Enzyme V in Fig. 1) by inhibiting the functionally similar enzyme (7) which synthesizes it (Enzyme I in Fig. 1).

The data above offer strong substantiation for the theory that diacetyl is produced by yeast as a by-product of its synthesis of valine.

It was now of interest to determine the valine levels in some brewery worts. The results are shown in Table III. Despite the similarity in total "protein" levels, there were appreciable differences in valine content. It is not known whether this relates to malt variety, modification, mashing procedure, or hopping rate. A yeast which has become adapted to living in a valine-deficient wort may be rich in valine-synthesizing enzymes, and so produce diacetyl as a by-product of this synthesis.

TABLE III
Valine and "Protein" Levels in Brewery Worts

	Valine	Protein (N × 6.25)
	ppm	%
Wort B	128	0.39
Wort D	112	0.49
Wort E-1	115	0.46

It was found also that yeasts differ significantly in their ability to absorb valine

from wort. For example, in a set of laboratory fermentations with brewery worts, a strain of *S. carlsbergensis* (D) in the first 2 days of fermentation, absorbed 110 ppm. of valine (85% of that available); whereas another strain (E) could absorb only 54 ppm. of valine (42% of the available supply) in the same time from the same wort (wort B). In another wort (wort E), yeast D absorbed 98 ppm. of valine (89%), whereas yeast E picked up only 60 ppm. of valine (55%) in 2 days of fermentation. Yeast E, which apparently is unable to absorb valine as rapidly as the other yeast, is, in fact, a diacetyl producer. The valine-absorption results are graphically shown in Fig. 3. It should be noted that these differences are largely eliminated by the end of fermentation, but since diacetyl is produced during the early stages, it is this time which is critical for the phenomenon under study.

A valine balance for these yeasts showed that much more valine is absorbed from their respective worts than is actually contained in the harvested yeasts (Table IV). It appears that valine enters extensively into metabolic processes other than intact incorporation into yeast substance.

TABLE IV
Valine Balance

	Valine Absorbed from 1 Bbl. of Wort	Valine Contained in Yeast From 1 Bbl. of Wort
	g	*g*
Yeast D	14.5	5.7
Yeast E	12.3	2.8

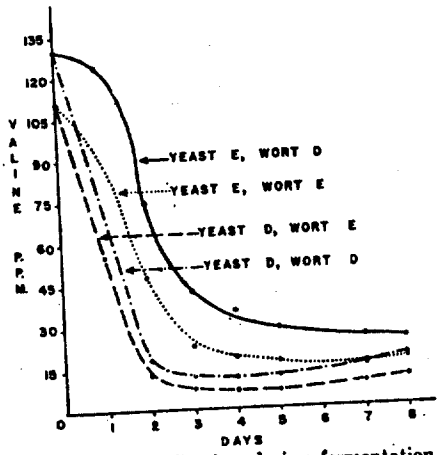

Fig. 3. Valine utilization during fermentation.

TABLE V
Effect of Acetolactate on Diacetyl Levels

Acetolactate	Diacetyl			
	Wort D		Wort E	
	Yeast D	Yeast E	Yeast D	Yeast E
ppm	*ppm*	*ppm*	*ppm*	*ppm*
0	0.30	1.38	1.77	2.24
50	0.24	1.29	0.24	2.30
100	0.31	0.17	0.19	0.29

The reason yeasts differ in their ability to absorb valine is not completely understood. However, it is well known that absorption of some amino acids occurs by active transport (16), and also, it has recently been shown that intracellular potassium levels are involved in amino acid transport (15).

Preliminary experiments in these laboratories indicate that a mechanism similar to the one proposed here for valine synthesis may also be in operation for isoleucine synthesis (21,22). A homolog of diacetyl, 2,3-pentanedione, would be the by-product of this synthesis. It is conceivable that some of the discrepancies between odor and actual diacetyl content of a beer may be attributed to this compound.

While not strictly a part of this paper, it may be of interest to examine several of the available hypotheses which explain the enzymatic removal of diacetyl. D. E. Green (5), in 1947, found a diacetyl mutase which converted two molecules of diacetyl to one of acetoin and two molecules of acetic acid:

$$2\ CH_3-CO\ .\ CO\ .\ CH_3 + 2H_2O \rightarrow CH_3 \cdot CHOH.COCH_3 + 2\ CH_3COOH$$

Juni, in 1956 (7), proposed a diacetyl cycle in which a molecule of diacetyl is split into two molecules of acetic acid, with diacetyl methyl carbinol as a key intermediate (Fig. 4).

Which of these, if either, is applicable to beer is not yet known. But regardless of mechanism, yeast can and does remove

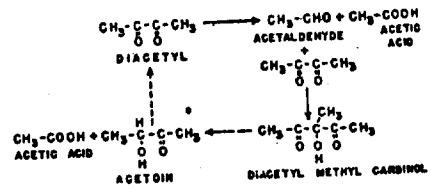

Fig. 4. Removal of diacetyl.

1505

diacetyl during the latter part of fermentation (see Fig. 1) and during storage (2).

Experimental Methods

Diacetyl was determined by the method West *et al.* (28) or by a modification of the Voges-Proskauer method (13).

Valine assays were done in accordance with the method of Barton-Wright (1), using *L. arabinosus* 17-5 and Difco valine assay medium.

L-valine was the product of Schwarz Laboratories, Inc., and 1-alpha-acetolactic acid (ethyl ester) was purchased from Krishell Laboratories, Portland, Oregon.

Discussion

A mechanism which connects diacetyl production by yeast to its synthesis of valine has been suggested. Several important practical implications follow from this theory.

First, it relates diacetyl formation to the anabolic existence of yeast, when the yeast is actively reproducing and metabolizing amino acids. Any treatment which invokes the Pasteur effect — the conversion of the anaerobic pathways to aerobic ones, such as pitching into aerated wort, rousing, or transfer of wort during fermentation — may predispose the yeast toward diacetyl formation.

Second, low valine levels in wort may, in time, lead to an adaptive change in the yeast which enhances its ability to synthesize valine. In so doing, it may produce diacetyl as a by-product. A reversal of such adaptation should also be possible. The practice of introducing fresh cultures of yeast periodically would mitigate against such changes.

In connection with low valine levels in wort, it should be noted that Sandegren (17) found an increase in valine after boiling with hops (from 119 to 160 ppm.). Whether the hops themselves, or mere boiling, were responsible for this increase is not known.

Third, the theory here suggested offers an important means of screening yeasts for their propensity to produce diacetyl. An isolate, or culture, which is found to be retarded in valine absorption should not be chosen for further cultivation where diacetyl is a consideration. Since the valine assay is done on 1-ml. samples, test tube cultures can be screened in this fashion.

And lastly, while bacteria are not directly involved in this scheme of diacetyl formation, it is to be expected that the presence of a valine-utilizing yeast contaminant could further deplete the available supply of valine and so indirectly promote diacetyl formation by yeast. Inconsistencies in diacetyl levels in successive brews may well be partly attributable to differences in contamination of the pitching yeasts. At least one bacterial companion of a pitching yeast has been found to absorb valine from wort.

Summary

1. A mechanism for the formation of diacetyl in beer by yeast is postulated.

2. Diacetyl formation is shown to be closely related to the synthesis of the amino acid, valine.

3. Possible mechanisms for the removal of diacetyl from beer are discussed.

4. Several practical suggestions for controlling diacetyl formation are made.

Acknowledgment

The technical assistance of Mrs. Gisela Frenkel is gratefully acknowledged.

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NITROGEN METABOLISM. II

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1509

Hydrogen Peroxide–Catalase Milk Treatment for Enhancement and Stabilization of Diacetyl in Lactic Starter Cultures [1]

J. Dairy Sci. 51(4) 511-516, (1968)

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Abstract

Hydrogen peroxide–catalase treatment of milk, prior to inoculation with starter cultures containing diacetyl-producing aroma bacteria, has been found to increase synthesis and stabilization of diacetyl in the cultured milk. From 0.015 to 0.03% H_2O_2 is mixed into pasteurized milk, followed by addition of sufficient food-grade catalase to decompose the peroxide. The milk then is inoculated (0.5 to 1.0%) and incubated (21 C for 14 to 18 hours) as usually done with cultured buttermilk. The treatment results in at least a 100% increase in diacetyl production and the flavor produced is stable for at least one week, whether or not the product is cooled when the desired acidity is reached. Because of its simplicity, effectiveness, and nontoxicity, the method may be used in the dairy industry for increasing the level and uniformity of flavor in cultured buttermilk and sour cream.

The most important single substance responsible for the desirable flavor of certain fermented dairy products is diacetyl, though the proper amount of other compounds (8) cannot be disregarded. Diacetyl is produced in milk primarily from citrate by aroma bacteria, the *Leuconostoc* species and *Streptococcus diacetilactis* (4, 5). Seitz et al. (22), however, found that the same aroma bacteria have a capacity to decompose the synthesized diacetyl. Recently, Seitz et al. (21) showed that the destruction of diacetyl was mediated by a microbial enzyme called diacetyl reductase. Psychrophilic spoilage bacteria were also found to exhibit diacetyl reductase activity (21). The same workers found wide variation in the level and activity of this enzyme between species and even between strains within the same species. Recent work by Pack (15) demonstrated that the over-all diacetyl level in a growing milk culture of lactic acid and aroma bacteria consisted of a biosynthetic (increasing) phase, followed by a reductive (decreasing) phase which culminated in a stationary phase at low flavor level (less than 0.5 ppm). Such diacetyl destruction results in the loss of desirable aroma and flavor in dairy products, and consequently lowers their marketable quality.

In the competitive market of today, measures should be taken to control undue loss of flavor to prevent lack of product uniformity, with consequent customer dissatisfaction and reduced sales. Furthermore, the recent report of Hempenius et al. (6) has re-emphasized the importance of diacetyl flavor in consumer acceptance. By proper in-plant sanitation (23) and sufficient cooling and refrigeration to minimize post-pasteurization contamination and growth, the psychrophilic bacteria capable of diacetyl destruction (3) can be controlled. However, diacetyl destruction by the aroma bacteria presents an even more difficult control problem and two possible approaches may be suggested. One would be the selection of aroma bacteria capable of producing high concentrations of diacetyl, but with low levels of diacetyl reductase activity. Another would be the stabilization of diacetyl at the desirable level by manipulation of temperature, pH, and oxidation-reduction potential to find cultural conditions unfavorable for diacetyl reductase activity.

Efforts to select aroma-bacteria with decreased diacetyl destroying ability have not been successful. Therefore, studies were conducted to develop a method which could be used in dairy plants to enhance and stabilize diacetyl in cultured products. The present report presents the results achieved with one such method (hydrogen peroxide–catalase milk treatment), and a companion paper (17) presents results obtained with another process (timed-cooling) effective in this regard.

Materials and Methods

Cultures. The single-strain *Streptococcus diacetilactis* 18-16 used was from the stock cul-

[1] Received for publication October 11, 1966.

Technical paper no. 2207, Oregon Agricultural Experiment Station. Supported in part by a grant from the American Dairy Association.

ture collection of the Department of Microbiology, Oregon State University. Mixed-strain starter Culture E, known to contain *Streptococcus cremoris, S. diacetilactis,* and *Leuconostoc citrovorum* strains (15), was obtained from commercial sources. The cultures were maintained in sterile nonfat milk by transfer of one per cent inoculum and incubation for 18 hours at 21 C.

Reagents. The solutions of hydrogen peroxide (35%) and beef liver catalase (Miles Chemical Co., Clifton, New Jersey) used in this study were obtained from Marschall Dairy Laboratory, Inc., Madison, Wisconsin. These reagents are presently in use in a number of dairy plants where milk is treated with hydrogen peroxide and catalase prior to Cheddar cheese manufacture. The catalase was used at a concentration of 100 Keil units per ml as determined by the Keil assay (11). Forty per cent aqueous potassium iodide (KI) in distilled water (w/v) was made fresh just before use to detect the presence of H_2O_2 in milk after treatment with catalase to decompose added H_2O_2.

Culture medium. Nonfat milk used to maintain cultures was made from Matrix Mother Culture Medium (Galloway-West Co., Fond du Lac, Wisconsin) by reconstitution to 11% solids, followed by heating at 121 C for 12 minutes.

Diacetyl production and destruction patterns in milk cultures. One hundred milliliters of sterile 11% nonfat milk in screw-cap bottles was inoculated at a concentration of 1% with the desired cultures and incubated at 21 C. A time-course study of culture diacetyl production and destruction over a period of 60 hours was conducted by determination of diacetyl levels in 20-ml samples collected at various intervals. In one instance, the diacetyl levels were measured over a period of eight days at various intervals. The method of Owades and Jakovac (14), as modified by Pack et al. (16), was used for diacetyl determinations.

Hydrogen peroxide–catalase treatment of milk. Screw-cap bottles containing 100 ml of 11% nonfat milk were autoclaved at 121 C for 12 minutes and cooled to 25 C. Hydrogen peroxide was added to the cooled milk at levels ranging from zero to 0.03%. After thorough mixing, a sufficient amount (Table 1) of catalase at 100 Keil units per ml was added and mixed thoroughly with the milk to decompose the hydrogen peroxide. One Keil unit is the amount of enzyme necessary to decompose one gram of 100% hydrogen peroxide in 10 minutes at 25 C under the standard assay conditions described previously (11).

TABLE 1. Amount of catalase required for decomposition of different levels of hydrogen peroxide added to 11% nonfat milk at 25 C.

Per cent catalase	Reaction with KI in milk treated with H_2O_2 at the rate of 0.015%[a]	0.03%[a]
0.000	+++	+++++
0.001	±	++
0.002	—[b]	+
0.004	∓	—
0.006	—	—

[a] + indicates yellow coloration due to the presence of H_2O_2. The number of symbols indicates the relative intensity.
[b] — indicates no discoloration and absence of H_2O_2.

The amount of catalase required for the rapid and complete decomposition of different levels of hydrogen peroxide was determined colorimetrically. The method consisted of adding five drops of freshly prepared 40% KI to 10.0 ml of hydrogen peroxide–catalase-treated milk in a test tube. The appearance of a yellow discoloration indicated the presence of hydrogen peroxide which had oxidized the KI, liberating free iodine to color the milk. Hydrogen peroxide–free samples did not exhibit any color change. Strips of paper saturated with KI also are available for conducting the test for residual H_2O_2 (11).

Effect of hydrogen peroxide–catalase treatment on culture diacetyl levels. Bottles containing 100.0 ml of 11% nonfat milk treated as described with various amounts of hydrogen peroxide and catalase were inoculated with different cultures at a concentration of 1% and incubated at 21 C. Samples were collected at various intervals for diacetyl determinations, and the results were plotted with time and compared with untreated controls.

Results

Table 1 lists the amounts of catalase required for the dissipation of hydrogen peroxide added to milk at 0.015 and 0.03% levels. It may be seen that 0.004% catalase completely decomposed 0.03% of hydrogen peroxide within a few minutes. Only one-half as much catalase was required to dissipate 0.015% of added hydrogen peroxide in the nonfat milk.

A remarkable effect of the hydrogen peroxide–catalase treatment of milk on the formation and stability of diacetyl in mixed-strain starter Culture E can be seen in Figure 1. The control culture exhibited the normal di-

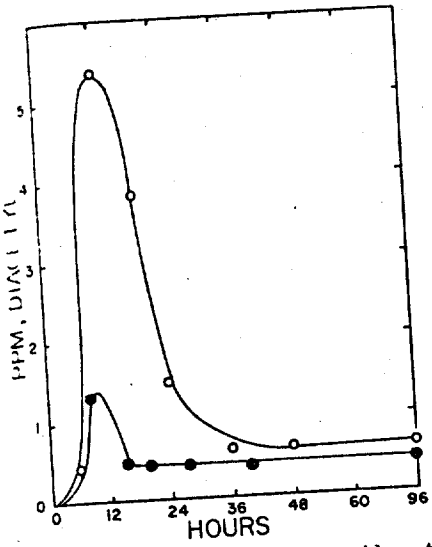

Fig. 1. Effect of the hydrogen peroxide–catalase treatment on diacetyl stability in mixed-strain starter Culture E. —○—: Sterile nonfat milk without treatment; —△—: Milk treated with hydrogen peroxide alone; —□—: Milk treated with 0.004% catalase alone; —●— and —▲—: Milk treated with 0.03% of hydrogen peroxide followed by an addition of 0.004% of catalase. These treatments were performed before incubation except one case (—●—), treated after the inoculation.

...l pattern in the milk medium. In contrast, ...same starter grown in hydrogen peroxide–catalase treated milk showed almost a threefold enhancement of diacetyl production, as well as stabilization of the maximum level for prolonged periods at 21 C. In the control culture, the maximum level of around 7.0 ppm diacetyl rapidly fell to one-third the value (around 2.0 ppm) within 24 hours. The decrease in the maximum diacetyl concentration in the treated culture was very small and gradual, amounting to only one-seventh the maximum value over a period of eight days. The above effects on the diacetyl production-destruction pattern in treated cultures occurred, irrespective of whether the medium was subjected to the treatment before or after inoculation of the starter cultures. In spite of a slight increase in the maximum concentration of diacetyl attained in the medium treated after inoculation, this procedure was discontinued in later studies, since a retardation in the rate of acid production was noted in such cultures. It is also seen from the diacetyl curves for cultures treated with catalase alone or with hydrogen peroxide only, that the desirable effects of the treatment were due to the combined use of hydrogen peroxide and catalase and not due to either one of the two ingredients alone. Also, when heat inactivated catalase was substituted for the active enzyme, the treatment did not enhance or stabilize diacetyl; the remaining hydrogen peroxide killed the cells and no flavor compound was produced. The diacetyl curve for culture treated with hydrogen peroxide alone especially illustrated the severity of the effect of hydrogen peroxide exposure on bacterial cells. Similar results were obtained with *S. diacetilactis* 18-16.

The optimum time for the hydrogen peroxide–catalase treatment for maximum enhancement and stabilization of diacetyl was determined by adding the reagents at various time intervals after inoculation of the culture. The results of such studies with mixed-strain Starter E and *S. diacetilactis* 18-16 are shown in Figures 2 and 3, respectively. The diacetyl patterns obtained in both cases indicated that the enhancing effect was operative at any stage during the ascending phase, but was lost immediately after the onset of the declining phase. The stabilizing effect of the treatment, however, was retained to a considerable degree throughout the entire period. From these re-

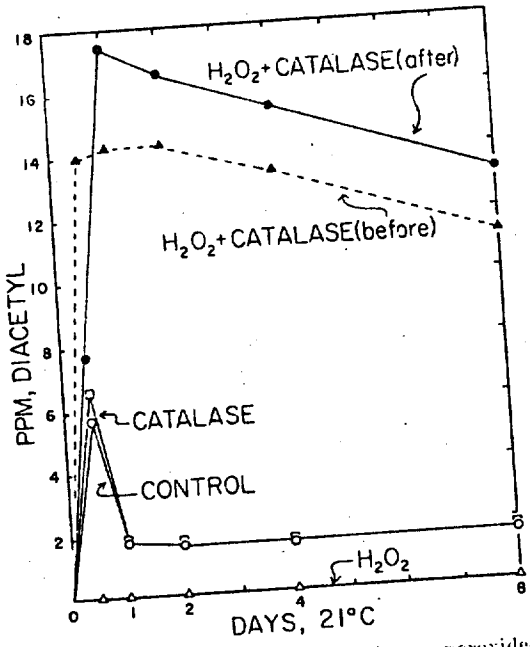

Fig. 2. Effect of time of the hydrogen peroxide–catalase treatment on diacetyl production and stability in mixed-strain starter Culture E. —○—: Ten per cent nonfat sterile milk without treatment; —●—: Milk was treated with 0.03% of hydrogen peroxide followed by an addition of 0.004% of catalase after 0, 9, 12, 15, and 18 hours of incubation at 21 C. Times of addition of catalase are indicated for each curve.

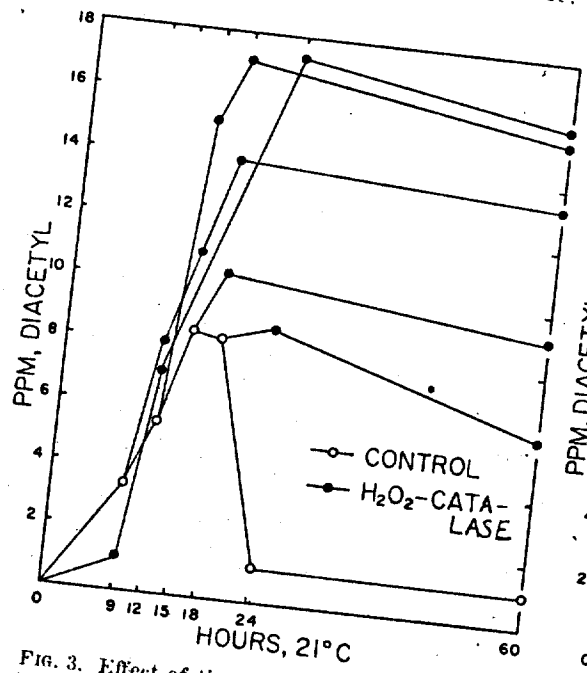

FIG. 3. Effect of time of the hydrogen peroxide–catalase treatment on diacetyl production and stability in a single-strain culture of *S. diacetilactis* 18-16. —○—: Ten per cent nonfat sterile milk without treatment; —●—: Milk was treated with 0.03% of hydrogen peroxide followed by an addition of 0.004% of catalase after 0, 9, 12, and 15 hours of incubation at 21 C. Times of addition of catalase are indicated for each curve.

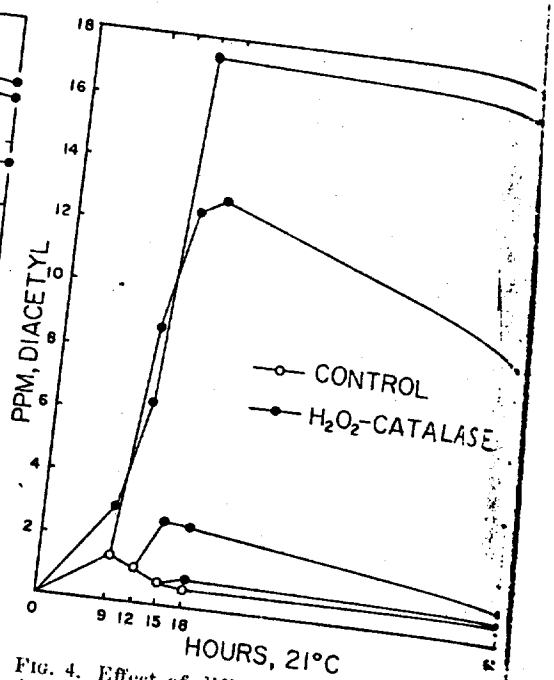

FIG. 4. Effect of different concentrations of the hydrogen peroxide–catalase on diacetyl production by mixed-strain Culture E. —○—: Ten per cent nonfat milk without treatment; —●—: Milk treated with 0.03% of hydrogen peroxide followed by addition of 0.004% catalase; —▸—: Milk treated with 0.015% of hydrogen peroxide followed by addition of 0.002% of catalase.

sults it appears that the product of the hydrogen peroxide–catalase reaction enhances the diacetyl level only when the cells are actively synthesizing the compound. It also is apparent that this product of the hydrogen peroxide–catalase reaction is relatively ineffective in enhancing flavor after synthesis of diacetyl ceases.

The maximum level of hydrogen peroxide used in these experiments was 0.03%, since this amount presently is in use for the treatment of milk in the Cheddar cheese industry. To determine the effect of amount of hydrogen peroxide on enhancement and stabilization of diacetyl, this and a lower level (0.015%) were compared using mixed-strain Starter E and *S. diacetilactis* 18-16. The results obtained for the mixed culture are shown in Figure 4. Greater enhancement and stabilization at higher levels of hydrogen peroxide were noted in both cases. The desirable effect at the higher hydrogen peroxide level was more pronounced in the case of *S. diacetilactis* 18-16 than in Culture E.

Although the higher hydrogen peroxide level was found to provide greater enhancement and stabilization of diacetyl in Culture E and *S. diacetilactis* 18-16, slight retardation of acid production was noted at this concentration. The retardation, however, was not sufficient to interfere with normal plant routine during, for example, the manufacture of cultured buttermilk. A study was made, however, to determine whether the use of a larger inoculum would overcome the retardation: the larger inoculum helped to correct the slight decrease in the rate of acid production at all levels (0.01 to 0.03%) of hydrogen peroxide treatment. For example, at 0.03% oxidant addition, 16 hours were required to produce 0.85% titratable acidity from a 1% inoculation of nonfat milk incubated at 21 C; 14.5 hours were required for production of the same amount of acid from 2% inoculum.

Discussion

Hydrogen peroxide–catalase treatment of milk has been used by the Cheddar cheese industry for the past several years instead of heat pasteurization. The treatment has also been used successfully in the manufacture of Swiss cheese (12) and canned sterile whole-

s (18). The purpose of this treatment is destroy pathogens and undesirable spoilage teria in raw milk. The treatment has been d in our study to enhance and stabilize cetyl aroma and flavor.

The effect of the hydrogen peroxide–catalase atment on diacetyl enhancement apparently ue to the oxidative effect(s) of the oxygen erated from the oxidant by catalase. The act nature of the effect will require further dy. There are several reports in the litera- e which indicate that oxygen may act on pha-acetolactate, resulting in the accumula- n of diacetyl. Pette (19) found that oxygen s effective in stimulating diacetyl formation inly during the period of vigorous fermenta- n of citric acid by mixed-strain starter cul- es. He postulated that an intermediate sub- ance formed from citric acid was oxidized to acetyl. DeMann and Pette (2) reported that pha-acetolactate which accumulated during fermentation of pyruvic acid by *B. cremoris* s transformed into diacetyl by decarboxyla- n on heating in the presence of air. They so found alpha-acetolactate decarboxylase in ls of *B. cremoris* and postulated that in xed-strain starters, sour cream and butter, pha-acetolactate was produced by aroma bac- ia and then oxidatively decarboxylated to acetyl in the presence of oxygen. Taufel and ke (26) demonstrated the conversion of thetic alpha-acetolactic acid to both diacetyl acetoin by cell-free extracts of aroma bac- ia in the presence of air. In light of these ts, it is noteworthy that the effect of the drogen peroxide–catalase treatment could not, our studies, be duplicated by bubbling air oxygen through the uninoculated culture It is known, however, that bubbling oxy- through cultures of aroma bacteria acidified th citric acid will enhance diacetyl produc- (11). Recently, Lundstedt and Fogg (9) monstrated an air requirement for diacetyl duction in Cottage cheese inoculated with *S. acetilactis* and held at 10 C. Recent evidence sented by Speckman and Collins (24, 25) owed that alpha-acetolactate was not a pre- sor of diacetyl in the diacetyl synthesis of *S. diacetilactis* or *L. citrovorum;* they reported that acetyl-CoA and active acetaldehyde con- dense to form diacetyl. The effect of oxygen this reaction apparently has not yet been determined.

Data from this study and from the work of Seitz (20) indicate diacetyl is irreversibly con- verted to acetoin by the action of diacetyl reductase in mixed-strain starter cultures under normal cultural conditions. However, it is possible that an excessively high concentration of oxygen could cause oxidation of acetoin back to diacetyl. This is in agreement with the mechanism of diacetyl production in *S. lactis* var. *maltigenes* and *S. paracitrovorus*, proposed by Nikkila (13). Kandler (7) also suggested that alpha-acetolactic acid was decarboxylated to acetoin which was then subsequently oxidized to diacetyl. However, the work of Speckman and Collins (24, 25) showed that acetoin was not a precursor of diacetyl. Furthermore, in the present work it was found that hydrogen peroxide–catalase treatment of a mixed-strain starter filtrate fortified with acetoin failed to show any increase in diacetyl, supporting similar findings by Michaelian and Hammer (10).

A possible means by which oxygen might enhance diacetyl production would be by elevating the rate of citrate or pyruvate metabolism. How this might occur is unclear at present and also would not afford any explanation of the increased stability of diacetyl under these conditions. In this regard, however, it is noteworthy that Brewer et al. (1) were able to enhance diacetyl production several hundred per cent by acidifying overnight cultures of aroma bacteria (*S. paracitrovorus*) with citric acid to pH 4.0, then subjecting them to aeration under at least 10.5 kg per cm² of air pressure; pressure without agitation was not effective. Lundstedt and Fogg (9) noted that Cottage cheese cooked out at a high temperature, such that the resulting cheese had a porous structure, would readsorb air during draining. When such cheese was creamed with citrated-whey culture of *S. diacetilactis*, diacetyl production occurred during refrigeration of the cheese; dense, whey-locked, rubbery cheese did not support good aroma development. It seems logical that the hydrogen peroxide–catalase treatment employed in the present studies enhances diacetyl production by providing oxygen to function as it did in the case of the work of Lundstedt and Fogg (9) and Brewer et al. (1). The exact nature of this effect will require further study.

Regardless of the mechanism involved, it appears that the hydrogen peroxide–catalase treatment offers a very simple, effective, and inexpensive method for the enhancement and stabilization of diacetyl in dairy products. Present prices for catalase and hydrogen peroxide allow use of the process without increasing the cost of cultured buttermilk more than one cent a gallon.

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1510

Effect of Temperature on Growth and Diacetyl Production by Aroma Bacteria in Single- and Mixed-Strain Lactic Cultures[1]

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J. Dairy Sci. 51 (3) 339-344, (March 1968)

Abstract

By the use of a differential plating medium, the growth of aroma bacteria (*Leuconostoc citrovorum* and *Streptococcus diacetilactis*) in mixed-strain lactic starter cultures was followed. Populations were greater at 30 C than at the temperature (21 C) normally used in the dairy industry for incubation of cultures for manufacture of fermented dairy products. At 30 C, peak level of diacetyl produced in mixed-strain lactic starters was lower than at 21 C; however, at the higher temperature there was an earlier initiation of the synthesis and the attainment of the peak level. Prompt cooling of cultures to 2 C inhibited diacetyl reductase activity and, consequently, loss of desirable flavor and aroma was prevented. The cooling, however, had to be carried out when the diacetyl level was high and before complete destruction of the flavor compound. By a careful combination of time and temperature of incubation and prompt cooling, the diacetyl flavor of milk cultures and Cottage cheese dressed with cultured cream could be stabilized and improved.

The flavor components of major importance in fermented dairy products are lactic acid and diacetyl. The former imparts the pleasant acid taste, while the characteristic butter-like flavor is attributed to the latter compound. Diacetyl in dairy products is formed by the fermentation of milk citrate by aroma bacteria, the *Leuconostoc* species and *Streptococcus diacetilactis* (5, 8). *Leuconostoc* species used in starter cultures have weak lactose-fermentative abilities (5, 11) and, due to the low pH optimum of citrate permease (6), milk citrate fermentation by these organisms occurs only after the pH is lowered below 6.0 by acid-producing species (*Streptococcus lactis* and *Streptococcus cremoris*). *S. diacetilactis*, on the other hand, is able to produce diacetyl from milk citrate even in the absence of acid-producing species, because of its lactose-fermenting ability (8).

In view of the associative growth conditions occurring in mixed-strain lactic starters, the factors affecting the individual strain populations in the mixed-strain cultures play an important part in day-to-day manufacture of uniformly, high grade dairy products with respect to body, texture, and flavor. Incubation and storage temperatures are factors that can be easily controlled in day-to-day operations and are known to have important influences on associative growth.

Hammer and Babel (5) suggested that to maintain a proper balance between the lactic acid-producing and aroma bacteria, cultures should be incubated at 21 to 22 C. They state: "Temperatures such as 70 to 72 F have advantages of considerable importance. With definitely lower temperatures growth of the organisms is delayed and it is difficult to obtain rapid ripening, whereas with significantly higher temperatures over-ripening often occurs and conditions are more favorable for development of heat-resistant organisms that survive pasteurization of the milk. At relatively high temperatures growth of *S. citrovorus* and *S. paracitrovorus* is influenced greatly and a lactic culture that has been through a very few transfers at 98.6 F may no longer contain these organisms." Due to the lack of proper medium for the direct enumeration of aroma bacteria in mixed-strain starter cultures, reports on the effect of temperature on the growth of aroma-bacteria under associative growth conditions have been largely speculative. In 1961, however, Galesloot et al. (3) described such a medium and more recently a similar differentiating medium for aroma bacteria in mixed-strain starters was described by Nickels and Leesment (9). In the present investigation, this latter medium was utilized to study the effect of in-

Received for publication July 29, 1967.

[1]Technical paper no. 2314, Oregon Agricultural Experiment Station. Supported in part by a grant from the American Dairy Association.

cubation temperature on growth and diacetyl production by aroma bacteria present in mixed-strain starters. The use of low temperature as a means of preventing enzymatic destruction of diacetyl also was investigated, since the report of Elliker et al. (2) and Prill and Hammer (13) indicated that time-cooling was effective in stabilizing culture flavor.

Materials and Methods

Cultures. The four mixed-strain starter cultures A, B, E, and G used in this investigation were obtained from commercial sources. Culture A contained strains of *S. cremoris* and *Leuconostoc citrovorum*, and Culture B was made up of *S. lactis*, *S. cremoris*, and *S. diacetilactis* strains. The remaining two cultures contained *L. citrovorum* for aroma production in addition to acid-producers. The cultures were obtained as lyophilized powders and were added as direct seed to 11% reconstituted Matrix Mother Culture Medium (Galloway-West, Fond du Lac, Wisconsin) for the various experiments. Direct seeding of cultures was adopted to prevent strain dominance (15). *L. citrovorum* L_2 was isolated from a mixed-strain starter culture and *S. cremoris* F_2d_2 obtained from Professor A. Zeilinger of Vienna, Austria.

For Cottage cheese dressing, a single-strain culture of *S. diacetilactis* 18-16 was used. The dressing was made as described by Elliker et al. (2).

N-L medium for the enumeration of aroma bacteria. The plating medium was made by mixing the following three formulations after tempering them in a 45 C water bath:

a) *Culture filtrate.* A single-strain milk culture of *S. cremoris* incubated for 48 hr at 22 C was filtered through a Whatman No. 1 paper. Ten-milliliter volumes of the filtrate dispensed into screw-cap test tubes were sterilized at 121 C for 15 minutes.

b) *Sodium citrate agar.* The medium consisted of 2.0% Tryptone, 0.5% yeast extract, 0.25% gelatin, 0.5% glucose, 0.5% lactose, 0.4% sodium chloride, 0.2% trisodium citrate · 2 H_2O, 1.0% calcium lactate · 5 H_2O, 1.5% Bacto agar, and 100 ml distilled water. The pH was adjusted to 6.7 and the agar medium sterilized by autoclaving at 121 C for 15 min.

c) *Calcium citrate solution.* Ten grams of sieved calcium citrate was suspended in 100.0 ml 1.5% (w/v) solution of carboxymethylcellulose (Uddeholm, CMC Cekol, MV, Uddeholm, Sweden). The dissolution of carboxymethylcellulose in distilled water was aided by warming (45 to 50 C). The solution was sterilized at 121 C for 15 min.

The agar medium was melted and after tempering at 45 C in a water bath, the culture filtrate and the calcium citrate solution were added and mixed together. The ratio by volume of agar medium to culture filtrate and calcium citrate solution in the final mixture was 85:10:5.

After pour plates (7 to 9 ml per plate) of suitable dilutions were made, the plates were incubated at 25 C. Colonies of aroma bacteria are distinguished from the acid-producing species on this medium by the presence of a clear zone around the former due to the utilization of citrate. *S. diacetilactis* strains appear as medium-sized colonies with a clear halo, usually within two days. *Leuconostoc* appear as small colonies with a clear halo only after three to five days. This distinction, however, is variable and for positive identification it is necessary to inoculate colonies into litmus milk which is reduced by *S. diacetilactis* but not by *Leuconostoc*.

Diacetyl determinations. The method of Pack et al. (12) was used for estimating diacetyl in milk cultures and in Cottage cheese. A 20.0 ml volume of homogenate, prepared by blending 150.0 g of cheese with an equal amount of distilled water, was used for diacetyl determinations on Cottage cheese samples.

For the study of temperature effect on diacetyl production-destruction patterns, cultures were incubated at the desired temperatures and samples were collected at various time intervals. The diacetyl concentrations were plotted against time to obtain time-course patterns at 21 and 30 C, respectively.

To determine effect of cooling on diacetyl stabilization, the milk cultures or dressed Cottage cheese were transferred to coolers and samples were collected at time intervals for diacetyl quantitation. The diacetyl concentrations were plotted against time, and comparisons were made between cooled and uncooled samples.

Hydrogen Peroxide–Catalase treatment of cultures. Details regarding this treatment have been described by Pack et al. (11). After treatment and initial incubation of cultures at 21 C, experimental flasks were transferred to a cooler held at 2 C; control flasks were held at 21 C. After holding for five days, the diacetyl concentrations were determined and compared.

Results

The characteristic colonial appearance of the citrate-fermenting aroma species present in mixed-strain starter Culture B plated on the

[illegible]ium is shown in Figure 1. The clear [illegible]ounding the *S. diacetilactis* colonies [illegible] visible and the non-haloed colonies [illegible] and *S. cremoris* also may be seen, [illegible] as readily. *Leuconostoc* colonies that [illegible] when the three other mixed-strain cul[illegible] plated were identical, though some[illegible] aller, especially when they first ap[illegible] and with smaller halos.

[illegible] counts on N-L medium showing the [illegible] *L. citrovorum* L_2 in nonfat milk in [illegible]sence of *S. cremoris* are shown in Table 1. [illegible]*nostoc*, it may be seen, apparently [illegible]ppressed by growth with *S. cremoris* [illegible]lar data were obtained with three other [illegible]*stoc* and *S. cremoris* pair combinations. [illegible]other experiment, where mixed-strain [illegible] Cultures A and B were incubated at 21 and 30 C, data (Table 2) indicated that the aroma bacteria grew better at 30 C than at 21 C, though in the case of the *Leuconostoc*-containing culture, incubation for 24 hours was necessary to show the difference. Furthermore, rates of CO_2 evolution measured manometrically in milk by several *Leuconostoc* strains were greater at 30 C. In view of this, it seemed advisable to examine the diacetyl production-destruction patterns at these two temperatures and typical curves for Cultures A and B at 21 and 30 C are shown in Figure 2. In both the cultures, a greater diacetyl concentration was obtained when the cultures were incubated at the lower temperature. However, the initiation of diacetyl synthesis was delayed considerably at the lower temperature. This also supported the plate count data suggesting better growth of aroma bacteria at the higher temperature.

Figure 3 illustrates the diacetyl production-destruction patterns in Culture E when samples of the milk cultures collected at various time intervals during incubation at 21 C were cooled and held at 2 C. Maximum diacetyl concentra-

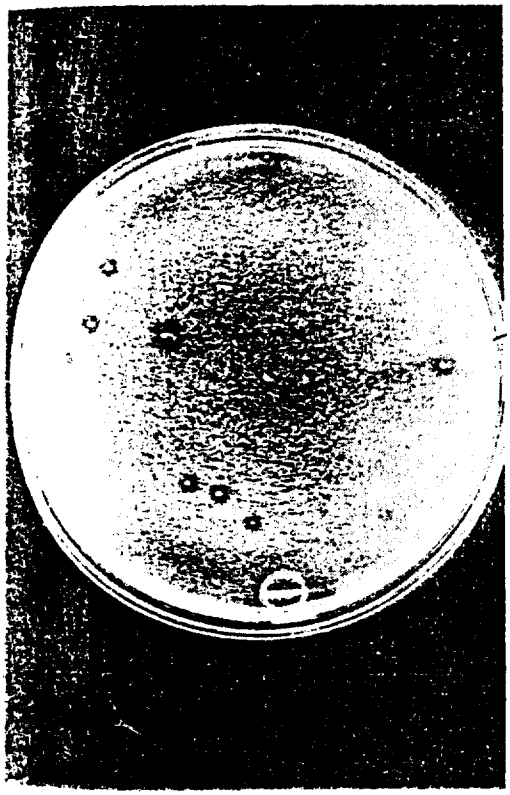
TABLE 1

Numbers of *Leuconostoc* present at various times in single-strain culture and in combination with *S. cremoris* as determined by plating on N-L medium[a]

Hours at 21 C	Single-strain *L. citrovorum* L_2 ($\times 10^6$)	*L. citrovorum* L_2 plus *S. cremoris* F_2d_2 ($\times 10^6$)
0	0.00198[b]	0.6
4	0.00650	1.4
8	0.0770	4.6
12	1.10	10.0
16	8.80	41.0
24	130.0	31.0
28	200.0	.....
30	.....	70.0

[a] Data provided by Ruth Brown, Department of Microbiology, Oregon State University.

[b] Culture diluted at 0 time to about 2,000 cells/ml.



[illegible]. Photograph showing *Streptococcus di*[illegible] colony growth from mixed-strain starter [illegible] B on N-L medium after incubation for [illegible]ays at room temperature. A clear halo [illegible] the colonies indicates utilization of calcium [illegible] present as a turbid suspension in the me[illegible] *Leuconostoc* sp. form smaller colonies with [illegible] zones of clearing around them and appear [illegible]fter incubation for three to four days at [illegible] temperature. Acid-producing species produce [illegible] without the clear halo, due to their in[illegible] to utilize citrate.

TABLE 2

Plate counts of aroma bacteria in mixed-strain starter Cultures A and B incubated at 21 and 30 C. Samples were collected at the indicated time intervals and plated on N-L medium

Hours	Count ($\times 10^6$) per ml of culture			
	Leuconostoc in mixed Culture A		*S. diacetilactis* in Culture B	
	21 C	30 C	21 C	30 C
0	0.014	0.014	0.009	0.009
12	.....	.....	1.0	5.0
18	1.0	1.0	9.0	16.0
24	8.0	42.0	10.0	14.0

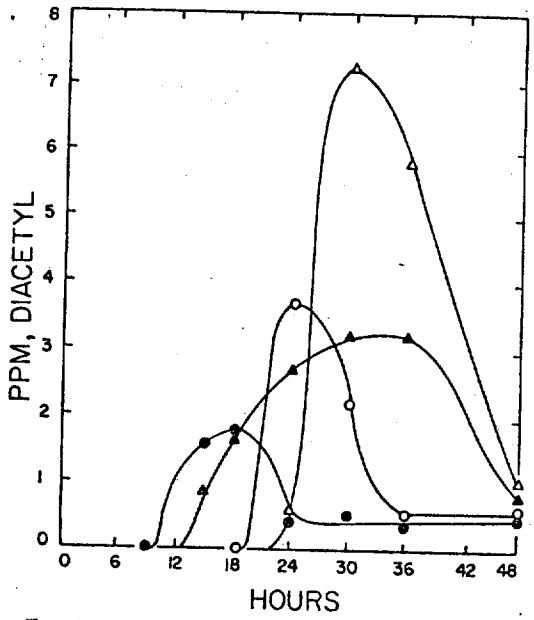


FIG. 2. The effect of incubation temperature on diacetyl production and destruction by mixed-strain starter Culture A at 21 C (open circles) and 30 C (solid circles) and by mixed-strain starter Culture B at 21 C (open triangles) and 30 C (closed triangles).

tion and stabilization were achieved when the sample was cooled to and held at 2 C after incubation for 12 to 14 hours at 21 C. The peak level of diacetyl in the milk culture was also observed between 12 to 14 hours during incubation at 21 C. There was a slight increase in the diacetyl level when a six-hour sample was cooled to 2 C and held at that temperature; the 24-hour sample, however, showed negligible increase when cooled to and held at 2 C. Results of similar experiments conducted with Cultures E and G over extended periods of storage up to seven days at 2 C are shown in Figure 4.

Increase and stabilization of diacetyl flavor in Cottage cheese creamed with dressing containing *S. diacetilactis* 18-16 as compared to a control sample dressed with noncultured cream when held at 0 and 5 C, are illustrated in Figure 5. It may be seen that a greater increase and stabilization could be obtained at 5 than at 0 C.

Table 3 summarizes results of an experiment performed to study the effect of the combined use of hydrogen peroxide–catalase treatment and cooling on the diacetyl concentration and stability in a mixed-strain starter culture. It may be seen that the combined treatment provided greater stability than the hydrogen peroxide–catalase treatment alone.

Discussion

By use of the N-L differential plating medium, data were obtained which indicated that the growth of aroma bacteria in mixed-strain lactic starter cultures prepared by the direct seed process occurs more rapidly at temperatures higher than 21 C, the temperature normally used for culture incubation in the dairy industry. Thus, the industry practice of incubating cultured buttermilk and sour cream at 21 C need not be based on the belief that this temperature is required for the best growth of the aroma bacteria. However, it also has been shown that at temperatures above 21 C, diacetyl is destroyed more rapidly and also reaches a lower level at the peak of production.

The diacetyl curves for Cultures A and B revealed an earlier initiation of diacetyl synthesis at 30 than at 21 C. This probably was due to

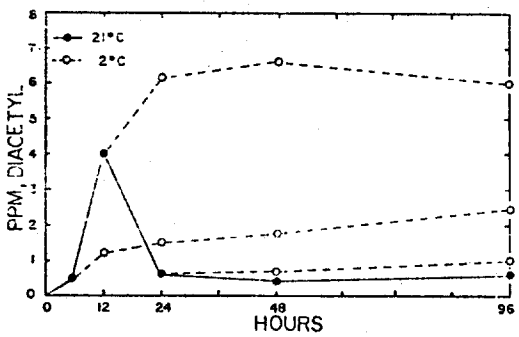


FIG. 3. The influence of culture age on effectiveness of cooling in stabilizing diacetyl in mixed-strain starter Culture E. The solid line is the control held continually at 21 C. The broken lines represent diacetyl levels found in cultures cooled to 2 C after 6, 12, or 24 hours.

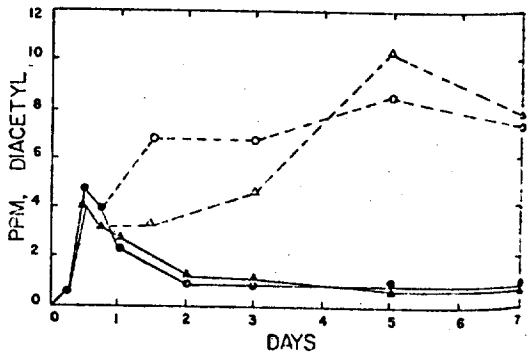
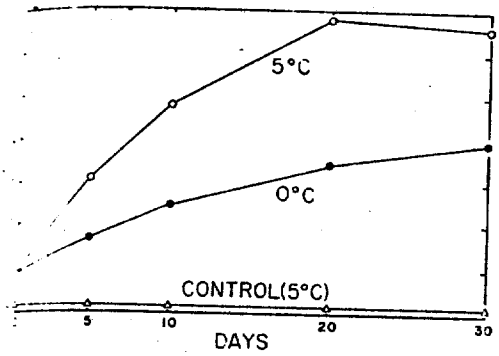


FIG. 4. Diacetyl levels in 100-ml milk cultures of Starters E and G held continuously at 21 C (solid circles and triangles respectively) and cooled to 2 C after 18 hours (open circles and triangles respectively).



[illegible] 5. The effect of cooling on diacetyl en-[illegible]ment in Cottage cheese creamed with cultured [illegible]ing. The top (—○—) and middle (—●—) [illegible] indicate diacetyl contents in cheese dressed [illegible] the cultured cream, and the bottom (—△—) [illegible] is that for cheese dressed with noncultured [illegible].

[illegible] faster rate of growth and acid production [illegible] the higher temperature and a consequent [illegible]lier reduction of pH below 6.0 necessary for [illegible] rapid uptake of citrate. Greater flavor de-[illegible]uction at 30 C might be due to enhanced di-[illegible]yl reductase activity at this temperature as [illegible]pared to 21 C. Since it is likely that diacetyl [illegible]thesis and destruction take place simultane-[illegible] (10), the stimulated reductase activity [illegible] prevent the rapid build-up of diacetyl, [illegible]ally during the last stages of the synthe-[illegible] phase, due to the depletion of diacetyl pre-[illegible]. As a result, during the final stages of [illegible]esis, an apparent steady state between [illegible]sis and destruction could occur, which is [illegible]ably manifested as a plateau in the diacetyl [illegible]uction-destruction curve. At 21 C, on the other hand, there is a rapid accumulation of diacetyl, indicating that due to its reduced activity, the reductase system is unable to keep pace with synthesis; however, when the diacetyl precursors are completely exhausted the destructive phase is dramatically expressed. Consequently, at 21 C there occurs a sharp peak between the synthesis and reductive phases in both Cultures A and B. Confirmatory evidence for these possibilities would consist of determining the temperature optima for diacetyl reductase systems in aroma bacteria. Work along these lines is in progress.

TABLE 3

[illegible]ined effect of hydrogen peroxide-catalase [illegible]tment and cooling on diacetyl stability in mixed-strain starter Culture E[a]

[illegible]tage of hydrogen [illegible]xide used for the treatment	ppm Diacetyl after 5 days[b] at	
	21 C	2 C
0.000	0.7	4.0
0.010	1.7	5.8
0.015	3.3	5.5
0.020	4.3	6.0
0.025	4.7	8.6
0.030	7.2	9.8

[a] The autoclaved milk medium was treated be-[illegible] inoculation at the rate of 1% and incubated [illegible] 21 C. After incubation for 12 hours one set of [illegible]ples was transferred to a cooler held at 2 C and [illegible] other left at 21 C. Diacetyl determinations [illegible] made after five days.

[b] The diacetyl concentration at 12 hours was 4.5 ppm.

The feasibility of arresting the loss of diacetyl by the diacetyl reductase activity in aroma bacteria through the prompt cooling of cultures was demonstrated by the results shown in Figures 3 and 4. As may be seen in Figure 3, the immediate cooling of Culture E at the peak level of the diacetyl in the culture not only stabilized the flavor over a storage period of four days but also resulted in one and one-half-fold increase in the diacetyl concentration. The increase in the diacetyl level at low temperatures was regarded to be the result of an oxygen-dependent enhancement of diacetyl production, as recently demonstrated by Landstedt and Fogg (7). Figure 3 also indicated that cooling of the culture at the early stages of initiation of diacetyl synthesis did not result in a very great increase in the diacetyl level. This might have been due to the retardation of acid production at the low temperature as a result of which the pH of the medium was not lowered sufficiently for optimal citrate permease activity. Hence, due to the lack of sufficient diacetyl precursors, there was very little chemical synthesis of the compound. At the end of the destructive phase, due to the absence of diacetyl precursors, the cooling effect failed to reverse the flavor loss in the culture. As seen in Figure 4, cooling of Cultures E and G halfway in the destructive phase resulted in the immediate stabilization of the flavor, in addition to an increase in the diacetyl concentration beyond the maximum levels attained in uncooled control cultures.

The initial increase and the stabilization at the peak level of diacetyl in Cottage cheese dressed with cream ripened with *S. diacetilactis* 18-16, when held at 5 and 0 C, respectively, indicated that the increase in the diacetyl level at low temperatures was due to the chemical or enzymatic conversion of diacetyl precursors to diacetyl. The combined use of cultured cream dressing ripened with *S. diacetilactis* 18-16 and low temperature not only enhanced and stabilized the desirable diacetyl flavor in Cottage cheese (Figure 5) but, as reported earlier (15)

also prevented the growth of spoilage bacteria and enterotoxigenic pathogens. Regarding the cooling effect, it is noteworthy that Prill and Hammer (13) found that butter cultures ripened 16 hours at 21 C, then fortified with citric acid (0.15%) and chilled by packing in ice produced more diacetyl than control cultures held continuously at 21 C.

The combined use of hydrogen peroxide-catalase treatment and cooling afforded even greater enhancement and stabilization of diacetyl as compared to hydrogen peroxide–catalase treatment alone. In view of this, the combined use of cooling and peroxide–catalase treatment could offer a suitable method of maintaining full flavor in cultured dairy products without upsetting the operational schedules in a dairy plant. These methods also could be used to advantage in preparing cultures to be used for manufacture of starter culture distillate, now widely used as a flavor additive in margarine and other foods.

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J. Dairy Sci. 47(9): 981-986, (1964)

OWADES AND JAKOVAC METHOD FOR DIACETYL DETERMINATION IN MIXED-STRAIN STARTERS[1]

(1511)

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194

SUMMARY

A method recently described for the determination of diacetyl in beer was applied to single- and mixed-strain lactic streptococcus starter cultures of *Streptococcus lactis, Streptococcus cremoris,* and *Streptococcus diacetilactis.* Twelve samples were assayed simultaneously, using a gassing manifold. Twenty-milliliter samples in a 65 C water bath were purged with nitrogen gas and the emerging diacetyl trapped as dimethylglyoxime in buffered hydroxylamine and converted to a colored complex by reaction with ferrous sulfate. Advantages of the method over two other procedures with which it was compared include greater accuracy and sensitivity and use of simple, inexpensive equipment. Chief advantage is that the procedure allows a number of samples to be tested at the same time, which allows accurate comparative data on diacetyl production by lactic streptococcus starter cultures to be obtained.

Diacetyl has long been known as a major contributor to desirable flavor and aroma in mixed-strain lactic streptococcus starter cultures and certain fermented dairy products. As a consequence, a number of methods for quantitative determination of this compound in dairy products have been reported (1-3, 6, 10, 11); the procedure used most widely at the present time is the steam distillation technique of Pien et al. (10), as modified by Elliker (3) to use a spectrophotometer.

During some recent studies employing the enzyme diacetyl reductase to remove diacetyl from beer and citrus juices (report in preparation), it was necessary to review the literature for methods of diacetyl assay in beer.

Received for publication January 24, 1964.

[1] Published as technical paper no. 1762, Oregon Agricultural Experiment Station. Supported by a grant from the American Dairy Association.

Several procedures were found (4, 5, 9, 13), including a new technique described recently by Owades and Jakovac (7, 8). This latter technique had several advantages over steam distillation procedures, so an attempt was made to apply it to mixed-strain starter cultures. Results of our findings are presented in this paper.

EXPERIMENTAL PROCEDURES

Cultures. Single-strain organisms were available from the stock culture collection in the Department of Microbiology, Oregon State University. These were *S. lactis* strains E and C2, *S. cremoris* strains 1 and C3, and *S. diacetilactis* strains 26-2 and 18-16. Mixed-strain starter cultures referred to in Table 4 as E, F, G, and H were obtained from commercial sources. All cultures were maintained in 10 ml of sterile (121 C for 12 min) inhibitor-free

TABLE 1

Duplicate absorbancy readings at 530 mμ of different amounts of diacetyl collected from water system and milk system by the Owades and Jakovac method[a]

System	Absorbancy at 530 mμ								% Recovery
	0.8 ppm		1.6 ppm		2.0 ppm		3.2 ppm		
	1	2	1	2	1	2	1	2	
Water	0.20	0.19	0.40	0.39	0.52	0.49	0.79	0.79	95 to 99
Milk	0.19	0.18	0.40	0.37	0.49	0.48	0.78	0.77	92 to 95
Control	0.21	0.20	0.42	0.41	0.53	0.50	0.82	0.81	100

[a] Control readings were obtained by adding color reagents directly (without sweeping by gas) to 1 ml of buffered hydroxylamine containing different amounts of diacetyl.

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Duplicate absorbancy readings at 530 mμ of different amounts of diacetyl collected from water system and milk system by the Owades and Jakovac method[a]

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Milk	0.19	0.18	0.40	0.37	0.49	0.48	0.78	0.77	92 to 95
Control	0.21	0.20	0.42	0.41	0.53	0.50	0.82	0.81	100

[a] Control readings were obtained by adding color reagents directly (without sweeping by gas) to 1 ml of buffered hydroxylamine containing different amounts of diacetyl.

TABLE 2

Effect of different sweeping gases on the amount of diacetyl found in a mixed-strain starter culture using the method of Owades and Jakovac

Gas	Absorbancy at 530 mμ 1	2	3	Average ppm diacetyl
Air	0.64	0.62	0.62	2.7
N_2	0.54	0.53	0.48	2.3
CO_2	0.50	0.48	0.48	2.2

11% nonfat milk by transfer of 1% inoculum every other day and incubation for 18 hr at 21.5 C.

Diacetyl determinations. The Owades and Jakovac method was used as originally described (7, 8), except for a few procedural modifications to insure accuracy. Twelve separate determinations could be run simultaneously through use of a gassing manifold as shown in Figure 1.

The reagents used were the same as those described by Prill and Hammer (11) and Owades and Jakovac (8) and their composition is repeated here for availability:

(1) Buffered hydroxylamine, prepared by mixing K_2HPO_4 (33 g K_2HPO_4 in 100 ml of distilled water), $NH_2OH \cdot HCl$ (11.0 g made to 250 ml with distilled water), sodium acetate (35.0 g made up to 100 ml with distilled water) 2:4:1.

(2) Acetone-phosphate, prepared by dissolving 29.0 g of K_2HPO_4 (38 g of $K_2HPO_4 \cdot 3 H_2O$) in distilled water followed by addition of 40 ml of pure acetone and dilution to 200 ml with distilled water; store in refrigerator.

(3) Alkaline tartrate, prepared by mixing saturated potassium sodium tartrate (100 g/150 ml of distilled water) with concentrated ammonium hydroxide 22:3.

(4) Ferrous sulfate prepared frequently (when slight yellow color begins to appear) by dissolving 5.0 g of $FeSO_4 \cdot 7 H_2O$ in 100 ml of 1% sulfuric acid.

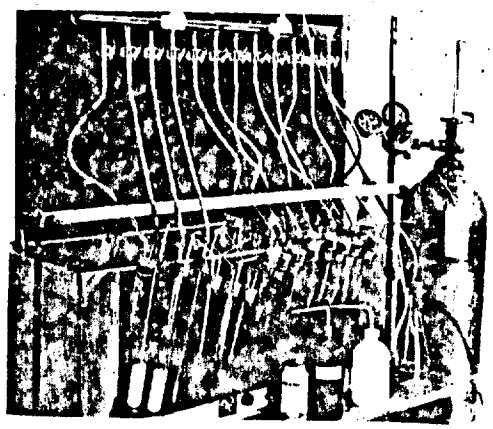

FIG. 1. Apparatus used for diacetyl determinations, showing gassing manifold (top), reaction, and trapping vessels.

Twenty milliliters of culture were placed in the large test tube (25 by 250 mm—Corning No. 9820) shown outside the water bath on the left of Figure 1 (actually immersed in the water during the determination). The test tube was fitted with a two-hole No. 5 soft rubber stopper, one hole occupied by a 1.1 ml milk dilution pipet running to the bottom of the tube. The top of this pipet was connected to the nitrogen supply as shown. The other hole in the No. 5 stopper was fitted with an inverted U-shaped tube which protruded only 1 cm or so below the base of the stopper. This tube led to a two-hole No. 6 stopper mounted in a 50-ml centrifuge tube (Corning No. 8100) which contained a 12-ml centrifuge tube (Kimax No. 54199) cushioned on a small piece of rubber about 0.5 mm square. The U-tube extended through the rubber stopper to the bottom of the inner 12-ml centrifuge tube, which contained 1.0 ml of buffered hydroxylamine. The second hole of the No. 6 stopper was occupied by a short piece of angle tubing, as shown in Figure 1.

Twenty milliliters of the 18-hr culture to be

TABLE 3

Effect of antifoam on absorbancy at 530 mμ of diacetyl collected from different systems and reacted by the Owades and Jakovac method

Analysis system	Absorbancy at 530 mμ: Minus antifoam 1	Minus antifoam 2	Plus antifoam 1	Plus antifoam 2
Water + 2.0 ppm diacetyl	0.45	0.45	0.45	0.45
Sterile milk	0.02	0.02	0.02	0.02
Sterile milk + 2.0 ppm diacetyl	0.48	0.47	0.45	0.45
Mixed-strain starter cultures	1.90	1.90	1.90	1.80
Water + 5.0 ppm AMC	0.005	0.001	.....	.....
Water + 5.0 ppm 2,3-BG	0.005	0.000	.....	.....

TABLE 4

Diacetyl produced (ppm) by different single- and mixed-strain lactic streptococcus starter cultures as determined by the Pien, Baisse, and Martin (PBM), Owades and Jakovac (O and J), and Prill and Hammer (P and H) methods

Cultures	ppm Diacetyl found by 3 methods					
	PBM [a]		O and J		P and H	
	A [b]	B	A	B	A	B
S. lactis E	1.03	0.48	0.00	0.05	0.05	0.08
S. lactis C_2	1.02	1.36	0.08	0.08	0.08	0.05
S. cremoris 1	0.55	0.36	0.03	0.05	0.05	0.05
S. cremoris C_3	0.72	0.72	0.00	0.01	0.05	0.07
S. diacetilactis 26-2	3.28	1.52	1.84	1.76		
S. diacetilactis 18-16	1.68	0.64	0.88	0.88	0.35	0.53
Mixed Culture E	0.72	0.30	1.84	2.36	0.54	0.42
Mixed Culture F	0.60	0.00	0.00	0.00	0.12	0.07
Mixed Culture G	0.36	0.48	1.28	0.96	0.55	0.58
Mixed Culture H	0.96	0.48	1.30	1.32	0.12	0.15

[a] As modified by Elliker (3).
[b] A and B determinations made in duplicate, but on different days.

tested were placed in the test tube and Foamkil (Nutritional Biochemicals Corp., Cleveland, Ohio) added from a pressurized spray can. The reaction unit was assembled and suspended over a wooden rod mounted over the water bath so that the culture-containing tube was immersed in the water (65 C) while the hydroxylamine trap was outside the bath. Gas from the nitrogen tank was allowed to enter the system by successive passage through a 20-liter carboy, a 500-ml suction flask, the manifold, the sample-containing tube, the hydroxylamine trap, and finally out the angle vent tube. A gas flow to produce about five to seven bubbles per second was used, but variation in the rate did not alter results; excessive bubbling was avoided. Owades and Jakovac (8) recommend a gas flow of about 100 to 150 ml per minute.

After gassing for 1.5 hr, the hydroxylamine trap was disconnected (with gas still flowing) and the tip of the inverted U-tube rinsed into the trap with a few drops of 33% K_2HPO_4; a few drops of rinse also were added to the inside of the other end of the U-tube and forced into the hydroxylamine trap by air pressure from an empty squeeze bottle.

The 12-ml centrifuge tubes then were immersed in a 75 C water bath for 10 min, removed and, while still warm, diluted with 0.5 ml of the acetone-phosphate solution and mixed. After cooling, 1.5 ml of alkaline tartrate were added, followed by mixing. Then 0.1 ml of ferrous sulfate was added and mixed immediately. The volume was adjusted to 5.0 ml with 33% K_2HPO_4 and absorbancy of the pink color read at 530 mμ, using a Bausch and Lomb Spectronic 20 colorimeter. The concentration of diacetyl was determined by comparison with a standard curve of dimethyl glyoxime. Blanks used to zero the colorimeter were sterile, uncultured nonfat milk treated exactly as the samples being tested for diacetyl; such blanks gave absorbancy readings of 0.01 to 0.02 when read against water reagent blanks at 530 mμ. Dimethyl glyoxime (K and K Laboratories) was used as the standard rather than diacetyl to avoid frequent distillation of diacetyl; dimethyl glyoxime is the compound actually measured in the colorimeter as a result of a reaction between diacetyl and hydroxylamine. The solution of glyoxime was prepared as described by Prill and Hammer (11), so that each milliliter was equivalent to 100 μg of diacetyl.

For comparison with the Owades and Jakovac method, diacetyl determination on single- and mixed-strain lactic streptococcus cultures were made using the procedure described by Prill and Hammer (11) and also the method described by Pien et al. (10), as modified by Elliker (3). Reagent blank used to zero the colorimeter with these two methods was sterile, uncultured nonfat milk, steam-distilled and otherwise treated exactly as the samples being analyzed. During the Pien method, distillate (50 ml) was collected in a volumetric flask from 20 ml of sample. When distilled water was used to zero the colorimeter, it was found that blanks from sterile milk gave absorbancy readings of about 0.3 at 425 mμ, due to heat-induced production of carbonyl compounds which reacted under acidic conditions with the 3-3′ diaminobenzidine tetrahydrochloride (K and K Laboratories); distilled milk previously heated at 62 C for 30 min gave absorbancy readings of about 0.1 under the same conditions. The Prill and Hammer method was used

as originally described (11), except the instrument was not flushed with CO_2.

RESULTS

Figure 2 shows the spectrum of the diacetyl-colored complex recovered from milk using the Owades and Jakovac method; diacetyl recovered from water showed an identical absorption spectrum. Maximum absorption occurred at 530 mμ and it also may be seen that the control milk gave negligible absorption at this wavelength. The absorption spectrum of the color complex of diacetyl and 3-3′ diaminobenzidine tetrahydrochloride is shown in Figure 3.

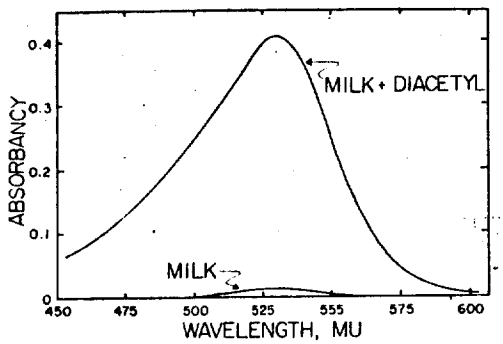

FIG. 2. Absorption spectra (450 to 600 mμ) of diacetyl-hydroxylamine complex obtained from sterile milk with 2.0 ppm added diacetyl (top curve) and from plain milk (lower curve) by the Owades and Jakovac method. Curves were obtained on a Cary No. 11 continuous recording spectrophotometer, using a reagent blank with plotting done on chart paper no. 1100.

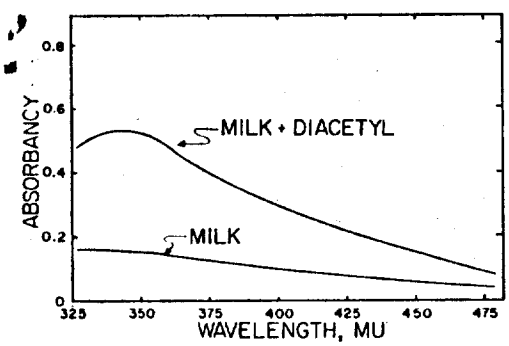

FIG. 3. Absorption spectrum of diacetyl-3-3′ diaminobenzidine · 4 HCl complex obtained from sterile milk with 2.0 ppm added diacetyl (top curve) and from plain milk (lower curve) by the method of Pien et al. Cary spectrophotometer was used as in Figure 2, except employing hydrogen lamp for wavelengths below 375 mμ.

Figure 4 shows a typical standard curve for diacetyl obtained from sterile, nonfat milk by the Owades and Jakovac method. Also shown for comparative purposes is the curve for diacetyl collected from distilled water, as well as the control curve (treated with color reagents directly). Data from which these curves were obtained are presented in Table 1, in order that the amount of variation normally encountered between duplicate tubes may be seen; at least 92% of diacetyl was recovered from sterile nonfat milk.

The effect of using different gases for sweeping diacetyl from a mixed-strain starter is shown in Table 2. The use of air resulted in high values, perhaps due to chemical oxidative decarboxylation of alpha-acetolactic acid to diacetyl. There appeared to be no difference between nitrogen and CO_2 and tanks of the former were used, since they were less expensive.

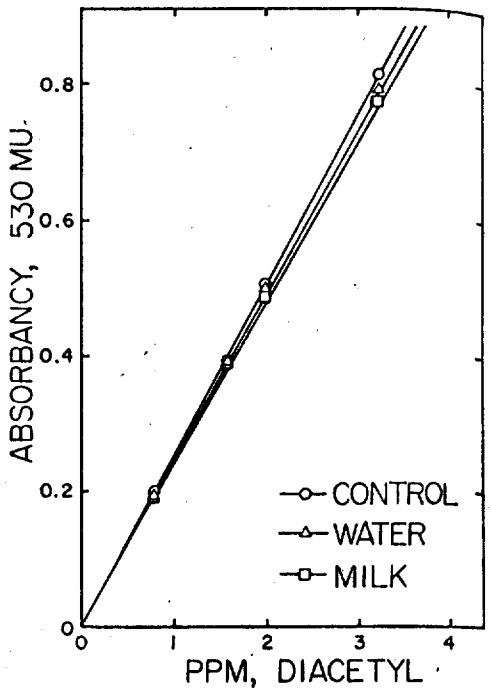

FIG. 4. Absorbancy at 530 mμ of different amounts of diacetyl collected from water (triangles) and from milk (squares) by the Owades and Jakovac method. Control curve (circles) obtained from reading for different amounts of diacetyl added directly to buffered hydroxylamine and reacted with color reagents.

To prevent undue foaming of milk cultures during analysis, it was necessary to use an

antifoam agent (Foamkil; Nutritional Biochemical Corp., Cleveland, Ohio). Evaluation of the effect of this agent on absorbancy readings under different conditions is shown in Table 3. It may be seen that the use of antifoam had no influence on the results. It also can be seen from this table that acetylmethylcarbinol (AMC) and 2,3-butanediol (2,3-BG) give essentially no color reaction with hydroxylamine and ferrous sulfate. Ethyl acetate and ethyl formate also gave no color.

Table 4 shows results comparing the different methods for diacetyl determination on single- and mixed-strain lactic streptococcus starter cultures. From the table it may be seen that, in general, the A and B determinations made on the same cultures but on different days were in better agreement by the Owades and Jakovac and the Prill and Hammer method than by the method of Pien et al. Also, the latter method usually gave higher results than the two other methods; duplicate samples of the same culture tested by this method also were more variable.

DISCUSSION

The advantage of the Owades and Jakovac method as applied to starter cultures in this study lies primarily in the fact that several samples can be analyzed simultaneously for diacetyl. This is a saving in time but, more important, this allows one to compare different cultures for flavor production without having to chill and hold samples until they are distilled. Such chilling practices may lead to inaccurate diacetyl values, due to the destruction of diacetyl by diacetyl reductase (12) while samples are cooling. Also, the apparatus required is simple, inexpensive, and should make it possible for more dairies and culture supply houses to examine the production and stability of flavor and aroma in their single- and mixed-strain lactic streptococcus starter cultures.

Diacetyl analyses using the color formed as a result of the reaction with hydroxylamine to form dimethylglyoxime are more accurate than the determination based on reaction with diaminobenzidine. This may be seen from Figures 2 and 3; the dimethylglyoxime complex (Figure 2) has a fairly sharp absorption maximum at 530 mμ which is essentially absent in milk controls. The diaminobenzidine complex (Figure 3) shows broad absorption in the ultraviolet range, with a maximum around 350 mμ; milk controls, however, also absorb significantly in this region. Workers in the with a UV light source, have used 425 mμ for reading this color absorbancy, and it may be seen from Figure 3 that such a practice would not be very satisfactory. Furthermore, to achieve an absorbancy reading of about 0.72 at 425 mμ, about 200 ppm of diacetyl is required. However, using the dimethylglyoxime end point, only about 3.0 ppm of diacetyl is required (Figure 4) for this absorbancy reading. Thus, the latter end point also provides much greater sensitivity in diacetyl determinations.

These advantages (accuracy and sensitivity) are reflected in Table 4; the Owades and Jakovac and Prill and Hammer methods were generally more reproducible, presumably due to the fact that they each employ the dimethylglyoxime end point. The accuracy of the Owades and Jakovac method as used in the present investigation also may be seen in Tables 1, 2, and 3, where repeated analyses show close agreement.

It is difficult to explain the higher diacetyl values found for the single and mixed cultures using the method of Pien et al. It is possible that carbonyl compounds other than diacetyl which react with diaminobenzidine are produced by some but not all lactic streptococcus organisms. Recent unpublished observations from these laboratories indicate that this may be the case. Also, since only 10 ml of distillate are collected in the Prill and Hammer method, values obtained by this technique may be low. Consequently, a sample high in diacetyl may contribute to a high value in the next sample distilled, due to diacetyl accumulation in condensate in the distillation apparatus. But, since it was not the intent of the present work to study other diacetyl determination methods per se, no attempt was made to verify these possibilities.

Since it is known that *S. lactis* and *S. cremoris* organisms produce little or no diacetyl, it is felt that the results from the Owades and Jakovac method shown in Table 4 are accurate reflections of diacetyl production for the cultures listed. This method, therefore, is now being used routinely in both the laboratories cooperating during this research.

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1511

RES. 25: 764-9, (1960) (1591)

VOLATILE CARBONYL COMPOUNDS OF COOKED CHICKEN. II. COMPOUNDS VOLATILIZED WITH STEAM DURING COOKING

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(Manuscript received October 26, 1959)

Flavor improvement and prevention or postponement of appearance of off flavor in poultry products is of primary importance to the poultry industry. A rational approach to these problems requires a thorough understanding of the flavor characteristics of the products. As a step in this direction, investigations of the fundamental nature of poultry flavor have been under way at this laboratory.

It was recently established *(8)* that a considerable amount and variety of volatile carbonyl compounds may be isolated from chicken cooked in water under atypical oxidation-favoring conditions. This work was extended to include a study of the approximate amount and type of steam-volatile carbonyl compounds which may be isolated from chicken cooked in water under more normal conditions. In the present study, raw cut-up chicken and water were brought to boiling and steam which evolved was condensed and passed into 2,4-dinitrophenylhydrazine reagent. Separation of the mixture of hydrazones obtained into components which permitted unequivocal or tentative identification of many of the carbonyl compounds is described. The significance of these findings to chicken flavor is discussed.

EXPERIMENTAL

Chickens. Hens, culled from laying flocks, were processed to the ready-to-cook point at a commercial processing plant. They were obtained shortly after coming off the processing line, were transported to the laboratory packed in crushed ice, and promptly transferred to individual polyethylene bags, frozen, and held at −30° F until used.

Isolation of steam volatile carbonyl compounds from chicken. Essential features of the apparatus used for isolation of carbonyl compounds as 2,4-dinitrophenylhydrazones are shown in Figure 1. The combination cooking and distillation pot (A) measuring 12 inches in diameter and 13 inches high, was of stainless steel. The lid was modified by providing it with a stainless steel outlet tube and a neoprene gasket that assured a lid-to-pot seal when the lid was held in place with "C" clamps. A stainless steel rack was provided to keep the chicken off the bottom of the kettle to avoid the possibility of burning the chicken by local overheating. The isolation procedure was a batchwise operation in which the cut-up carcasses of five birds (average weight, 5.6 kg) were placed on the rack. Water, 1.07 times the weight of the chicken, was added. The lid was clamped in place and its outlet tube connected to the condenser and traps as shown. The pot was then heated with a gas burner and after gentle boiling started, this state was continued for 4 hr. Steam escaping from the pot was condensed and passed into the 2,4-dinitrophenylhydrazine reagent in trap B. Low boiling carbonyls which might escape this trap were captured by a second trap, C. During the 4 hr cooking, about 4 liters of water distilled. Following a run, contents of the traps were permitted to stand 2 days. Then the hydrazones were filtered off through a fine

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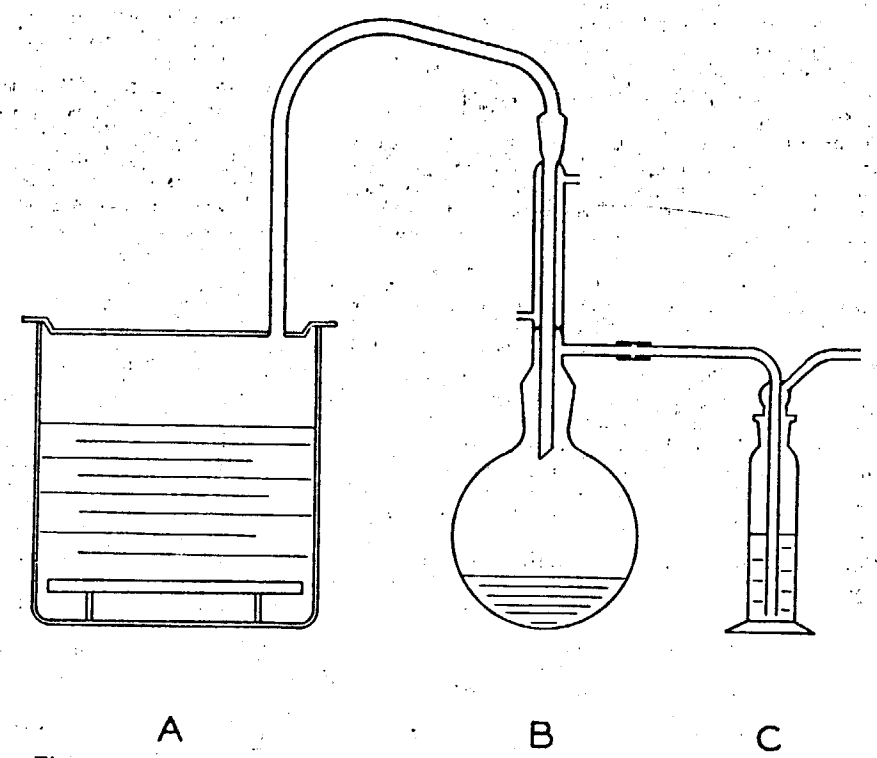

Figure 1. Apparatus used to isolate steam volatile carbonyl compounds from chicken. A—Stainless-steel kettle containing chicken and water heated to boiling. B—5-liter flask containing 500 ml of 2,4-dinitrophenylhydrazine solution (2 g/liter in 2 N HCl). C—Trap containing 200 ml of 2,4-dinitrophenylhydrazine solution (2 g/liter in 2 N HCl).

sintered glass filter. Hydrazones obtained from each trap and from each batch were combined to give a total of 651 mg of 2,4-dinitrophenylhydrazones from 50 birds (56 kg).

Fractionation and identification of 2,4-dinitrophenylhydrazones. A rough preliminary fractionation of the hydrazones was accomplished by taking advantage of solubility differences as previously described *(8)*. The "polycarbonyl" fraction was recrystallized 3 times from nitrobenzene before characterization. The nature of hydrazones, other than the material that crystallized from nitrobenzene, which may have been present in this fraction, was not investigated. The monocarbonyl fraction was divided into approximately 3 equal portions and each was chromatographed on a silicic acid-Celite column as previously described *(8, 9)*. Corresponding preliminary fractions so obtained were combined and rechromatographed on silicic acid-Celite columns until no further separation occurred or the size of the fraction became so small that it was not profitable to continue with this technique. At this point, those fractions which were of sufficient size and of reasonable purity were recrystallized one or more times from ethyl alcohol and characterized by the methods shown in Table 1. Those fractions which were too small to permit unequivocal characterization and which could be further separated by paper chromatography were tentatively identified by their migratory behavior and spectral properties on the paper chromatograms. Methods employed in carrying out these operations have been described elsewhere *(5, 8)*. Authentic n-alkanal, n-alk-2-enal, and 2-alkanone 2,4-dinitrophenylhydrazones required were available from previous studies and are described elsewhere *(5, 8, 9)*. Authentic n-alka-2,4-dienals and the 2,4-dinitrophenylhydrazone derivatives required are those described by Pippen and Nonaka *(10)*.

1591

Estimation of carbonyl in chicken broth. Carbonyl in broth was estimated by modifying the methods of Iddles and Jackson *(1)* and Lohman *(3)*. Hydrazone formation was accomplished by mixing 25 ml of broth and 25 ml of 2,4-dinitrophenylhydrazine reagent (a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl) and permitting this solution to stand 1 to 18 hr at room temperature. To prevent emulsion formation it was necessary to deproteinize before extracting the hydrazones. This was accomplished by adding 10 ml of 10% sodium tungstate solution followed by centrifugation. The supernatant was extracted with hexane in a liquid-liquid extractor for 1 hr. The precipitate, after treatment with 5 g of sodium bicarbonate, was extracted 4 times with hot ethanol (55 ml total). The ethanol extract, after addition of 25 ml of water, was extracted in a liquid-liquid extractor for 1 hr. Hexane extracts were combined, anhydrous sodium sulfate added, and the extracts permitted to stand overnight at room temperature. The extract was filtered, diluted to 200 ml and the absorbancy determined at 340 mμ on a Beckman DU spectrophotometer. A blank was run, with water substituted for broth. From the net absorbancy, the concentration of carbonyl in broth was calculated by using the molar absorption coefficient, 22,500, as determined for authentic n-heptanal-2,4-dinitrophenylhydrazone in hexane. n-Heptanal and cyclohexanone, added to chicken-broth samples at concentrations of 1.3×10^{-6} and 1.5×10^{-6} moles per liter, respectively, were recovered by this method at 79 and 93% levels, respectively. Carbonyl values obtained by the method described here are obviously estimates, because carbonyl compounds in chicken broth are known to include those whose 2,4-dinitrophenylhydrazone derivatives show molar absorption coefficients different from that of n-heptanal-dinitrophenylhydrazone. Also some carbonyl 2,4-dinitrophenylhydrazones, such as the bis derivative of diacetyl and possibly others yet unidentified, have very limited solubility in hexane. This consideration, together with less than theoretical recovery of carbonyl added to broth, means that estimates of carbonyl in chicken broth obtained in this study probably represent minimum values.

RESULTS AND DISCUSSION

In confirmation of previous results [Pippen *et al (8)*], it was found that a 2,4-dinitrophenylhydrazone identical to the bis-2,4-dinitrophenylhydrazone of diacetyl and/or acetoin could be crystallized directly from the "polycarbonyl" fraction. The properties of the recrystallized material are listed at the bottom of Table 1. The relatively large amount of this fraction indicates that diacetyl and/or acetoin occurs as one of the most abundant carbonyl compounds.

TABLE 1

Principal carbonyl compounds isolated

Carbonyl compound identified	Yield of crude fraction, mg	Properties of purified 2,4-dinitrophenylhydrazones		
		Melting point, °C	λ max, mμ	Additional methods of characterization
Acetaldehyde	37	166 –168	355.5 [1]	3, 4, 5
n-Hexanal	125	105 –108.5	357.5 [1]	3, 5
C_{16} and C_{18} aldehydes	16	100 –104.5	357.5 [1]	4, 6
n-Deca-2,4-dienal	32	141.5 –144.5	391 [2]	3, 4, 5
Diacetyl and/or acetoin	191	[4] > 300	392.5 [2]	4, 7

[1] Chloroform solvent.
[2] Benzene solvent. Authentic diacetyl-bis-2,4-dinitrophenylhydrazone in benzene solvent exhibited at a maximum at 392 mμ.
[3] Melting point not depressed by mixing unknown with authentic sample.
[4] The infrared spectrum of the unknown was identical, except for minor differences, with the infrared spectrum of the authentic sample.
[5] The migratory and spectral properties of the unknown on paper chromatography *(5)* were compared to authentic samples.
[6] Paper chromatography *(5)* of this fraction revealed two spots having R_h values of 2.56 and 2.85 and λ max., mμ of 369 and 368 respectively.
[7] The unknown exhibited a blue-violet color in alkaline ethanol.

Table 1 also lists the most abundant monocarbonyl 2,4-dinitrophenylhydrazones which were identified. Carbonyl compounds unequivocally identified include acetaldehyde, n-hexanal, and n-deca-2,4-dienal. The remaining fraction, m.p. 100 to 104.5° C, resisted further efforts to purify it by means of column chromatography and recrystallization. Its rapid movement on column chromatography suggested that it consisted of a derivative of considerable chain length. As indicated in Table 1, the evidence strongly suggests that this fraction consisted of the mixed derivatives of the C_{16} and C_{18} n-alkanals.

It was evident that 2,4-dinitrophenylhydrazones of many carbonyl compounds, in addition to those listed in Table 1, were present to a lesser extent. This was apparent on the one hand by observing that, after no additional hydrazones could be eluted from the column with the petroleum ether-ethyl ether mixture employed, additional bands could be eluted with chloroform. These consisted of only a fraction of a milligram each and gave violet to blue colors in alkaline ethanol, indicating that they were derivatives of dicarbonyl compounds in which the carbonyl functions are on adjacent carbon atoms *(4)*. Since methods for separating homologues of these compounds have not been developed and authentic samples were not available, these fractions were not investigated further.

On the other hand, it was found that some portions of the mono-carbonyl-2,4-dinitrophenylhydrazone fraction could be profitably investigated by a paper chromatography method previously described *(5)*. This method provides both migratory and spectral data, thus enhancing the probability of assigning the correct identification to a component separated on the paper chromatogram as compared to dependence on migratory data alone. However, it is recognized that identities so assigned are not necessarily unequivocal. Table 2 lists migratory and spectral properties and tentative identity of 2,4-dinitrophenylhydrazones separated by paper chromatography from the mono-

TABLE 2

Tentative identity of carbonyl compounds which occurred in minor amounts. Identity is based on the mobility (R_h) of the 2,4-dinitrophenylhydrazone on paper chromatograms and determination of spectral properties of hydrazones directly on the developed chromatogram

R_h [1]	λ max, mμ	Carbonyl compound indicated
0.56	369	n-Butanal
0.68	370	n-Pentanal
1.00	369	n-Heptanal
1.21	370	n-Octanal
0.57	374	Acetone
1.03	373	2-Pentanone
1.40	374	2-Heptanone
0.33	390	2-Butenal
0.62	388	2-Hexenal
0.74	389	2-Heptenal
0.89	389	2-Octenal
1.01	389	2-Nonenal
1.21	389	2-Decenal
0.46	401	2,4 Heptadienal

[1] R_h equals distance moved by n-heptanal-2,4-dinitrophenylhydrazone/distance moved by unknown 2,4-dinitrophenylhydrazone.

1591

carbonyl 2,4-dinitrophenylhydrazone fraction. It may be seen that these include representatives from the n-alkanals, 2-alkenals, 2,4-alkadienals, and 2-alkanones.

It is of interest to compare total carbonyl in chicken broth to the amount recovered in the steam distillate. Analysis of 4 different chicken-broth samples showed carbonyl concentrations ranging from 9.4 to 16.8 x 10^{-5} moles of carbonyl per liter, the average being 14 x 10^{-5}. The total amount of broth prepared, as a minimum estimate, would be 56 liters. Hence, the total amount of carbonyl present would be 784 x 10^{-5} moles, which, in terms of acetaldehyde 2,4-dinitrophenylhydrazone would amount to 1.75 g. Since only 651 mg of 2,4-dinitrophenylhydrazones were isolated in the steam distillate, it is apparent that considerably less than half of the total carbonyl in the broth was recovered in the distillate.

These results establish for the first time that volatile carbonyl compounds can be isolated from fresh, good-quality chicken, cooked in water for a 4-hr period. Qualitatively, carbonyl compounds identified show similarity to those found in a previous study *(8)* in which diacetyl and/or acetoin was found to comprise the major portion of the polycarbonyl fraction, and n-hexanal, acetaldehyde, and a C_{10} to C_{12} 2,4-dienal were major components of the monocarbonyl fraction along with lesser amounts of other aldehydes and ketones. The most striking difference between results reported here and those previously obtained *(8)* is the appearance of the longer chain aldehydes. Their appearance in the present study is attributable to their volatility with steam, whereas in the air entrainment method of isolation *(8)* they were apparently not sufficiently volatile to escape from the chicken-water mixture. Although the long chain derivatives isolated show properties which are indistinguishable from authentic C_{16} and C_{18} n-alkanal 2,4-dinitrophenylhydrazones, the possibility remains that they may contain unsaturation in one or more positions not conjugated with the carbonyl function. This possibility must be kept in mind until authentic samples and methods become available for establishing the presence or absence of isolated double bonds in the chain of the carbonyl moiety of a 2,4-dinitrophenylhydrazone.

It is clear from these results that fresh, good-quality chicken gives rise to a complex volatile carbonyl fraction when cooked in water. The obvious implication is that these carbonyl compounds contribute to flavor. Much remains to be determined before it can be concluded in detail what contribution they make to flavor. Impossible to evaluate at present is the possible flavor significance of secondary products which could arise through polymerization, oxidation, browning reactions, etc., involving the reactive carbonyl compounds.

However, it is possible to make some general observations concerning the flavor significance of these compounds. Authentic samples of all carbonyl compounds identified in this study were obtained or synthesized. None of these had flavor characteristics like that of cooked chicken. This observation, and the complex nature of the carbonyl fraction revealed by this study, suggests that typical chicken flavor consists of a blend of a broad spectrum of substances. No attempt was made to evaluate the flavor characteristics of a blend of carbonyl compounds identified in this study because the qualitative and quantitative picture is still incomplete. It is of interest to note that the concentration of total carbonyl reported above in chicken broth exceeds considerably the concentration reported *(2, 6, 7, 11)* as the threshold level for similar types of compounds in various media. Consequently, it seems plausible that carbonyl compounds in chicken broth make a significant contribution, collectively, and possibly individually, to flavor. Of particular interest is the recent report *(6)* that n-deca-2,4-dienal, one of the principal monocarbonyl compounds identified in this study, has unusually potent and significant flavor characteristics.

SUMMARY

Carbonyl compounds comprising representatives of the n-alkanals, 2-alkenals, 2,4-alkadienals, 2-alkanones, and 1,2-dicarbonyl compounds were identified in the steam distillate of fresh, good quality chicken cooked in water for four hours. The distillate from 56 kg of chicken yielded 651 mg of 2,4-dinitrophenylhydrazones. Principal carbonyl compounds unequivocally identified include diacetyl and/or acetoin, acetaldehyde, n-hexanal, and n-deca-2,4-dienal. The tentative identity of 16 other carbonyl compounds, occurring in lesser amount, was established. Evidence was obtained indicating the presence of additional carbonyl compounds which were not identified. None of the authentic carbonyl compounds identified in this study had flavor characteristics resembling the complete flavor spectrum of cooked chicken. However, the concentration of total carbonyl found in chicken broth is sufficient to make it plausible that one or more carbonyl compounds contribute significantly to the over-all flavor.

Acknowledgment

The authors thank Glen F. Bailey for assistance in determining and interpreting the spectral data required for this study.

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1591

THE INFLUENCE OF OXYGEN ON THE PRODUCTION OF DIACETYL DURING FERMENTATION AND CONDITIONING

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Received 24th January, 1966
J. Inst. Brew. 72(5) 458-61, (1966)

Although varying concentrations of oxygen in wort at pitching do not affect the uction of diacetyl, its availability during fermentation has a significant effect. enhanced production of diacetyl resulting from access of oxygen during fermen- n is caused by its effect in increasing yeast growth and in altering the balance of o acids relative to sugars and not by a direct oxidation of acetoin. Oxygen has fect upon the concentration of diacetyl during storage and conditioning, pro- d that the beer is free from contamination.

Introduction and Discussion

influence of oxygen as an important r affecting the concentration of diacetyl r has been suspected for several years. 57, Burger *et al.*[2] noted that the produc- of diacetyl was greatest in fermenting s situated in positions exposed to hts and suggested that the enhanced ction of diacetyl might be due to the sed availability of oxygen. Denschikov also observed that an increase in the ction of diacetyl occurred when oxygen reely available.

previous paper on factors affecting the ction of diacetyl[10] was concerned ly with its production during fermenta- However, the possibility existed that, ll as affecting the production of diacetyl g fermentation, oxygen also affected of diacetyl during storage and con- ing. Accordingly, the influence of n has been studied in three circum- es.

Influence of varying concentrations of n in the wort at pitching.—An investiga- n which streams of air or carbon dioxide passed through duplicate samples of ame wort until they were saturated d that initial concentrations of oxygen no effect on the production of diacetyl g fermentation (Table I).

Availability of oxygen during fermenta- —Duplicate fermentations in which slow ms of either air or carbon dioxide were d through the fermenting worts pro- l widely differing concentrations of diacetyl. Table II shows that the fermenta- tion through which air was passed produced a concentration of diacetyl 5–6 times as much as that produced when carbon dioxide was used.

TABLE I

Influence of Initial Concentrations of Oxygen on the Production of Diacetyl during Fermentation

Initial treatment of wort		Diacetyl in beer (p.p.m.)
Saturated with air	A	0·20
	B	0·14
Saturated with CO_2	A	0·19
	B	0·18

TABLE II

Effect of Oxygen on the Production of Diacetyl during Fermentation

Gas passed through fermenting wort	Diacetyl in beer (p.p.m.)	Yeast in beer (g., wet weight)
Air	2·04	20·2
CO_2	0·36	4·4

It can be seen that the increased growth of yeast in the aerated fermentation is comparable with the increased concentration of diacetyl.

In further trials, streams of carbon dioxide or of air were allowed to pass through the gas

spaces above the fermentations but not through the liquids. Table III shows that the fermentation which had air passed through the head space produced twice as much diacetyl as that under carbon dioxide and the increased yeast growth in the aerated fermentation was again comparable with the increased production of diacetyl.

TABLE III

Effect of Air in Headspace on the Production of Diacetyl during Fermentation

Gas passed through headspace	Diacetyl in beer (p.p.m.)	Yeast in beer (g., wet weight)
Air	0·93	8·1
CO_2	0·52	4·6

These conditions are obviously like those pertaining in open and covered fermenting vessels used in commercial breweries and the results suggest that the concentration of diacetyl produced in commercial fermentations could be reduced by covering the fermenting vessels.

Thus, it can be seen that the availability of oxygen during fermentation has a significant effect on the concentration of diacetyl produced. It has been suggested by Schultze & Antelmann[11] that the influence of oxygen in enhancing the production of diacetyl is due to the direct oxidation of acetoin, although Brenner *et al.*[1] observed no relationship between changes in the concentrations of these two substances.

However, estimations of acetoin have relied upon its oxidation by ferric chloride and measurement of the diacetyl thus produced. A conventional distillation technique was used and the concentration of acetoin was computed from the difference between the concentrations of diacetyl measured before and after oxidation. Recent work using gas chromatography[5] has shown that methods of estimating diacetyl which include a distillation procedure are invalid when applied to samples of fermenting wort and this circumstance obviously weakens the reliability of the derived estimations of acetoin.

In order to examine the possibility that acetoin is oxidized directly to diacetyl, 20 p.p.m. of acetoin were added to fermenting wort and a current of air was passed over the surface for 4 days. Analysis of the beer at the end of this time showed that the sample to which the acetoin had been added contained no more diacetyl than the untreated control (Table IV). These concentrations of diacetyl were measured by gas chromatography in order to eliminate artefacts which might have arisen from the distillation of unchanged acetoin.

TABLE IV

Effects of Additions of Acetoin on the Concentration of Diacetyl Produced

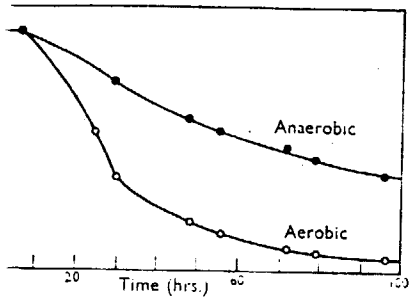
	Diacetyl (p.p.m.)
Beer untreated	0·18
Beer + 20 p.p.m. acetoin	0·19
Water + 50 p.p.m. acetoin (oxygenated)	0·11
Water + 50 p.p.m. acetoin (carbonated)	0·13

A further trial showed that the passage of a fast stream of pure oxygen through an aqueous solution of acetoin failed to convert it to diacetyl. It is therefore clear that the enhanced production of diacetyl during fermentation is not due to a direct oxidation of acetoin.

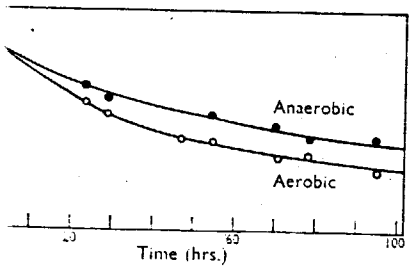
Recent work[3] has also shown that, when fermenting under aerobic conditions, yeasts which have little tendency to produce diacetyl may nevertheless produce high concentrations of acetoin. It would thus seem that the inter-relationship of diacetyl and acetoin is less direct than has previously been supposed.

Previous work[10] showed that increased concentrations of yeast result in enhanced levels of diacetyl and it is known that oxygenation during fermentation enhances yeast growth. Additional studies were therefore designed to clarify the relationship between oxygenation, yeast growth and the production of diacetyl. It has been shown[10] that the ratio of amino acids to fermentable sugars in wort has a major influence on the concentration of diacetyl produced during fermentation and that any decrease in the concentration of amino acids or increase in the concentration of sugars enhances s production.

Figs. 1 and 2 show that the availabilit oxygen during fermentation has a signific



-Utilization of amino acids during aerobic anaerobic fermentations.
ffects of passing air (○—○) or carbon ide (●—●) over the surface of fermenting t on the rate of uptake of amino acids.



-Utilization of sugars during aerobic and robic fermentations.
ffects of passing air (○—○) or carbon ide (●—●) over the surface of fermenting on the rate of uptake of sugars.

pon this balance. The sugar con-on falls slightly more rapidly in fermentation than under anaerobic ns. This decrease is entirely ac- for by the increase in yeast growth minimized by the more efficient on of sugars in aerobic respiration. r, in agreement with previous work,[12] centration of amino acids was found rapidly under aerobic conditions and so in anaerobic fermentations. Again is a result of the utilization of amino y the rapidly multiplying yeast. iently the valine originally present in t is exhausted more rapidly under conditions and it has been shown is effect leads to the premature ion of diacetyl and to an increase in l level.[10]

other trial a solution of valine was continuously into an aerated fer-on at a rate calculated to be slightly in excess of its maximum rate of utilization by the yeast. The production of diacetyl was almost entirely suppressed although it was formed in normal concentration in the control fermentation (Table V).

TABLE V

SUPPRESSION OF THE FORMATION OF DIACETYL BY ADDITION OF VALINE

Treatment	Diacetyl in beer (p.p.m.)	Yeast in beer (g., wet weight)
Valine solution added ..	<0·05	8·3
Water added ..	0·27	7·9

An examination of the amino acids present in the beer at the end of these fermentations showed that the addition of valine partly suppressed the uptake of a number of amino acids (Table VI), all of which fall into the Group C classification of Jones & Pierce.[6]

TABLE VI

EFFECT OF ADDITION OF VALINE ON THE UPTAKE OF AMINO ACIDS DURING FERMENTATION

Amino acid	Concentration in beer (μmoles/ml.)	
	Control	With added valine
Aspartic	Trace	Trace
Threonine ..	Trace	Trace
Serine and amides	Trace	Trace
Glutamic	Trace	0·04
Proline	0·78	1·05
Glycine	Trace	0·13
Alanine	Trace	0·13
Valine	Trace	1·67
Methionine ..	nil	nil
Isoleucine ..	Trace	Trace
Leucine	Trace	Trace
Tyrosine	Trace	0·17
Phenylalanine ..	Trace	0·12
Tryptophan ..	Trace	0·15
Lysine	Trace	0·04
Histidine	Trace	0·03
Ammonia ..	Trace	0·19
Arginine	nil	0·05

A large initial concentration of valine added to one of a pair of aerated fermentations produced a similar result.

It is concluded that oxygen is a secondary factor influencing the production of diacetyl during fermentation and that its influence

is not due to a direct oxidation of acetoin but that it affects the metabolism of the yeast, as is reflected by a change in the relative rates of utilization of sugars and amino acids.

(3) *Availability of oxygen during storage and conditioning.*—Table VII shows that beer which was fermented using pure culture yeast and subsequently transferred to sterile storage vessels has a constant concentration of diacetyl regardless of the availability of oxygen.

TABLE VII

CONCENTRATION OF DIACETYL DURING STORAGE

Treatment	Diacetyl (p.p.m.) after:		
	2 days	4 days	6 days
Air passed through headspace	0·68	0·70	0·71
CO_2 passed through headspace	0·72	0·67	0·70

Thus it would appear that increases in the concentration of diacetyl which occur during storage or conditioning may result from the continued presence of living organisms and that the availability of air does not promote the production of diacetyl in uncontaminated beer.

EXPERIMENTAL

All worts were brewed using Proctor malt and were adjusted to a gravity of 1040; they contained 0·7 μmoles per ml. valine (±0·05 μmoles per ml.). Fermentations were carried out using pure culture yeasts (N.C.Y.C. Nos. 1026 and 1200) in one-litre flasks in sterile conditions. Gas passed through or over the fermenting wort was directed through a microbiological filter to ensure sterility.

Estimations of diacetyl were by the method of Brenner *et al.*[1,9] (Tables I and II) or by gas chromatography of the head space gas using a gas chromatograph fitted with an electron capture detector (Wilkens Co. Ltd.).

Estimations of amino acids[7] and of sugars[8] were performed using a Technicon Auto-analyser.

Acknowledgement.—The author wishes to thank Dr. A. H. Cook, F.R.S., for his advice and interest.

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1614

SOME FACTORS AFFECTING THE CONCENTRATION OF DIACETYL IN BEER

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Received 2nd August, 1965
J. Inst. Brewing 72(2) 193-6 (1966)

Investigations of factors affecting the production of diacetyl during fermentation have shown large differences in the abilities of different strains of brewer's yeast to produce diacetyl. Studies on pilot scale and on small laboratory fermentations have established a connection between the concentration of amino acids in the wort and that of diacetyl in the fully fermented beer. Control of the ratio of amino acids to sugars in wort is thus an important factor in maintaining concentrations of diacetyl in beer at an acceptable level. The effects of increased pitching rate and of high-temperature fermentation in enhancing the production of diacetyl are demonstrated.

Introduction and Discussion

The problem of diacetyl in beer has of late become more acute. It seems that the recent trend to lighter beers has resulted in levels of diacetyl which were previously not prominent becoming more discernible and less acceptable. Moreover, the introduction of accelerated systems of fermentation has resulted in enhanced levels of diacetyl.

Recent work by Harrison *et al.*, who used a gas chromatograph fitted with an electron capture detector[3] has shown that measurements of the concentration of diacetyl during fermentation are inaccurate, when the procedure involves distillation of fermenting wort, owing to the formation of considerable quantities of diacetyl during boiling. Further investigations[5] have shown that the large peak in diacetyl concentration reported[1,2] as occurring between 18 and 48 hr. from the commencement of fermentation is greatly exaggerated. This discovery offers an explanation for the frequently reported failure to detect diacetyl by taste or smell in fermenting worts. Further work shows that there is little discrepancy between results by the two methods when beer is analysed for diacetyl; moreover, there is good agreement between gas chromatographic and distillation results after a period of approximately 4 days from the commencement of fermentation.

The important tendency of different yeast strains to produce differing amounts of diacetyl from the same wort and under identical conditions was clearly demonstrated during a series of laboratory fermentations in which a standard all-malt wort was fermented by a number of commercial strains of brewing yeasts (Table I). The results suggest that other factors affecting the production of diacetyl are of secondary importance to the choice of yeast strain selected for the fermentation.

TABLE I

Diacetyl Concentration Produced from the Same Wort by Different Strains of Brewery Yeasts

Yeast N.C.Y.C. No.	Concentration of diacetyl in beer (p.p.m.)
1005	0·54
1026	0·42
1046	1·07
1057	1·51
1068	0·76
1082	0·44

In 1959 Owades *et al*[7] suggested a possible pathway for diacetyl formation as a by-product in the synthesis of valine and showed that the addition of valine to wort resulted in a reduction of terminal levels of diacetyl. This effect has been re-examined and it has been found that the reduction in final levels of diacetyl due to the addition of valine stems from a delay in the onset of its

TABLE II

The Effect of Valine on the Production of Diacetyl

Valine concentration in wort (μmoles per ml.)	Time of commencement of diacetyl production (hr. from pitching)	Concentration of diacetyl in beer (p.p.m.)
0·7	6	0·62
1·4	17	0·25
2·1	25	0·07
2·8	31	0·02

formation during fermentation (Table II). Addition of several other individual amino acids, including leucine and isoleucine, was found to have no effect on the production of diacetyl.

Subsequently fermentations of worts produced from different grists were examined to investigate the relationship between the amounts of valine normally present in boiled worts and the concentrations of diacetyl produced during their fermentation. Table III shows the difference in levels of diacetyl produced by fermentation of worts prepared entirely from pale and coloured malts. Wort from coloured malt gave rise, on fermentation, to significantly more diacetyl than did that from pale malt and it is noteworthy that the coloured malt gave rise to wort with a valine content which was considerably lower both in absolute amount and relative to the total amino acids present.

Addition of glucose to worts prior to fermentation (thus increasing their sugar content in relation to their amino acid content) also has the effect of raising concentrations of diacetyl in the beer (Table IV). Thus it may be stated that the ratio of amino acids, specifically valine, to fermentable sugars in wort is an important factor in influencing the level of diacetyl in the beer. It was concluded from this that the use of pale malt and small proportions of adjuncts is likely to result in lower concentrations of diacetyl than the use of coloured malts and high proportions of adjuncts, which were shown by Jones & Pierce[4] to give rise to worts with reduced amino acid concentrations.

Fermentation of worts brewed from mixtures of malt with adjuncts which yield little nitrogen, by a yeast possessing considerable propensity to form diacetyl, showed that the reduction in concentration of amino acids in wort due to the use of adjuncts results in enhanced levels of diacetyl (Table IV).

TABLE III

Diacetyl Produced on Fermentation of Pale and Coloured Malt Worts

Type of wort	Gravity	Tint (E.B.C.)	Total amino acid (μmoles per ml.)	Valine (μmoles per ml.)	Diacetyl (p.p.m.) final level
Coloured malt wort ..	1040	8·5	9·48	0·37	1·04
Pale malt wort ..	1040	2·5	12·60	0·70	0·77

TABLE IV

Effect of Adjuncts on Diacetyl Formation

Grist mixture	Original gravity	Concentration of valine in wort (μmoles per ml.)	Concentration of Diacetyl in beer (p.p.m.)
100% pale malt (control) ..	1040	—	0·28
95% malt + 5% glucose ..	1040	—	0·40
90% malt + 10% glucose ..	1040	—	0·49
100% pale malt (control) ..	1040	0·71	0·30
75% malt + 25% wheat flour	1040	0·55	0·48
50% malt + 50% wheat flour	1040	0·34	0·55

The adjunct used in this experiment was wheat flour which contained a very low concentration of amino acids. The enhancement in the production of diacetyl can be compensated by the use of wheat flour which has a relatively high nitrogen content and which results in a wort amino nitrogen concentration comparable to that of a wort brewed entirely from malt.

Further studies show that other adjuncts which give rise to low concentrations of amino nitrogen have similar effects which may be off-set by the use of a malt containing a high nitrogen concentration, thus maintaining a balanced concentration of amino acids in the wort.

Yeast concentration has been shown to have a considerable effect on the production of diacetyl in that high concentrations of yeast result in relatively high levels of diacetyl in beer. (Table V). This effect was

TABLE V

EFFECT OF PITCHING RATE ON DIACETYL LEVELS

Pitching rate (lb. per brl.)	Concentration of diacetyl (p.p.m.)
1	0·27
2	0·40
4	0·48
8	0·65

observed in both laboratory and pilot scale fermentations. In the latter case a fermentation pitched at four times the normal rate produced a beer containing 0·42 p.p.m. diacetyl compared with 0·18 p.p.m. in the control beer. It is of further interest to note that these beers were obtained using a yeast which normally produces very little diacetyl.

The effect of temperature on diacetyl production may be seen in Table VI.

TABLE VI

Temperature of wort at pitching (° F.)	Concentration of diacetyl in beer (p.p.m.)
55	0·26
60	0·29
65	0·42
70	0·51
75	0·66

Increases in temperature at pitching resulted in a more rapid fermentation accompanied by enhanced production of diacetyl.

The observed effects of increases in pitching rate and of increases in temperature are identical to those caused by reduced concentrations of amino acids. It is reasonable to assume that both increased concentrations of yeast and the increase in metabolic rate at higher temperatures result in a more rapid uptake of amino acids and thus parallel the effect of low concentrations of amino acids. Thus it is possible that control of the level of amino acids in wort might be adjusted to compensate for these factors.

From this evidence it appears that control of diacetyl is most easily achieved by the selection of a strain of yeast known to have little tendency to produce it. Where existing yeasts which are known to produce diacetyl freely are favoured, control of the balance between the amino acids and fermentable sugars in the wort can be of help in maintaining concentrations of diacetyl at acceptable levels. Similar control might well be employed where accelerated systems of fermentation are in use.

EXPERIMENTAL

All fermentations were carried out using pure culture yeasts (N.C.Y.C. Nos. 1026 and 1200) except where otherwise stated. Apart from the use of special grists, all worts were brewed using Pale Proctor Malt; they were adjusted to a gravity of 1040 and contained 0·7 μmoles of valine per ml.

Estimations of diacetyl were effected using a modification of the technique of Brenner *et al.*[1,3] or, in the case of the yeast concentration trials, by the non-specific method of Owades *et al.*[6] modified to include distillation using a Markham still.

Amino acid estimations and checks on the purity of added valine were by the method of Spackman *et al.*[9]

Batch fermentations were carried out in twin-necked Woolfe bottles under sterile conditions. The bottles were ventilated through a microbiological filter and samples were withdrawn through a silicone rubber septum by means of a hypodermic syringe. The fermentations used to test the effect of temperature on the production of diacetyl were carried out on the laboratory scale using

glass fermenting vessels lagged with thick layers of cloth in order to minimize the effect of air temperature.

Acknowledgement.—The author wishes to thank Dr. A. H. Cook, F.R.S., for his advice and encouragement.

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1615

1618

Intern. Dairy Congr. Proc. 17th 3:49-6 (1966)

Microbiological and Biochemical Processes in the Sour Cream Butter Produced by the Separation Method

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The object of the present paper is to study microbiological and biochemical processes as well as the methods of retaining aroma substances in sour cream butter in the course of long time storage in conditions of plus and minus temperatures.

Experimental batches of sour cream butter with fourfold reproduction were manufactured by the separation method in the pasture period. The butter was manufactured from cream with a fat content of 28—37 % and acidity of 12—15°T. The initial cream was pasteurised at 87—90°C in a tubular pasteurizer with automatic control of the pasteurisation temperature. Pasteurized cream was pumped into separators to obtain high fat cream. The high fat cream, with moisture content of 13.7—13.8 %, was cooled to 45°C and 2 % of the pure culture starter and citric acid in quantities of 180 g per 1 ton of butter were added. The composition of the starter was Str. lactis (strains 217, 810). Str. cremoris (strains 466, 913), Str. diacetilactis (strain "Apple"), the latter producing large quantities of diacetyl. In order to prepare the starter the whole milk was pasteurized in an autoclave by means of a steam flow for 20—30 min. was cooled down to 28—30°C and then 5 % of the mixed cultures was introduced. The ratio of acid forming and aroma forming cultures was 1 : 1. The milk with the cultures introduced was thoroughly stirred and incubated at 25 C for 16—17 hrs. After incubation the starter was cooled to 5—7°C in a refrigerator.

The starter, ready for the manufacturing of butter, had the following characteristics: its acidity was 96°T, the volatile acids content was 1.6 ml 0.1 N NaOH, the diacetyl content was 0.411 mg%. The total count of lactic acid bacteria was 1625 mil/ml, the aroma forming strepfococci comprising 732.5 mil/ml, a ratio of aroma forming to acid forming of 1 : 2. Microscope examination showed actively developed cocci, diplococci and short chains. The starter had a pure lactic acid flavour and a pleasant aroma.

After fermentation and thorough agitation the high fat cream was pumped into a plate transmutator with a capacity of 600 kg butter per 1 hr. The outlet temperature of butter was 14°C. The butter was packaged into standard boxes weighing 25.4 kg and stored at 5°C and —12° to —15°C.

Organoleptic evaluation of butter was carried out by a group of experts by the 100 score system, 50 being given for flavour and aroma.

Physical and chemical characteristica of fresh butter are summarized in Table 1.

TABLE 1

Physical and Chemical Properties of Fresh Butter

Physical and chemical properties	*Average*	*Fluctuations*
Moisture content %	15.6	15.2 — 15.8
S. N. F. %	1.62	1.2 — 1.9
Air content %	0.48	0.33 — 0.64
Iron mg/kg	0.92	0.4 — 1.5
Hardness g/cm	46.5	38 — 64
Deformation coefficient	0.9	0.82 — 0.92

A. BUTTER STORED AT O ÷ 5°C

Sour cream butter stored for 5—6 months at 5°C was studied for changes in the microflora and for biochemical and organoleptic changes. The volume and dynamics of microbiological changes are shown in Table 2, Fig. 1.

TABLE 2

Changes in Microflora of Butter During Storage (0 ÷ 5° C)

Decription	*Bacteria content in 1 fresh butter mil. ml.*	*Survival of bacteria in butter during storage as % of that in fresh butter*			
		1 month	*2 months*	*3 months*	*5 months*
Total quantity of bacteria	43.1	97.0	49.2	39.7	7.2
Aroma forming bacteria among them	14.1	56.0	27.8	9.9	5.7
Acidforming bacteria among them	29.0	116.5	59.0	50.7	7.9

It can be clearly seen that in the course of storage at 0 ÷ 5°C microbiological processes in the sour cream butter are slowed down and the amount of microflora diminishes.

In the course of the first month of storage a slight decrease in the total count of microflora is observed (by 1.4 mil/ml) while during the further storage the decrease is more pronounced. After three months of storage only 40 % of the

1618

original microflora (43.1 mil/ml) survived and after 5 months only 7.2 %. Aroma forming bacteria (Str. diacetilactis) died out more rapidly than acid forming bacteria such as Str. lactis and Str. cremoris. In the first month of storage 44 % of aroma forming streptococci died and after three and five months of storage they comprised only 9.9 and 5.7 % respectively of the initial count (14.1 mil/ml).

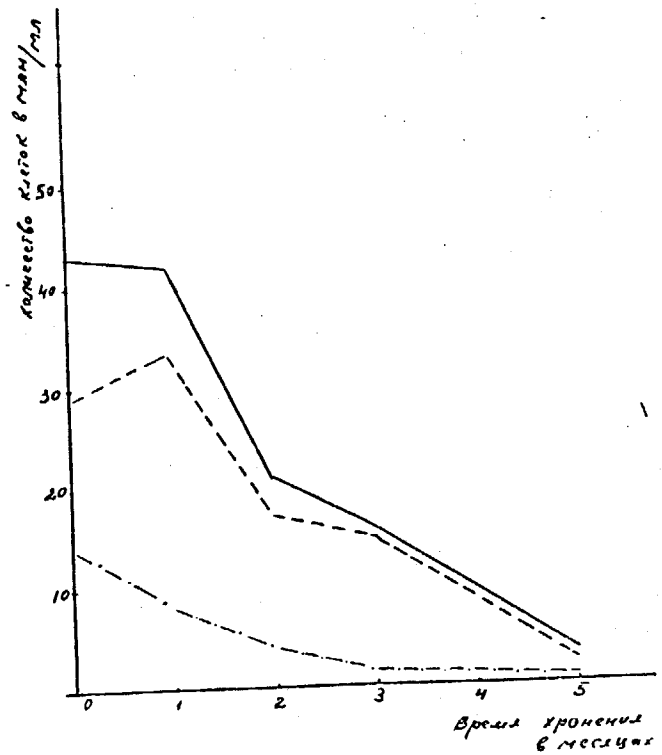

Fig. 1. *A change in microflora content in butter during storage (0, + 5 C).*
1. Period of storage, months. 2. The number of cells.
— — — total count of bacteria
— — — aroma forming bacteria
— — — acid forming bacteria

Acid forming cultures Str. lactis and Str. cremoris grow only in the first month of storage (by 4.8 mil/ml). Their number is gradually reduced after 2—3 months of storage (by 41—49 % of the initial count 29.0 mil/ml). After 5 months of storage the reduction was 92 %.

The different rates of death of the lactic acid microflora affects the ratio of aroma forming and acid forming cultures in the butter during its storage. Thus, in the fresh butter acid forming bacteria dominate the aroma forming streptococci by

2.1 times, after 3 months of storage by 4.3—10.5 times and after 5 months by only 2.9 times.

In spite of the fact that the microflora markedly decreases during storage a high content of viable lactic acid bacteria survive. Thus, after 5 months the total count of lactic acid microflora is 3.1 mil/ml and of aroma forming bacteria is 0.8 mil/ml and after 6 months storage the figs. are 2.86 and 0.12 mil/ml respectively. Viable lactic acid microflora produces a certain amount of lactic acid, diacetyl and the like, which affect the flavour of butter, though its activity at 5 C is considerably inhibited.

Titrable and active acidity in the butter serum increased in the course of 5 months storage at 0 ÷ 5 C, the total increase of titrable acidity in the serum being 9.1 °T. Active acidity increased by 5.9 % as compared with that in the fresh butter (Table 3). This increase in acidity was due to viable lactic microflora at the storage temperature.

TABLE 3

Changes in Acidity in Butter Serum During Storage (0 ÷ 5 C)

Time of analysis	*Acidity of the serum °T*	*pH of serum*
Fresh butter	39.2	5.44
After 3 months	45.3	5.23
After 5 months	48.4	5.09
As % of the fresh butter	123.5	94.1

The fresh butter contained the full complex of aroma forming substances which is clearly seen in Table 4. In the course of storage partial accumulation or destruction of certain aroma substances occurred which was a result of viability of aroma forming bacteria and redox conditions in the butter. In the first period of storage (up to 3 months) accumulation of diacetyl and volatile acids (by 78.1 and 14 % respectively) was observed. This period was characterised by the decrease in ester content (by 55.6 % as compared with that in the fresh butter).

TABLE 4

Changes in Aroma Substances in Butter During Storage (0 ÷ 5 C)

Time of analysis	*Diacetyl mg %*	*Acetoin mg %*	*Volatile acids mg % (in terms of acetic acid)*	*Esters mg %*
Fresh butter	0.283	1.241	3.888	2.605
After 3 months	0.504	1.392	4.433	1.157
After 5 months of storage	0.372	1.195	6.898	2.364
As % of the fresh butter	131.4	96.3	177.4	—

1618

Acetoin content remained practically unchanged. It should be noted, however that further storage (up to 5 months) brought about a decrease of aroma especially ester substances, except volatile acids. After 5 month storage esters were found only in one experimental sample of butter, while the content of diacetyl, acetoin and volatile acids was quite sufficient (0.372 mg%; 1.195 mg% and 6.898 mg% respectively). In the course of the whole storage period reduction reactions lagged behind the oxidation ones as was proved by redox potential measurements which were increased by 45.1% as compared with the fresh butter (Fig. 2). Nevertheless, the redox processes were more intense in the first period of storage. Positive redox potential reached by that time + 353 mv. This figure remained practically unchanged until the end of the storage period. Changes in redox condition of the media affected, in our opinion, the retention of aroma substances

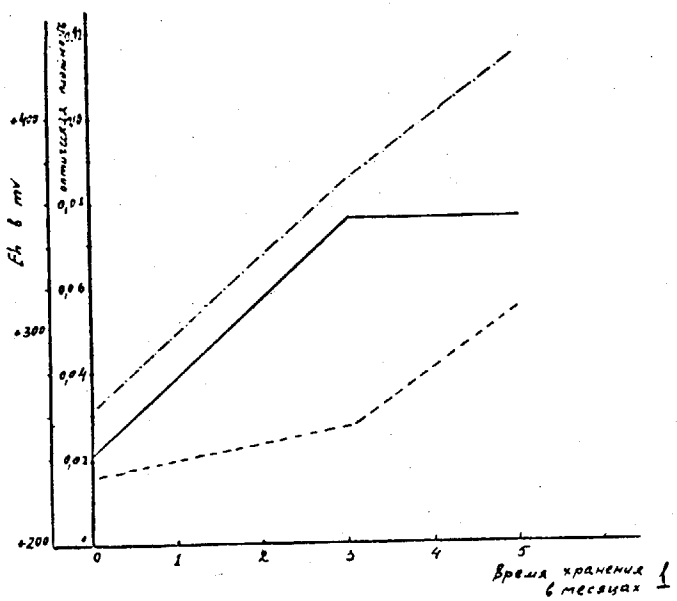

Fig. 2. *Changes in redox potential, oxidation of butter and fat during storage (0, + 5°C).*
1. Period of storage. 2. Optical density:
a — redox potential; b — TBA-test for fat ; c— TBA-test for butter.

and the changes in the butter and butter fat oxidation. The oxidation of butter and butter fat tended to increase in the course of the storage, which was proved by the 2 thiobarbital acid test (TBA). After 5 month storage the TBA-value increased 3.6 times in butter and 3.4 times in butter fat as compared with those in the fresh butter (Fig. 2). Nevertheless, it should be noted that the oxidation of both fresh and stored butter was twice as high as the TBA-value in butterfat.

Resistance to oxidation of butter fat during storage was determined by the induction period. Butter fat was artificially spoiled by ultra-violet irradiation with the help of lamp "SVD-120 A". Induction period for the fresh butter fat was 114 min and for the stored butter it was 90 min after 3 month storage and 86 min after 5 month storage.

Biochemical characteristics of the butter stored 6 months was practically the same as for the butter stored 5 months. Biochemical changes affected the flavour of the butter. Flavour of the butter was slightly worse (by 1.25 points already after 3 months storage. After 5 months storage the butter did not have the desired aroma typical of sour cream butter. Some samples of butter were graded as having slightly unclean flavour. After 6 month storage butter had such faults as "slightly tallowy and oily". The change in organoleptic evalution is given in Fig. 3.

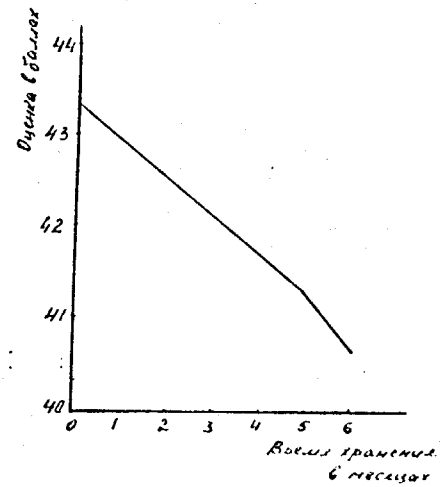

Fig. 3. *A change in flavour evaluation during storage (0, + 5 C).*
1. Period of storage, months. 2. Scores.

B. BUTTER STORED AT MINUS 12—15 C

The nature of changes in microbiological processes in sour cream butter in the course of its storage at the temperatures —12 to —15°— differed but slightly from that stored at 0÷5 C. About 95—96% of lactic acid microflora died after 6—7 months of storage. After 9 month storage its content comprised only 0.6—1.7% of that in the fresh butter. The aroma forming streptococci died out more rapidly than acid forming streptococci so that by the end of 7 months the latter were 2.8 times higher and 6.1 times higher after 9 months. (See Table 5.)

Biochemical changes in butter stored at minus temperatures are practically the same as in the butter stored at 0 ÷ 5° C, the difference being only in the intensity of the biochemical processes. Biochemical changes in the butter stored at —12 to

—15°C were not so intensive as in the butter stored at 0÷5°C. Aroma substances (diacetyl, acetoin, volatile acids and esters) were retained in butter a longer period. Their loss, with the exception of esters, comprised only 13.8—16.7 % after 9 months of storage (see Table 6). A slight increase in titrable acidity in the butter plasma (3.3—1.5 T) and in active acidity (pH - 0.23—0.16) was also observed (see Table 7).

TABLE 5

Changes of Microflora Content in Butter During Storage (—12.0 to —15.0° C)

Description	Number of cells in butter: In fresh butter	After 6—7 months: mil/ml	After 6—7 months: as % to the fresh butter	After 9 months: mil/ml	After 9 months: as % to the fresh butter
Total number of bacteria, mil/ml	43.1	2.1	4.87	0.57	1.3
Aroma forming bacteria, mil/ml	14.1	0.55	3.9	0.08	0.6
As % of the total number	32.7	26.2		14.0	
Acid forming bacteria, mil/ml	29	1.55	5.3	0.49	1.7
As % of the total number	67.3	73.8		86.0	
Ratio of aroma forming bacteria to acid forming bacteria	1 : 2.2	1 : 2.8		1 : 6.1	

TABLE 6

Changes in Aroma Substances in Butter During Storage (—12.0 to —15.0° C)

Time of analysis	Diacetyl mg%	Acetoin mg%	Volatile acids (in terms of acetic acid) mg%	Esters mg%
Fresh butter	0.283	1.211	3.888	2.605
After 6—7 months	0.293	1.080	4.031	2.675
After 9 months	0.240	1.070	3.240	1.810
As % of the fresh butter	84.8	86.2	83.3	69.5

TABLE 7

Changes in Acidity in Butter Serum During Storage (—12° C, —15° C)

Time of analysis	Acidity of the serum °T	pH of serum
Fresh butter	39.2	5.11
After 6—7 months	42.5	5.18
After 9 months	40.7	5.25
As % of the fesh butter	103.8	97.0

TABLE 8

Changes in some Characteristic Features in Butter During Storage (—12° C, —15° C)

Time of analysis	Eh mv	Butter oxidation according to TBK optical density	Fat oxidation according to TBK optical density	Induction period min.
Fresh butter	+ 244	0.032	0.016	114
After 6—7 months of storage	+ 364	0.095	0.048	72
After 9 months of storage	+ 341	0.102	0.051	63
As % of the fresh butter	139.7	318.7	318.7	55.3

This is due to the effect of minus temperatures upon the activity of lactic acid microflora. The redox potential and the oxidation of butter and butter fat increased in the course of storage (Table 8) while the induction period decreased. After 9 months of storage the redox potential increased by 39.7 % and the TBA-value in the butter and the butter fat 3 times as compared with the fresh butter; the induction period was 63 min.

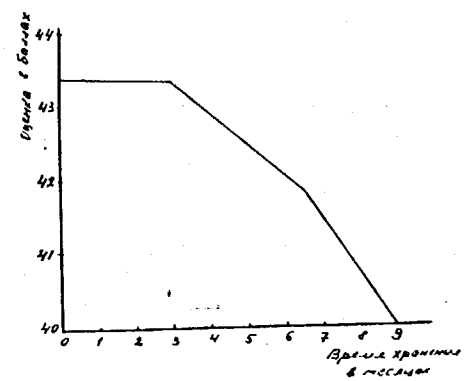

Fig. 4. *Changes in flavour evalution of butter during storage (—12°C, —15°C).*
1. Period of storage. 2. Scores.

When stored at minus temperatures the quality of the butter remained unchanged after 3 months storage and only after 6 months some changes in the flavour and aroma were observed (the butter received by 1.55 points less). During further storage some faults affecting the quality of the butter were observed. It became unclean and sligthly tallowy; Fig. 4 shows changes in the organoleptic evaluation of the butter and the scores given for its flavour and aroma.

8191

Reprinted from
Iowa State College Journal of Sciences
Vol. XII No. 3, April, 1938, pp. 385-395

Iowa State College J. of Sciences - 12(3): 385-395 (1938)

A COLORIMETRIC METHOD FOR THE MICRODETERMINATION OF DIACETYL[1]

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Accepted for publication March 24, 1933

Diacetyl is a flavor contributant of butter and various other food products. Although there have been extensive studies of this substance and its apparent biochemical precursor, acetylmethylcarbinol, relatively few data are available on the diacetyl content alone (as distinguished from acetylmethylcarbinol plus diacetyl) of dairy products or other foods. The reason for this has been the lack of suitable analytical methods for measuring the small amounts of diacetyl which suffice to influence the flavor of a food product.

The gravimetric method for the determination of diacetyl, more usually acetylmethylcarbinol plus diacetyl, as nickel dimethylglyoximate is satisfactory when fairly large amounts are present, but owing to the slight solubility (1) of this derivative attempts to measure 1 or 2 mg. of diacetyl in 100 ml. of distillate yield low results or even fail in the qualitative detection of it. Kunze (7) recently described a micromodification of this method, but recommended colorimetric determinations for amounts less than 0.2 mg.

At the Iowa Agricultural Experiment Station a colorimetric procedure has been developed which can be applied to the determination of very small amounts of diacetyl in butter culture, cream, butter and other materials. A description of it is given herein.

REVIEW OF COLORIMETRIC METHODS

Testoni and Cluza (16) appear to have been the first to use a colorimetric method for diacetyl. They oxidized the nickelous dimethylglyoximate and obtained a soluble red complex in which the nickel has a higher valence number.

Barnicoat (1) dissolved the precipitated nickel dimethylglyoximate in chloroform and used the resulting yellow solution for comparisons. Mohr and Wellm (10) improved this method by extracting with chloroform the nickel complex which still remained in solution as well as that which was precipitated.

Pien, Baisse and Martin reacted the diacetyl with certain orthodiaminobenzene derivatives and made use of the yellow colors which the resulting quinoxaline derivatives exhibit in the presence of strong acid as the bases of their methods. In their first procedure (12) they used 3,4-diaminotoluene. Later (13), they used diaminobenzidine and obtained a stronger yellow color which they assumed resulted through the formation of a bisquinoxaline derivative and which they claimed to be useful for measuring as little as 0.05 mg. of diacetyl in 10 ml. of distillate.

The Voges-Proskauer reaction, as improved by Barritt (2), although very sensitive, is not applicable to the quantitative determination of diacetyl because the color developed is not proportional to the concentration; furthermore, acetylmethylcarbinol also gives the test. Certain color reactions reported by Smith (15) might possibly be made the bases of other color tests.

Titrimetric methods for diacetyl are also possible such as that of Ruehe and Corbett (14), but it is doubtful whether these could be as sensitive and specific as a suitable colorimetric method for microdeterminations.

DEVELOPMENT OF THE METHOD

Tschugaeff and Orelkin (17, 19) developed a procedure for the colorimetric determination of very small amounts of iron by the use of dimethylglyoxime and ammonium hydroxide. Just as the converse of the procedure for the gravimetric determination of nickel with dimethylglyoxime provided the basis for the gravimetric method for diacetyl, so the converse of the procedure for iron constitutes the basis for a colorimetric method for small amounts of diacetyl. The intensely rose-red colored, water-soluble complex that is produced under the conditions of the test is an ammono-ferrous dimethylglyoximate having the probable formula

$$DH\text{-}Fe\text{-}HD \cdot 2NH_3$$

(DH_2 indicates that dioxime and DH- indicates that the H of one of the two oxime groups has been replaced by metal). The compounds which iron as well as nickel and several other metals form with certain dioximes belong to the class of substances now often known as chelate ring compounds. These are discussed in recent reviews (3, 8).

The diacetyl is first converted into diacetyl dioxime (dimethylglyoxime). In the present procedure this is accomplished by the use of a solution containing approximately equimolar quantities of hydroxylamine hydrochloride and sodium acetate and which is therefore essentially the same as a solution containing hydroxylamine acetate and sodium chloride. Such a solution is slightly acidic, because hydroxylamine is a very weak base. Preliminary investigations (unpublished) on the formation of the dioxime show that this reagent is satisfactory under the prescribed conditions (concentration and period of heating which are indicated later) and that a neutral, alkaline, or strongly acidic solution of this reactant is less satisfactory for the present purpose.

For the subsequent color producing reactions to be satisfactory, it was found necessary to remove the excess hydroxylamine remaining after the completion of the formation of the dioxime. In the trials performed before this fact was appreciated, and in which the excess of hydroxylamine was not removed before applying the subsequent reactions, the colors obtained faded rapidly and developed a brownish tinge. The reason for this is that hydroxylamine in alkaline solution oxidizes ferrous iron to ferric iron (9). The excess of hydroxylamine can be fixed by the addition of acetone, whereby it is converted into acetoxime which does not

[1] Journal Paper J543 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 127.

[2] The development of the method was largely financed through a grant from Chas. Pfizer and Co., Inc., which provided particularly for the services of Dr. Prill.

1626

interfere. It was suspected, however, that the acetone also might react with the dioxime of diacetyl to produce acetoxime and the monoxime of diacetyl, thereby partly undoing that which was accomplished in the first step of the method.

When acetone was added to a test solution, in which the dioxime formation had been completed and which was slightly acid, and the solution then heated at 60° C. for 1 hour, the color which developed after the addition of the color producing reagents was somewhat less intense than that which developed in a comparison test solution which had been heated only 5 minutes after the addition of acetone. When the proper amount of dipotassium phosphate was added along with the acetone so that the resulting solution was nearly neutral (pH 6.7) it was found that the intensity of the final color obtained was not affected by the period of heating following the addition of the acetone. This indicates that acetone reacts with the dioxime in acid solution but not to a detectable extent in nearly neutral solution.

In the next step it is necessary to add some reagent to prevent the precipitation of the excess iron as iron hydroxide when ammonium hydroxide and ferrous sulfate are added to the solution containing the dioxime. Potassium sodium tartrate was found to be very effective. A small amount of the tartrate prevents the formation of a precipitate but the solution is colored intensely blue due to tartrate-soluble ferrous-ferric hydroxide (slight oxidation of the iron by air is unavoidable in alkaline solution). By using a relatively large amount of the tartrate this color is suppressed so that only a very faint green tint remains. Kershek (6) made similar observations in connection with the development of his titrimetric method for iron. The high concentration of tartrate in the to fade because of the oxidation of the excess ferrous iron by the air.

It was found that the ferrous sulfate should be last in the sequence in which the several reagents are added. It was noted also that a relatively small amount of it was sufficient, which is fortunate because this makes it possible to so reduce the slight green color caused by the reagents that it is negligible. On the final addition of the ferrous sulfate the beautiful rose-red color appears instantly. The color attains its full intensity in a few minutes and is stable for several hours, but then begins to fade because of the oxidation of the excess ferrous iron by the air.

Some explanation of the other complexes that iron forms with dimethylglyoxime seems pertinent to the reasons why the ferrous sulfate should be added last. This reagent might be contaminated with a trace of ferric salt. Ferric iron under certain conditions also forms a complex with dimethylglyoxime (5), but if the addition of tartrate or of tartrate plus ammonium hydroxide precedes the addition of the iron salt the ferric complex does not appear to be formed. There are also other ferrous complexes. It was observed that when a very minute quantity of ammonium hydroxide is added to a solution containing the dioxime, tartrate and ferrous sulfate, the color becomes dark brown, which appears to be due to the same dark brown ferrous complex (13) that would be formed if sodium hydroxide were added in place of the ammonium hydroxide. On the subsequent addition of an excess of ammonium hydroxide the resulting color is a mixture of red and brown; the brown complex appears to be so stable when once formed that it persists even on the addition

of an excess of ammonium hydroxide. If the ammonium hydroxide were added last in the analytical procedure, a condition similar to this might be obtained in the lower part of the test tube because of the low concentration of the ammonium hydroxide that had reached the lower part of the tube by diffusion. This would be true especially if the contents of the tube were not mixed immediately.

It should be mentioned also that if certain amines are substituted for the ammonium hydroxide in the regular analytical procedure, different reddish or purplish colors are developed.

REAGENTS

Blank determinations should be performed on the chemicals used in the preparation of the reagents. The acetone and the methyl alcohol certainly should be tested since conceivably these might contain traces of diacetyl or one of its homologs. Distilled water should be used throughout.

A. Hydroxylamine acetate

Dissovle 70 g. of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) in water to make 200 ml. of solution. Dissolve 35 g. of hydroxylamine hydrochloride in water to make 800 ml. of solution. Prepare a day's supply of the reagent by mixing the two solutions in the proportion of 1 part of the first to 4 parts of the second by volume.

B. Acetone-dipotassium phosphate mixture

Dissolve 144 g. of anhydrous dipotassium phosphate or 190 g. of its hydrate ($K_2HPO_4 \cdot 3H_2O$) in water, add 200 ml. of pure acetone and make up to a liter with water.

C. Ammonium hydroxide

Concentrated C.P. ammonium hydroxide (sp. gr. 0.90) is used.

D. Saturated tartrate solution

As needed, dissolve 90 g. of potassium sodium tartrate (Rochelle salt) in 50 ml. of water by warming. Keep warm (about 35° C.) in order to prevent crystallization.

CD. Mixture of C and D

In treating a number of samples at a time it is more convenient to add C and D together. Just before it is needed, prepare the mixture in the proportion of 100 ml. of the warm tartrate solution to 13.5 ml. of concentrated ammounium hydroxide. Use immediately so that crystals do not separate from the solution due to cooling.

E. Ferrous sulfate

Dissolve 5 g. of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 100 ml. of a 1 per cent solution of sulfuric acid. This solution should be discarded whenever it shows evidence of oxidation.

F. Dilution solution

Ordinarily this solution is not required, but if dilutions are found to be necessary after the color has been developed they should not be made with water but rather with a freshly prepared mixture of reagents made up in the following proportion: Distilled water—83 ml.; B—10 ml.; C—3 ml.; D—22 ml.; and E—2 ml.

1626

G. [illegible]etyl standard

It is not practical to use diacetyl itself as the standard because absolutely pure diacetyl is not readily available, it is inconvenient to weigh accurately, and its solution has been reported to be unstable. Since the diacetyl in the unknown is actually converted into dimethylglyoxime in the course of the analytical procedure, there should be no objection to its use in place of diacetyl in making the standard solution, provided that the standard and the unknown solutions are given identical treatment in all other respects.

Weigh out 0.1349 g. of purified dimethylglyoxime (recrystallized from alcohol or from a large volume of water) and dissolve in 50 ml. of pure methyl alcohol. When all the crystals have dissolved add 30 ml. of reagent A (hydroxylamine acetate) and make up to a liter with water. One ml. of this solution contains 0.1349 mg. of the dioxime, which is equivalent to 0.100 mg. of diacetyl. The dilutions needed are freshly prepared each time.

The function of the hydroxylamine acetate in this solution is to repress any possible hydrolysis of the dioxime, to fix any trace of aldehyde that may possibly be in the solvent, and to inhibit any microorganisms which might otherwise grow in the solution.

DISTILLATION APPARATUS AND DISTILLATION PROCEDURE

The quantitative collection of the diacetyl from a sample in a small volume of distillate requires a special distillation apparatus and very carefully regulated distillation. After trying various types of apparatus which did not give entirely satisfactory results, the apparatus shown in figure 1 was devised and was found to be satisfactory.

It would perhaps be better if ground glass connections were used, but the present apparatus can be easily made from the usual laboratory equipment. Ordinary rubber stoppers, however, should not be employed since tests on shavings from such stoppers after being used for distillations showed the presence of detectable quantities of absorbed diacetyl. The connections which come in contact with the vapor, B_1 and B_2, may be made with synthetic rubber (neoprene). Shavings from neoprene stoppers after being used in distillations gave negative tests for diacetyl.

The inlet tube D is for the introduction of CO_2 and steam; either of the openings, E for CO_2 or F for steam, can be closed by means of a clamp on the rubber tubing connections when the other is being used. The tube D can be rotated in the stopper so that the CO_2 or the steam can be passed at will either through or above the sample in the flask. The water connections to the condenser jackets J_1 and J_2 should be so arranged that J_1 is always filled with water while J_2 can be easily filled or emptied, thus allowing the tube C to be used at will either as a reflux condenser or as a fractionating column.

In the tube C are placed some glass discs, G, about 15 in number, which are held about 20 mm. apart by means of short sections of glass rod attached to them. The lowest disc is attached to a longer section of rod (about 100 mm.), which rests on the wall of the bent section of tube C and thereby supports the column of discs. When C is used as a reflux the discs act as baffles and minimize the escape of diacetyl. When C is em-

[illegible]l as a fractionating column it is essentially a simple form [illegible] the [illegible] (20) column. The inner diameter of tube C may be 10 to [illegible] mm. and the discs should be ground so that their diameters are just slightly less than this (about 1 mm. less). Whether the discs fit properly or whether the apparatus as a whole is satisfactory can only be determined

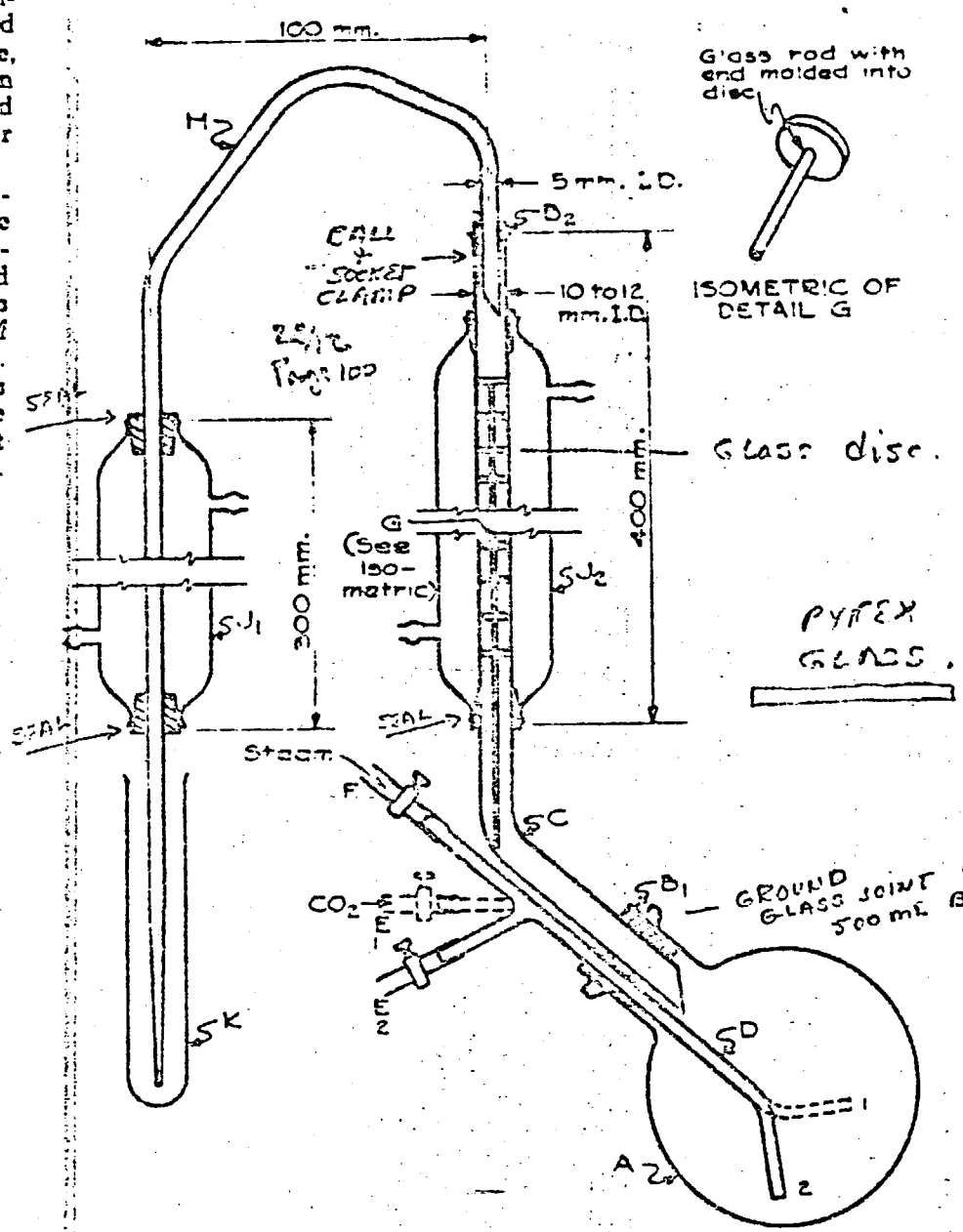

Fig 1. Diagram of distillation apparatus (Need two.)

DONOT INCLUDE BOIL. FLASK IN QUOTE.

1626

by actual trial distillations, wherein a solution containing about 1 mg. of diacetyl is distilled in the manner described in the following paragraphs, and several successive 5 ml. portions of the distillate collected. If all the diacetyl is consistently found in the first portion of distillate, the apparatus has been constructed in the proper manner.

Before an actual distillation is performed the apparatus should be cleaned by distilling water through it. The sample is weighed into the 500 ml. round bottom flask A; 50 g. is usually a sufficient and convenient amount, but for materials low in diacetyl a 100 g. sample may be used. The sample should be slightly acidic in reaction, which is usually the case, but with materials which could possibly have a reaction of pH 7 or higher, for example, butter from neutralized cream, the reaction should be made slightly acidic by the addition of a small amount of some weak non-volatile acid, such as lactic (blank determinations on the acid should show the absence of interfering substances). An alkaline reaction is avoided because even a slight alkalinity will tend to cause the destruction of diacetyl (11, 4), especially on heating. The flask is attached to the apparatus with the inlet tube D in position 1. Water should be running through both jackets J_1 and J_2. The narrow test tube K, which is calibrated at 10 ml. and also marked at approximately 6 ml., is used as the receiving vessel. One ml. of reagent A (hydroxylamine acetate) is pipetted into the test tube which is held so that the constricted end of tube H extends to the bottom of the absorbing liquid.

A slow stream of CO_2 is passed over the sample and through the apparatus for 5 minutes. Then with D still in position 1 the CO_2 inlet is closed and the steam is slowly admitted until the vapor starts to condense in the reflux C, whereupon D is carefully turned to position 2. The steam should be so regulated that the CO_2 which it displaces bubbles through the liquid in K at a slow rate.

There are reasons for employing these preliminary operations. If the air is not previously driven from the apparatus with CO_2, the atmospheric oxidation of the acetylmethylcarbinol (which is usually present in larger amounts than the diacetyl) to diacetyl on heating may lead to results which are too high. On introducing steam above the surface of the sample, the CO_2 and any remaining air are displaced by steam before the sample is greatly heated. It was observed also that when CO_2 was bubbled through a liquid sample or when the distillation was performed without the preliminary slow displacement of the CO_2 by steam under a reflux, a considerable fraction of the diacetyl was carried over with the CO_2 before any liquid actually distilled over and a certain percentage of this diacetyl was lost by being carried away with the bubbles of gas passing through the absorbing solution in K. With the procedure outlined very little of the diacetyl is prematurely carried over with the CO_2 and if several per cent of this small amount escapes absorption the loss is negligible. Refluxing is also often advantageous because after the sample has been refluxed until the initial frothing has subsided the distillation can proceed more smoothly.

When the rate of bubbling through the liquid in K indicates that the CO_2 has been displaced from the flask, the water is let out of J_2. After the column C has become warm the steam pressure should be so regulated that the distillation proceeds at a uniformly slow rate. If the rate of distillation is too rapid the column becomes flooded and its efficiency is de-

stroyed; also the required volume of distillate is soon collected but all of the diacetyl is not contained therein. The necessity for slow distillation cannot be overemphasized. If the volume of liquid in the test tube increases at a barely perceptible rate and the tube H is hot below the point where it starts to bend downward into the jacket J_1, the distillation is progressing effectively. This slow rate of distillation is maintained until the volume of liquid in the test tube K is between 6 and 8.2 ml. During the collection of the last 1 ml. the rate of distillation is increased slightly, the test tube K is lowered, and the distillate allowed to drip into it. The tube of distillate can be stoppered and kept until it is convenient to develop and compare the colors of a series of samples. The distillation proper should take approximately 30 minutes. One analyst can take care of several distillations at a time.

PROCEDURE FOR THE DEVELOPMENT AND COMPARISON OF THE COLORS

The tubes of distillate which have been collected each contain 1 ml. of reagent A (hydroxylamine acetate) and 6 to 5.2 ml. of distillate. The stoppers of the tubes are loosened and the tubes heated in a water bath at 65° C. for 1 hour in order to complete the formation of the dioxime.

The tubes are then removed from the water bath and while they are still warm 1 ml. of reagent B (acetone-dipotassium phosphate) is added to each and allowed to react for 5 minutes. This fixes the excess of hydroxylamine. The tubes are cooled and may be kept at this stage if desired.

To each of the tubes are next added 0.8 ml. of reagent C (ammonium hydroxide) and 2.2 ml. of reagent D (saturated tartrate solution) and the contents of each tube mixed; or, if more convenient, 2.5 ml. of the mixed reagent CD is added in place of C and D separately.

Each tube is then treated with 0.2 ml. of reagent E (ferrous sulfate) and the contents of the tube mixed immediately by inverting. The volumes are brought to 10 ml. by the addition of distilled water and again mixed. Although the color develops very rapidly, about 15 minutes should be allowed for it to attain its full intensity. The colors should be compared within 2 hours.

The color standards are made by adding the desired amount of solution G (diacetyl standard) or one of its dilutions to 1 ml. of reagent A (hydroxylamine acetate) and bringing the volume up to 6 ml. with water. These standards are then treated in exactly the manner described for the unknowns. For color standards ranging from 0 to 0.010 mg. of diacetyl, a series of tubes varying by 0.001 mg. should be prepared for direct visual comparisons. For the higher concentrations to be used with a colorimeter a series increasing in appropriate amounts, for example, 0.010, 0.015, 0.025, 0.040, 0.060, 0.100, 0.150 mg., etc., is prepared; the comparisons are more easily made with a blue filter in the colorimeter.

If it is desirable to collect a different volume of distillate and have a final volume other than 10 ml., the quantities of all the reagents used for the unknowns and for the color standards, with the obvious exception of the solution G (diacetyl standard), should be changed proportionately.

SENSITIVITY, ACCURACY AND SPECIFICITY

With the method described it is possible to detect a difference between 0.001 mg. and no diacetyl in 5 ml. of water. Throughout the range

1626

of diacetyl contents studied, the color appears to be proportional to the concentration of diacetyl, and in the range that can be conveniently measured with a colorimeter, which varies from about 0.01 to 0.5 mg. of diacetyl in the final volume of 10 ml., the proportionality is clearly evident.

Table 1 presents a comparison of the results obtained by the colorimetric and the gravimetric methods; the values were selected as representative of the many comparisons made. Each unknown solution of 200 ml was divided, using 1 per cent for the colorimetric and the remainder for the gravimetric determination. The data show a good agreement in the results of the two methods, the differences ranging from 0.6 to 4.5 per cent.

Practically quantitative recoveries were obtained when 0.2 mg. diacetyl was added to 50 ml. of fresh skim milk, and the milk acidified with 1 ml. of 85 per cent lactic acid and distilled.

It would be expected that certain volatile homologs of diacetyl would also produce colors with this new procedure as well as with the other colorimetric procedures for diacetyl to which references have been made in this paper. Glyoxal, methylglyoxal and acetylpropionyl were tested according to the present procedure and all were found to produce intense red colors. In addition, pyruvic acid, which in a sense is a 1,2-dicarbonyl compound also, was found to produce a yellow color with the reagents used. Since these other substances ordinarily would not be expected in dairy products, the method is applicable to the analysis of these products for diacetyl.

Pure acetylmethylcarbinol does not produce a color with the reagents used. The method can, however, be applied to the determination of this compound by first oxidizing to diacetyl.

SUMMARY

A sensitive colorimetric method has been developed which can be applied to the determination of very small amounts of diacetyl in dairy products and perhaps other materials.

The method is based upon the formation of the intensely colored ammono-ferrous dimethylglyoximate. The diacetyl is first converted into dimethylglyoxime; the excess of hydroxylamine is fixed with acetone in phosphate buffered solution; then ammonium hydroxide, a large amount of tartrate and, finally, a small amount of ferrous sulfate are added. The rose-red color develops very rapidly.

An apparatus and a procedure for the distillation of the diacetyl from the sample are described.

TABLE 1. *Comparison of the results obtained by the colorimetric and by the gravimetric methods*

Colorimetric p.p.m.	Gravimetric p.p.m.	Difference Percentage	Colorimetric p.p.m.	Gravimetric p.p.m.	Difference Percentage
171	170	+ 0.6	177	173	+ 2.2
297	294	+ 1.4	173	170	+ 1.7
197	103	− 1.0	137	120	− 2.4
160	162	− 1.2	43	43	+ 4.5
154	143	+ 4.0	63	63	+ 3.0

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CHANGES IN THE DIACETYL AND THE ACETYLMETHYLCARBINOL CONTENTS DURING THE MANUFACTURE OF BUTTER[1]

E. A. PRILL[2] AND B. W. HAMMER

Results of various investigations indicate that diacetyl is an important flavor constituent of butter. It is contributed by the butter culture used and by the cream. Apparently, in the fermentation characteristic of butter cultures the diacetyl is formed through an oxidation of acetylmethylcarbinol which is produced from citric acid, both the production and oxidation being brought about primarily by the citric acid fermenting streptococci (*Streptococcus citrovorus* and *Streptococcus paracitrovorus*) normally present. Sour cream contains diacetyl and acetylmethylcarbinol as a result of bacterial action, but traces of these products also appear to be present in sweet cream which presumably has not undergone any considerable change due to organisms.

Since butter culture and cream contain much less diacetyl than acetylmethylcarbinol, and also because often citric acid is still present, there is an opportunity for changes in the diacetyl and the carbinol contents during the manufacture of butter. Data on this point are presented herein.

METHODS

Manufacturing Procedure

The butter studied was made during the spring and early summer of 1938. The cream was delivered to the butter laboratory of the Iowa State College from farms in the vicinity, and was made into butter in the usual operation of the laboratory[3] (6).

Both the sweet and sour cream contained approximately 30 per cent fat. The cream was pasteurized at 150° F. for 30 minutes, the sour cream being neutralized to about 0.25 per cent acid prior to pasteurization; following cooling, both the sweet and sour cream were neutralized to approximately 0.10 per cent acid. The churnings in which aerated or modified aerated butter culture was used and some of those in which regular butter culture was employed with sweet cream were made with 150 lb. portions of cream, while the remainder were of commercial size.

Received for publication August 6, 1938.

[1] Journal Paper J-572 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 127.

[2] The studies reported were largely financed through a grant from Chas. Pfizer and Co., Inc., which provided particularly for the services of Dr. Prill.

[3] The churnings were made under the supervision of Dr. N. E. Fabricius who is in charge of the butter laboratory.

Analytical Procedures

Several investigators have reported on certain aspects of the changes in diacetyl and acetylmethylcarbinol contents during the manufacture of butter. Barnicoat (1) used his colorimetric method (2) which is based on a comparison of the yellow chloroform solutions of nickel dimethylglyoximate, Mohr and Wellm (9) employed their modification (10) of this procedure, Brioux and Jouis (4) made use of the colorimetric diaminobenzidine method (11), while Fabricius and Hammer (6), Davies (5), Krenn (8), and Virtanen (14) employed some form of the gravimetric procedure. The results reported by these investigators do not permit of close comparisons because of differences in the methods of buttermaking in the various countries and perhaps also because of the different analytical methods used.

In the present work the diacetyl was conveniently determined by the colorimetric procedure recently developed by Prill and Hammer (12) which was designed to avoid the difficulties encountered with some of the other methods, particularly when relatively small amounts of diacetyl are involved. The principle of the method is the conversion of the diacetyl into dimethylglyoxime and then into the intensely colored ammono-ferrous dimethylglyoximate.

The acetylmethylcarbinol plus diacetyl was also determined colorimetrically. Small aliquots of the distillate from the cream or culture distilled with added ferric chloride were used under special conditions (13). Samples of butter (20 g. each) with ferric chloride added were distilled in a special apparatus (12).

The results are recorded as milligrams of diacetyl or of acetylmethylcarbinol plus diacetyl per kilogram of the sample, which is synonymous with expressing them as parts per million.

RESULTS

In some of the first trials it was noted that the neutralized and pasteurized cream frequently had a lower diacetyl content than the raw cream and, accordingly, a more detailed study of this point was made with several lots of sour cream. The results, which are shown in Table 1, indicate some rather unexpected changes. Shortly after the preliminary neutralization of the cream to 0.25 per cent acid (the original acidity was often 0.5 to 0.6 per cent), or during the first part of the heating, the diacetyl content fell to a very low value and the carbinol content commonly de slightly. This may have been due to a reduction of these compou 2,3-butylene glycol (7) in the less acid medium. When the crea been heated to pasteurizing temperature and in the subsequent te diacetyl contents showed very significant increases which were pr actually even greater than shown by the analyses because of loss t

1627

TABLE 1

Changes in diacetyl and acetylmethylcarbinol contents during neutralization and pasteurization of sour cream

Cream	Trial 1		Trial 2		Trial 3		Trial 4		Trial 5	
	Milligrams per kilogram									
	Ac_2*	Amc† $+Ac_2$	Ac_2	Amc $+Ac_2$	Ac_2	Amc $+Ac_2$	Ac_2	Amc $+Ac_2$	Ac_2	Amc† $+Ac_2$
Raw	1.11	39.0	0.61	37.0	0.71	22.0	0.94	26.0	1.10	27.0
Neut. to 0.25% acidity	.24	37.0	.15	24.0	.20	26.0	.30	26.0	.10	29.0
Heated to 100° F.							.16	23.0	.12	22.0
Heated to 150° F.							.55	33.0	.39	27.0
After holding 30 min.							.52	32.0	.43	29.0
Cooled					.48	28.0	.75	33.0	.48	30.0
After final neutralization to 0.10% acidity			.55	36.0	.56	28.0	.74	32.0	.43	29.0
After holding about 1 hr.	.51	40.0	.35	32.0			.86	32.0	.57	28.0

* Ac_2 = diacetyl.
† Amc = acetylmethylcarbinol.

volatilization. The carbinol content showed an increase when the cream had reached the pasteurizing temperature and then remained fairly constant in the subsequent tests. The increase in diacetyl may possibly have been caused by non-biological oxidation but the increase in the carbinol through non-biological reactions seems improbable. The net result of the processing of the cream was a decrease in the diacetyl content and relatively little change in the acetylmethylcarbinol content.

Barnicoat (1) reported that during neutralization and pasteurization of cream (presumably the flash method) the carbinol is partly destroyed while the diacetyl is practically unchanged. Apparently, generalizations on these points which are applicable to all methods of processing cream cannot be made at present.

For the churnings used in studying the changes in the diacetyl and acetylmethylcarbinol contents, the butter culture was made with addition of 0.15 per cent citric acid (monohydrate) to the skim or whole milk employed, and from 5 to 10 per cent was added to the pasteurized and cooled cream. Except in one case, which is indicated, the mixture was held cold over night (36° to 44° F. so there would be no significant increase in acidity and churned the next morning. Samples of the processed cream, the butter culture, the mixture of cream and culture immediately after its preparation, the mixture just before churning, the mixture partly churned, the buttermilk, and the finished salted butter were analysed within 1 or 2 hours; they were kept cold during this interval. From the percentages of culture used and on the basis that 30 per cent cream plus 5 to 10 per cent culture yields approximately 1 part of butter (80 per cent fat) and 2 parts of buttermilk, the milligrams of diacetyl and of the carbinol plus

1637

TABLE 2

Changes in diacetyl and acetylmethylcarbinol contents during manufacture of butter from sweet cream with regular culture added

Trial no.	Per cent culture added to cream	Compounds determined	Diacetyl (Ac_2) and acetylmethylcarbinol plus diacetyl ($Amc + Ac_2$) in mg. per kg.							Estimated per cent of the total Ac_2 or $Amc + Ac_2$ retained in butter
			Past. neut. cream	Butter culture	Cream plus culture: at once	Cream plus culture: just before churning	Cream plus culture: partly churned	Buttermilk	Butter	
1	10	Ac_2	.03 (*.03*)*	1.53 (*.15*)	.32			1.60 (*1.07*)	.33 (*.11*)	
		$Amc + Ac_2$	5.1 (*4.6*)	315. (*31.5*)	34.			58. (*39.*)	8.0 (*2.7*)	
2	10	Ac_2	.10 (*.09*)	.69 (*.07*)	.39	.80	.82	1.33 (*.89*)	.28 (*.09*)	11.0
		$Amc + Ac_2$	2.8 (*2.5*)	278. (*28.*)	27.	37.		46. (*31.*)	5.5 (*1.8*)	4.9
3	7	Ac_2	.08 (*.07*)	.90 (*.06*)	.17	.26	.71	.81 (*.54*)	.14 (*.05*)	7.0
		$Amc + Ac_2$	3.3 (*3.1*)	270. (*19.*)	20.	21.	18.	27. (*18.*)		
4	10	Ac_2	.08 (*.07*)	.90 (*.09*)	.24	.50	.62	.86 (*.57*)	.18 (*.06*)	9.7
		$Amc + Ac_2$	3.3 (*3.0*)	270. (*27.*)	30.	30.		51. (*34.*)		
5	7	Ac_2	.18 (*.17*)	.83 (*.06*)	.32	.56	.60	.96 (*.64*)	.18 (*.06*)	10.0
		$Amc + Ac_2$	7.7 (*7.1*)	300. (*21.*)	24.	26.		38. (*24.*)	4.0 (*1.3*)	5.1
6	7	Ac_2	.25 (*.23*)	.52 (*.04*)	.42	1.18		1.66 (*1.10*)	.30 (*.10*)	
		$Amc + Ac_2$	12.5 (*11.6*)	232. (*16.*)	30.	50.		70. (*47.*)	5.5 (*1.8*)	3.6
7	10	Ac_2	.16 (*.14*)	2.56 (*.26*)	.41	.54	.61	.61 (*.40*)	.18 (*.06*)	9.8
		$Amc + Ac_2$	7.7 (*6.9*)	147. (*15.*)	24.	25.		30. (*20.*)	3.6 (*1.2*)	4.8
8	10	Ac_2	.05 (*.04*)	3.60 (*.36*)	.47	.83	.97	1.35 (*.90*)	.21 (*.07*)	7.2
		$Amc + Ac_2$	2.5 (*2.2*)	260. (*26.*)	29.	36.		53. (*35.*)		
9	10	Ac_2	.09 (*.08*)	2.16 (*.22*)	.49	1.05	1.17	1.47 (*.97*)	.29 (*.10*)	8.5
		$Amc + Ac_2$	3.7 (*3.3*)	306. (*31.*)	35.	49.		68. (*45.*)	5.8 (*1.9*)	3.9
10	10	Ac_2	.10 (*.09*)	.77 (*.08*)	.40	1.05	1.10	1.66 (*1.10*)	.33 (*.11*)	10.0
		$Amc + Ac_2$	4.2 (*3.8*)	360. (*36.*)	39.	56.		85. (*56.*)	7.8 (*2.6*)	4.7
11	10	Ac_2	.33 (*.30*)	.70 (*.07*)	.87	1.37	1.55	1.97 (*1.31*)	.38 (*.13*)	8.1
		$Amc + Ac_2$	16. (*14.5*)	250. (*25.*)	47.	60.	61.	74. (*49.*)	7.3 (*2.4*)	4.0
12	10	Ac_2	.09 (*.08*)	.56 (*.06*)	.32	1.30	1.64	1.48 (*1.00*)	.36 (*.12*)	7.3
		$Amc + Ac_2$	3.9 (*3.5*)	257. (*26.*)	26.	53.		74. (*49.*)	7.9 (*2.7*)	5.1

* Numbers in parentheses and italics denote the estimated mg. of Ac_2 or of $Amc + Ac_2$ contributed to, or derived from, 1 kg. of the churning mixture by the materials indicated.

1627

diacetyl contributed to, or derived from, 1 kg. of the churning mixture were estimated. The percentages of the diacetyl and of the carbinol plus diacetyl in the churning mixtures that were retained in the butter were also estimated.

Data obtained when regular butter culture was used with sweet cream are given in Table 2. The mixture of cream and culture at once usually contained approximately the amount of acetylmethylcarbinol contributed by the cream and the culture, whereas the diacetyl content was regularly higher than the calculated amount. The incorporation of air during the mixing presumably made it possible for the organisms to effect considerable oxidation. The next morning the diacetyl had increased further, often more than doubling over night, and the carbinol also showed some increases but the percentage increases were not as great as in the case of the diacetyl. During churning there were further increases in diacetyl; the carbinol, however, would not be expected to change greatly in the short period involved.

Fabricius and Hammer (6) found that when mixtures of cream and culture were held for a time before churning, the acetylmethylcarbinol plus diacetyl contents increased and the resulting butter scored higher than butter made by churning soon after the addition of the culture. Other investigators (8, 9, 14) also noted significant increases in the diacetyl and the carbinol contents during holding and churning of cream. Recent work (13) shows that similar increases occur in butter culture under analogous treatments.

The diacetyl and acetylmethylcarbinol contents of the butter and buttermilk are considered later with the values from the other series of churnings.

Table 3 presents results obtained when regular culture was used with sour cream. More diacetyl was contributed to the mixtures of cream and culture by the neutralized and pasteurized cream than by the regular culture, and acetylmethylcarbinol was contributed in comparable amounts from the two sources. However, the culture supplied active organisms which made possible a further accumulation of these two products. Except for a few irregularities, the changes occurring in the diacetyl and acetylmethylcarbinol contents were similar to those obtained with mixtures of sweet cream and regular culture.

Results obtained when aerated (3) or modified aerated cultures were used with sweet cream are given in Table 4. The aerated cultures were grown with aeration under pressure for 12 to 13 hours and had extremely high diacetyl contents (up to 37.4 mg. per kg.); the acetylmethylcarbinol contents were also much higher than those of regular cultures. The modified aerated cultures were aerated under pressure for 3 hours after the usual ripening period; they contained relatively large amounts of diacetyl and the carbinol contents were somewhat higher than those of regular

TABLE 3

Changes in diacetyl and acetylmethylcarbinol contents during manufacture of butter from sour cream with regular culture added

Per cent culture added to cream	Compounds determined	Diacetyl (Ac_2) and acetylmethylcarbinol plus diacetyl ($Amc + Ac_2$) in mg. per kg.						Estimated per cent of the total Ac_2 or $Amc + Ac_2$ retained in butter
		Past. neut. cream	Butter culture	Cream plus culture			Buttermilk	Butter	
				at once	just before churning	partly churned			
5	Ac_2	.54 (.52)*	.94 (.05)	.54	.42	1.00	1.25 (.84)	.20 (.07)	6.7
	$Amc + Ac_2$	7.7 (7.3)	250. (13.)	27.	39.		55. (37.)	6.1 (2.1)	5.3
10	Ac_2	.15 (.14)	.65 (.07)	.70	.79		1.18 (.79)	.37 (.12)	
	$Amc + Ac_2$	9.6 (8.7)	305. (31.)	47.	55.		60. (40.)	5.5 (1.8)	3.3
5	Ac_2	.43 (.41)	.33 (.02)	.47	1.13	1.20	1.20 (.80)	.32 (.11)	8.9
	$Amc + Ac_2$	20.7 (19.6)	230. (12.)	38.	42.	40.	52. (35.5)	5.3 (1.8)	4.5
5	Ac_2	.36 (.34)		.38	.36	.43	.55 (.37)	.16 (.05)	12.3
	$Amc + Ac_2$	14.5 (13.8)		20.	17.	15.	21. (14.)	2.8 (.9)	6.2
5	Ac_2	.40 (.38)	.38 (.02)	.52	.51	1.05	1.22 (.83)	.27 (.09)	8.5
	$Amc + Ac_2$	19. (18.)	266. (13.)	30.	46.		74. (50.)	5.0 (1.7)	3.7
5	Ac_2	.47 (.45)	.59 (.03)	.46	1.00		1.07 (.72)	.36 (.12)	
	$Amc + Ac_2$	28. (27.)	270. (14.)	39.5	53.		74. (50.)	6.5 (3.2)	6.0
7	Ac_2	.51 (.47)	.34 (.03)	.46	1.10	1.08	1.10 (.73)	.40 (.13)	12.0
	$Amc + Ac_2$	40.5 (37.)	262. (18.)	51.	69.		79. (53.)	7.1 (2.4)	4.0
	Ac_2	.35 (.32)	.33 (.03)	.66		1.00	1.10 (.73)	.25 (.08)	8.3
		32. (29.)	130. (10.)	40.		41.	55. (37.)	4.4 (1.5)	3.6

See 2.
In as prepared and churned the same day.

1627

cultures. Analyses of the mixtures of cream and culture at once do not indicate an increase in diacetyl during mixing but rather some of them suggest a slight decrease, especially in the case of the aerated cultures. It appears that after having grown in a strongly aerated medium the organisms did not immediately effect further oxidations leading to production of diacetyl on transfer to the less aerated medium. On holding until the next morning and during churning, the organisms usually produced additional diacetyl and acetylmethylcarbinol and although, in most cases, the actual increment in diacetyl was as great (or greater) as with regular culture, the percentage increase was not, because of the relatively large amount of diacetyl originally present. Consequently, in comparisons involving the addition of equal percentages of regular and aerated or modified aerated culture to pasteurized sweet cream, the butter made with the latter types of culture invariably had the higher diacetyl content (0.38 to 1.00 mg. per kg. as compared to 0.14 to 0.38 mg. per kg.), but these higher values were really much lower than they would be if the diacetyl contents of the butter were proportional to those of the cultures used. In case the cream is churned soon after the addition of the culture, however, aerated or modified aerated cultures probably would show a greater practical advantage over regular cultures.

In the distribution of the diacetyl and the acetylmethylcarbinol between the butter and the buttermilk, certain general relationships apply to all the churnings studied. The estimated milligrams of diacetyl in the buttermilk and the butter derived from 1 kg. of the churning mixture agree fairly well, in most cases, with the diacetyl content of the partly churned mixture. Also, the estimated milligrams of the carbinol plus diacetyl in the two products agree fairly well with the content just before churning. Except for a few irregularities, the diacetyl contents of the butter were very nearly 1/5 of the diacetyl contents of the corresponding buttermilk. Since butter contains about 1/5 serum it would appear at first sight that the diacetyl is contained entirely in the serum of the butter. However, the carbinol plus diacetyl content of the butter was, in most cases, only about 1/10 that of the buttermilk, which is much less than would be expected because acetylmethylcarbinol is presumably even more preferentially dissolved in the water phase than is the diacetyl. The average of the estimated percentages of the diacetyl in the churning mixtures that were retained in the butter was 8.8 per cent, and the corresponding value for the carbinol plus diacetyl was 4.5 per cent.

Barnicoat (1) reported that 0 to 33 per cent (average 15 per cent) of the diacetyl and 7 to 22 per cent (average 14 per cent) of the carbinol in cream were retained in the butter, and on the basis of the averages of these divergent values postulated that diacetyl and the carbinol were contained in the butter serum. Actually, a number of his analyses of individual churnings

TABLE 4

Changes in diacetyl and acetylmethylcarbinol contents during manufacture of butter from sweet cream with aerated or modified aerated culture added

Per cent and type culture added to cream	Compounds determined	Diacetyl (Ac_2) and acetylmethylcarbinol plus diacetyl ($Ame+Ac_2$) in mg. per kg.							Estimated per cent of the total Ac_2 or $Ame+Ac_2$ retained in butter
		Past. neut. cream	Butter culture	Cream plus culture			Buttermilk	Butter	
				at once	just before churning	partly churned			
10 at	Ac_2	.10 (.09)*	16.8 (1.68)	1.55	1.39	2.22	2.44 (1.65)	.40 (.13)	6.0
	$Ame+Ac_2$	2.8 (2.5)	410. (41.)	36.	34.		47. (31.)	4.8 (1.6)	4.7
10 a	Ac_2	.08 (.07)	25.0 (2.50)	2.30	2.40	2.90	3.60 (2.40)	.60 (.20)	6.9
	$Ame+Ac_2$	3.3 (3.0)	570. (57.)	51.	68.	68.	69. (46.)		
10 a	Ac_2	.16 (.14)	26.8 (2.68)	2.60	3.25	3.70	3.90 (2.60)	1.00 (.33)	9.0
	$Ame+Ac_2$	7.7 (6.9)	750. (75.)		90.		118. (79.)	13.4 (4.5)	5.0
10 a	Ac_2	.05 (.01)	37.4 (3.74)	3.85	3.85	3.35	5.30 (3.53)	.93 (.31)	9.3
	$Ame+Ac_2$	2.5 (2.2)	920. (92.)	85.	97.		120. (80.)		
10 mat	Ac_2	.09 (.08)	12.7 (1.27)	1.43	1.58	1.43	2.31 (1.54)	.44 (.15)	10.2
	$Ame+Ac_2$	3.7 (3.5)	410. (41.)	43.	46.		72. (48.)	6.5 (2.2)	4.8
10 ma	Ac_2	.10 (.09)	10.0 (1.00)	.91	1.14	1.40	2.00 (1.33)	.38 (.13)	9.0
	$Ame+Ac_2$	4.2 (3.8)	308. (31.)	35.	40.		59. (39.)	5.6 (1.8)	4.7
10 ma	Ac_2	.33 (.30)	15.5 (1.55)	1.96	2.60	2.72	3.90 (2.60)	.68 (.23)	8.5
	$Ame+Ac_2$	16.0 (14.5)	417. (42.)	53.	75.	74.	98. (65.)	9.3 (3.1)	4.2
10 ma	Ac_2	.09 (.08)	17.5 (1.75)	1.47	2.22	2.70	2.72 (1.81)	.56 (.19)	6.9
	$Ame+Ac_2$	3.9 (3.5)	323. (32.)	37.	62.	69.	82. (55.)	6.8 (2.3)	3.3

* ...ble 2.
culture.
...ed butter culture.

1637

showed a ratio of approximately 2 to 1 for the percentages of diacetyl and of the carbinol retained in the butter. The results obtained by Mohr and Wellm (9) indicate a lower percentage retention of diacetyl and a still lower percentage retention of the carbinol. They attributed this to the removal of some of these products by the wash water, presumably, relatively more of the carbinol than diacetyl being washed out. Krenn (8) made similar claims in regard to the washing. In the present work the analyses of a few samples of wash water showed only fractions of a milligram of the carbinol plus diacetyl per kilogram. While the total amount present in the wash water may be significant in comparison with the amount present in the butter, it is difficult to differentiate between material which comes from the film of buttermilk remaining between the butter granules and that which might possibly be removed from the granules themselves. If the diacetyl and the carbinol are both held entirely in the serum droplets in the same manner, they should be retained in the butter in the same proportion as they exist in the churning mixture at the time the butter is formed, and any possible washing out of these products should also be in this proportion. Actually, the distribution of the diacetyl and the carbinol in butter is probably more complex than has been assumed. The serum in the form of minute droplets may possibly have solvent properties different from those of larger volumes of this fluid. The possibility of the adsorption of the diacetyl at the enormous water-fat interface should also be considered, as well as the possibility of the solution of a portion of the diacetyl in the fat.

With the churnings made by adding any of the types of culture to sweet cream it was not possible to note a close relationship between the diacetyl contents of the culture and of the finished butter. Undoubtedly other factors, such as acetylmethylcarbinol content, acidity, oxygen tension, and numbers and activity of the organisms present are also involved. It would be even more difficult to detect a relationship when sour cream is used because of the greater variation in the quality of such cream and the comparatively large amount of diacetyl it contributes to the mixture of cream and culture.

SUMMARY

Marked changes in diacetyl content, both decreases and increases, were noted during the neutralization and pasteurization of sour cream. The changes in the acetylmethylcarbinol content were relatively small.

Progressive increases in diacetyl were found after mixing regular butter culture with pasteurized cream (either sweet or sour), after holding the mixture at a low temperature over night, and after the mixture had been partly churned. The acetylmethylcarbinol content increased somewhat during the holding period. The results when aerated or modified aerated butter culture was added to sweet cream were similar to those when regular culture was employed, except that no increases in diacetyl were noted

immediately after mixing, and the subsequent increases, while quite significant in actual amounts, were small on a percentage basis because of the relatively large amounts of diacetyl already supplied by these cultures.

It was not possible to note a close relationship between the diacetyl contents of the butter cultures added to the cream and those of the resulting butter. Aerated or modified aerated cultures, as compared with equal percentages of regular cultures, yielded butter having higher diacetyl contents, but these values were really much lower than they would have been if a proportionality existed between the diacetyl contents of the cultures and the butter.

On the average, 8.8 per cent of the diacetyl and 4.5 per cent of the acetylmethylcarbinol plus diacetyl in the churning mixture were retained in the butter.

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1637

1652

VEGETABLE FLAVORS

Flash Exchange Gas Chromatography for the Analysis of Potential Flavor Components of Peas

J. Agr. Food Chem. 8: 141-3, (1960)

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The technique of flash exchange gas chromatography was applied to the determination of aldehydes, ketones, and acids in the steam distillate of peas. The compounds identified were acetaldehyde, acetone, propionaldehyde, *n*-butyraldehyde, *n*-valeraldehyde, biacetyl, formic acid, acetic acid, and isovaleric acid. All these components occurred at a concentration of less than 3 p.p.m. on a fresh-weight basis. A mixture of these compounds at the concentration levels found did not reproduce the characteristic odor of pea steam distillate.

The ultimate goal of flavor research is to improve flavor so that abundant, nutritious foods will be consumed at an increased rate. It is most desirable that rapid techniques be developed which could be applied to small, experimental quantities of material. Accuracy need not be greater than that required for a starting point in taste panel evaluation. With this type of research tool, studies of plant breeding, crop selection, harvesting practice, processing, and storage could be made with improved flavor as an objective.

A useful technique for the determination of carbonyl compounds and acids was developed which required only a few milligrams of compounds (3). Aldehydes, ketones, and acids are isolated as solid derivatives, regenerated in rapid exchange reactions, and volatilized

Table I. 2,4-DNPH Prepared from Steam Distillate of Peas

Run No.	2,4-DNPH, Mg. 1958[a]	1959[b]
1	25.4	11.7
2	21.2	11.5
3	23.6	8.9
4	15.1	11.3

[a] Insoluble 2,4-DNPH collected by filtration.
[b] Extracted. Filtration gave 0.7 to 2.8 mg. of 2,4-DNPH.

Table II. Concentration of Volatile Carbonyl Compounds in Initial Steam Distillate of Peas

Compound	Av. Concn., Mg./Kg. Fresh Wt 1958	1959
Acetaldehyde	2.4	0.56
Acetone	0.35	0.12
Propionaldehyde	0.17	0.00
n-Butyraldehyde	0.13	0.06
n-Valeraldehyde	0.00	0.44

Table III. Change in Volatile Carbonyl Composition of Steam Distillate of Peas (1958) with Time

Time, Min.	Volume Distillate, Ml.	Weight 2,4-DNPH, Mg.	Weight Due to Biacetyl	Carbonyl Content, Mg./Kg., Fresh Wt.	
				Biacetyl	0.61
				Acetaldehyde	0.62 (0.70)
				Acetone	0.14 (0.52)
				Propionaldehyde	0.046 (0.10)
				n-Butyraldehyde	0.055 (0.11)
33	275	15.1	6.27		
57	263	5.05	4.04	Biacetyl	0.39
81	310	3.59	3.58	Biacetyl	0.34
99	258	1.96	3.02	Biacetyl	0.29

directly into a gas chromatography unit for separation and identification. Aldehydes and ketones are determined by regeneration from 2,4-dinitrophenylhydrazones (2,4-DNPH) with α-ketoglutaric acid. Monobasic, carboxylic acids are transformed from their potassium salts into ethyl esters by heating with potassium ethylsulfate.

Experimental

The peas used were portions of large lots of blanched, frozen, Perfection (canners) peas prepared on June 25, 1958, and June 19, 1959, at Milton, Ore. The only difference in the two lots was the earlier maturity and longer blanching time for the 1959 lot. The peas (2-kg. samples) were thawed for 4 hours, ground with 1000 ml. of distilled water in a food blender, and steam distilled at atmospheric pressure with a boiling water bath as a heat source. A heating period of 1 hour was necessary to reach distillation temperature. A first distillate of 250 ml. was collected by passing in clean steam for about a half hour. A gas trap containing 2,4-dinitrophenylhydrazine reagent was used to ensure that all volatile carbonyl compounds were trapped. The distillate was treated with a solution of 200 mg. of 2,4-dinitrophenylhydrazine in 20 ml. of concentrated hydrochloric acid. The turbid suspension was heated to boiling for 5 minutes, cooled slowly to room temperature, and stored overnight in a refrigerator. The 2,4-DNPH was collected by filtration, washed with 2*N* hydrochloric acid, then water, and dried to constant weight at reduced pressure over phosphorus pentoxide. The volatile C_2 to C_6 aldehydes and ketones in the mixed 2,4-DNPH were determined by the flash exchange reaction with excess α-ketoglutaric acid (*3*).

After the predominant derivatives had been removed by filtration, the filtrate and washings were diluted to 450 ml. and extracted continuously with purified petroleum ether for 16 hours. The yellow-colored extract, 250 ml., was washed with three 50-ml. portions of water, dried over anhydrous sodium sulfate, and evaporated.

For the determination of acids, the steam distillate was treated with 0.1*N* potassium hydroxide to pH 8.5 and evaporated. The resulting mixture of potassium salts was dried over phosphorus pentoxide at room temperature and pressure. The volatile C_1 to C_6 monobasic, carboxylic acids were determined by flash exchange with potassium ethylsulfate.

Results and Discussion

Carbonyl compounds are present in the steam distillate of peas at a very low concentration. Indeed, if blanched, frozen, intact peas are heated with water, there is almost no detectable carbonyl compound in the distillate. It was necessary to steam distill the pulverized peas to get sufficient carbonyl derivative for analysis. The results of several such steam distillations are shown in Table I. These results are in marked contrast to those reported by Silberstein (*5*) and Wager (*6*). Silberstein obtained 60 to 300 mg. of 2,4-DNPH from 2 kg. of peas, by steam distillation under pressure, which may account for part of the difference in results. The discrepancy is due in part to such factors as variety of pea, maturity at harvest, type of blanching used, and storage conditions. Silberstein demonstrated that blanched peas had a much lower volatile carbonyl content than fresh peas.

The results of the analyses of the mixed 2,4-DNPH from pea distillates are shown in Table II. The predominant carbonyl compounds are acetaldehyde, acetone, propionaldehyde, *n*-butyraldehyde, *n*-valeraldehyde, and biacetyl. The latter compound was determined separately by colorimetric methods and the results are shown in Table III. The differences in amount and distribution of the carbonyl compounds in the 1958 and 1959 pea lots is interesting. The 1959 material had a much lower volatile carbonyl content and was different from the 1958 material by the absence of propionaldehyde and the presence of *n*-valeraldehyde.

A brief study of the variation of carbonyl content with time of distillation was made and the data are shown in Table III. It was found that after the first distillation fraction, the major part of the weight of the precipitated 2,4-DNPH was due to biacetyl bis-(2,4-dinitrophenylhydrazine). The concentration of the biacetyl appears to decrease to a limiting value with time. A separate study (*1*) of acetoin and biacetyl in peas showed that acetoin is the predominant material with a concentration of 260 mg. per kg. of fresh weight. The acetoin is not volatile with steam under the conditions of the distillation.

Extraction of the filtrate after collection of the precipitated 2,4-DNPH produced considerable material (11.7 mg. from the first fraction of 1958 run No. 4). Analysis of the extracted material is shown within parentheses in Table III. It was expected that some trace constituents would appear in the extracted material. This did not occur, because only the four primary carbonyl compounds were found at both the 90° and the 150° C. column temperatures.

Volatile acids in the steam distillate

Table IV. Volatile Acids in Steam Distillate of Peas (1959)

Acid	Concn., Mg./Kg. Fresh Wt. Run No. 1	2	Av.
Formic	0.33	0.46	0.39
Acetic	0.024	0.029	0.027
Isovaleric	0.067	0.060	0.061

1652

in 1959 peas are shown in Table IV. The acid composition is relatively simple. Formic acid is the primary constituent with small amounts of acetic and isovaleric acids also present. Formic acid has been detected previously in canned peas (*1*).

The steam distillate of peas has the pleasant, characteristic odor of cooked peas. If the compounds responsible for the odor can be identified, a substantial part of pea flavor would be understood. Earlier work in this field has indicated that carbonyl compounds are an important part of pea odor (*2*). Mixtures of the carbonyl compounds identified in this study do not approach the cooked pea odor. Work on the isolation and identification of the odorous components of pea steam distillate is being continued.

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Received for review June 5, 1959. Accepted November 2, 1959. Division of Agricultural and Food Chemistry, 135th Meeting, ACS, Boston, Mass., April 1959. The author as collaborator was employed by the National Canners Association, with which this work was conducted cooperatively.

Vitis 8, 129—134 (1969)

(1655)

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1031

Diacetyl in Australian dry red wines and its significance in wine quality

by

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Introduction

Diacetyl is becoming recognised as an important flavour compound in dry red table wines, and when present in sufficient concentration, can impart a butter-like aroma to wine. Diacetyl is known to be an important component of the aroma of butter (1, 2), and its presence in wine is associated with the presence of lactic acid bacteria which promote a malo-lactic fermentation.

The study of diacetyl in wine is part of a continuing investigation into the influence of micro-organisms on the composition and quality of wines. Studies on the influence of wine yeasts have recently been reported (3), together with their effect on the growth of bacteria which bring about the malo-lactic fermentation (4), and considerable attention has been given to the role of these bacteria (5, 6). The study of diacetyl has arisen directly from this work.

This paper reports the results of a survey of the diacetyl content of Australian dry red wines. In the past, diacetyl has sometimes been measured together with acetoin (acetyl methyl carbinol) as one value, but since diacetyl is the important flavour compound, this has been measured directly. As far as we are aware, the results herein represent the most comprehensive survey yet reported of diacetyl in wine.

Taste thresholds of diacetyl were measured in two dry red table wines made from different grape varieties, by a panel of tasters, to assess the significance of the diacetyl values obtained.

Materials and Methods

In 1968 a total of 466 dry red table wines of current vintage was obtained from ineries in South Australia, New South Wales and Victoria, and represented all ie major wine growing areas in these three States, which together produce over 5 per cent of the total Australian production.

The wines were received at the Institute from 3 to 5 months after making, and ere stored at 15° C and analysed as soon as possible. As the chemical method for iacetyl measurement described previously (6) was too slow for the number of wines nvolved, a more rapid gas-chromatographic method was developed. This method vas based on headspace sampling, of which one of the authors had previous experience (8), and the use of the electron capture detector. The use of this detector had been reported for diacetyl measurements in beer (9, 10), but as far as could be ascertained, its use in wine analysis has not been reported. Accordingly, the method developed is described below.

10 ml wine, 1.0 ml of 10 per cent acetone in water (internal standard) and 2.0 ml water were pipetted into a 60 ml bottle, which was closed with a rubber Suba seal and completely immersed in a 35° C water bath for 30 minutes. The bottle was then raised so that the rubber seal was exposed, the seal was dried and 2 to 5 ml

(depending on diacetyl content) of head-space vapour were withdrawn with a 10 ml Hamilton gas-tight syringe after repeated aspiration, and injected into the gas chromatograph (Perkin-Elmer 801).

Operational parameters were as follows:

Column: Porapak Q 80—100 mesh 6 ft × 1/8 in stainless steel.

Temperatures: Injection port 175°, column oven 150° (isothermal), detector 110° C.

Carrier gas: Nitrogen 30 ml per minute through the column and 140 ml per minute through a diluent bypass leading to the detector.

Attenuation: 100.

Recorder: Leeds and Northrup Speedomax G 1 millivolt full-scale deflection, chart speed 15 in per hour.

Peaks appeared in the following order and retention time, and a typical analysis is shown in Fig. 1.

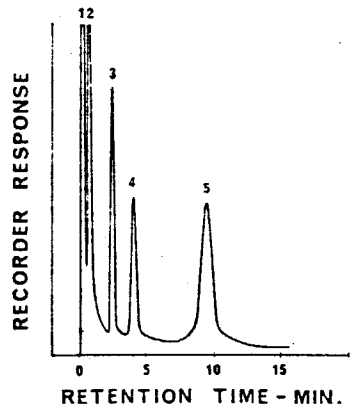

Fig. 1

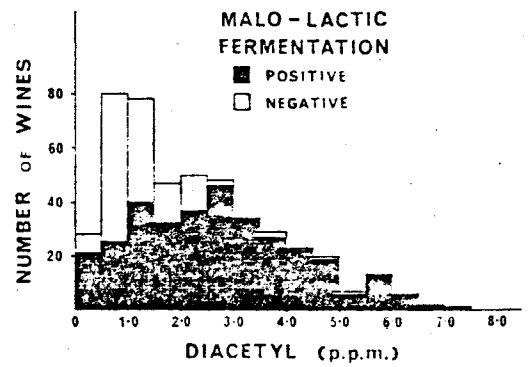

Fig. 2

Fig. 1: Typical gas-chromatographic trace of diacetyl analysis.

Fig. 2: Diacetyl content and occurrence of malo-lactic fermentation in 466 Australian dry red table wines.

1	air	0.1 min.
2	water	0.5 min.
3	ethanol	2.2 min.
4	acetone	4.0 min.
5	diacetyl	9.5 min.

The identity of the diacetyl peak was confirmed on two other stationary phases of differing polarity.

The product of peak height by retention time was measured for diacetyl and acetone, and the ratio calculated and calibrated for diacetyl concentration. The calibration was carried out with redistilled diacetyl analysed by the dimethyl-glyoxime method (6), both as pure solutions and added to wine in place of the 2 ml of water. Pure diacetyl was very difficult to prepare and the redistilled product assayed higher than 85 per cent on a weight basis.

Two syringes were used alternately. After use they were dried in a hot a at 60° C and returned to a compartment in the water bath to attemperate. means an analysis could be carried out every 15 minutes, and duplicate minations usually agreed to within ±5 per cent.

1655

7 mical dimethyl-glyoxime method and the gas chromatographic metho were red using a range of wines differing widely in amounts of diacetyl, and the two methods gave results which agreed closely, e. g. for six wines with diacetyl content ranging from 0.4 to 7 ppm the mean values were 3.8 ppm (dimethyl-glyoxime) and 3.9 ppm (gas chromatography). Above about 8 ppm the gas chromatographic method gave somewhat lower values than the chemical method, but these levels did not occur naturally in the wines examined. Recoveries of added diacetyl in the range of 0 to 8 ppm were accurate by both methods.

The occurrence of malo-lactic fermentation was measured by paper chromatography using the method of Ribéreau-Gayon (11).

Triangular taste tests (12) were used for the taste threshold measurements, and statistical evaluation of the results was measured by χ^2 tests. Solutions of purified and assayed diacetyl were prepared in two dry red table wines of 1967 vintage, which differed in the amounts of diacetyl which they contained, and presented in a range of concentrations to 11 tasters on a minimum of five different days for each concentration. Tasting was continued until the threshold level at a statistical significance of $P < 0.01$ could be obtained for each taster.

Results

The results of the analyses are shown in Fig. 2, which presents in histogram form the diacetyl content of the wines in class intervals of 0.5 ppm, together with the occurrence of malo-lactic fermentation.

The diacetyl values ranged from 0 to 7.5 ppm with a mean of 2.4 ppm. Malo-lactic fermentation had occurred in 71 per cent of the wines, and the mean diacetyl value for these wines was 2.8 ppm. The fermentation had not taken place in 29 per cent of the wines, and the mean diacetyl value for these wines was 1.3 ppm.

The change in diacetyl content of red table wines with time was measured with a range of these wines made from *Vitis vinifera*, variety Shiraz (the Syrah of the Rhone Valley and the Petite Sirah of California) in the Institute's experimental winery, and stored at 15° C during the course of the measurements. The diacetyl content at the end of malo-lactic fermentation was taken as zero time, and the mean loss of diacetyl as a percentage of that present at zero time was 19 per cent at 4 months (3 wines), 22 per cent at 8 months (8 wines), 26 per cent at 12 months (7 wines) and 28 per cent at 18 months (5 wines).

The diacetyl content of a range of dry white table wines made in the Institute's experimental winery from the grape varieties *V. vinifera* vars. Riesling, Clare Riesling and Semillon by different yeasts was measured, but no diacetyl was detected in any of the wines. (The minimum detectable amount measurable by the method was 0.1 ppm).

The results of the taste threshold measurements with diacetyl added to two dry red table wines are given in Table 1, which shows the minimum statistically detectable addition of diacetyl to the two wines. For the wine containing 0.3 ppm the lowest detectable addition was 1 ppm, and for the wine containing 3 ppm the lowest detectable addition was 4 ppm. The first wine was an Australian "flagon-style" red wine and was lighter in colour and had less aroma than the second wine, which was of higher quality.

Discussion

The amount of diacetyl present in some of the wines was surprisingly high, and this was closely related to the occurrence of malo-lactic fermentation, and to the

Table 1

Taste thresholds of diacetyl added to two dry red table wines

Wine	Grenache 1967	Shiraz 1967
Diacetyl content	0.3	3.0
Taster	Minimum detectable added diacetyl ppm	
A	6	10
B	1	6
C	1	4
D	6	10
E	4	4
F	4	4
G	>10	>10
H	>10	>10
I	>10	>10
J	>10	10
K	—	>10

L.S.D. ($P < 0.01$).

presence of a butter-like aroma in these wines. The results in Fig. 2 are of considerable interest in that they appear to be the summation of two separate sets of data, depending on whether or not the wines had undergone a malo-lactic fermentation. As expected, the wines which had undergone malo-lactic fermentation contained a wide range of diacetyl, the amounts probably being related to the type of bacteria present in the various wines, as it is known that diacetyl formation is influenced by the strain of bacteria (6, 7). However, an appreciable number of wines, which had not undergone malo-lactic fermentation, contained up to 2.5 ppm diacetyl and a few contained up to 5.5 ppm. We infer that the diacetyl in these wines was produced by yeast, and it is known that yeasts differ in this regard (13), but the range of values was surprisingly large.

A greater range of diacetyl concentration was present than was generally reported in the literature. Peynaud (15) and Peynaud and Lafon (16) found amounts up to 3 ppm in a range of red Bordeaux wines, and up to 6 ppm in six Algerian wines, whilst Kielhöfer and Würdig (17) and Dittrich and Kerner (18) found that normal German wines, mainly white, contained less than 1 ppm. Dittrich and Kerner considered that wines containing above 0.9 ppm were faulty, and their highest value for such wines was 4.3 ppm. Kunkee *et al.* (19) found diacetyl levels in 9 Southern California wines ranging from less than 2 to 8 ppm.

Various factors have been reported in the literature as influencing the amount of diacetyl formed by micro-organisms. These include the strain of micro-organism, amounts of citric acid, pyruvic acid and amino acids (particularly valine) in the medium, aeration, agitation, and temperature of fermentation. Further wo[illegible] progress in our laboratory on the influence of some of these factors on the [illegible] content of wine.

The reduction in diacetyl content of wines stored at constant temperat[illegible] dicates an initial decrease, amounting to 19 per cent reduction in the fir[illegible] months following the termination of malo-lactic fermentation, when diacet[illegible]

1655

tent is at its maximum level, and a gr[illegible] of 28 percent [illegible] 13 months. Considering the chemical reactivity of diacetyl, its [illegible] stability in dry red wine is surprising. Possibly the anthocyanins and tannins present have a protective function, because we have found that diacetyl added to white wines disappears rapidly.

The taste threshold tests showed that ability to detect a difference in diacetyl level depended on the type of wine and the original diacetyl content. Tasters differed widely in their ability to detect differences in diacetyl levels, which was expected from the results of previous threshold measurements with other compounds (8). It has been our experience that a small amount of diacetyl, 1 to 4 ppm depending on the wine, added complexity to the aroma and improved quality. Amounts above 3 to 4 ppm (again depending on the wine), became increasingly evident as diacetyl and at higher levels such as 5 to 7 ppm the wines had a distinct butter-like aroma, which was considered objectionable. It is interesting to note that 0.5 ppm is regarded as a high value for beer (20).

Summary

The diacetyl content of 466 Australian dry red table wines ranged from less than 0.1 ppm to 7.5 ppm with a mean of 2.4 ppm. Malo-lactic fermentation had occurred in 71 per cent of the wines, which had a mean diacetyl level of 2.8 ppm. In wines which had not undergone malo-lactic fermentation the mean diacetyl level 1.3 ppm.

Taste threshold tests showed that a difference of as little as 1 ppm could be detected in a light dry red wine containing 0.3 ppm diacetyl. In a full flavoured darker wine of higher quality containing 3 ppm the minimum detectable addition was 1.3 ppm.

It is considered that diacetyl in amounts up to 2 to 4 ppm, depending on the wine, improved quality by adding complexity to the flavour. Above these levels the aroma of diacetyl became identifiable as such and resulted in a reduction in quality. The diacetyl content of a range of red table wines stored at 15° C showed a mean decrease of 19 per cent in diacetyl content in 4 months, 22 per cent in 8 months, 26 per cent in 12 months and 28 per cent in 18 months.

Acknowledgements

The authors wish to express their indebtedness to the many wine Companies which kindly submitted wines for this study, and to the staff of The Australian Wine Research Institute and the Division of Horticultural Research, CSIRO, who participated in the tastings.

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Eingegangen am 20. 5. 1969

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1655

Food Technology Vol 15 p 137 — 40 1694

The Use of Starter Distillate for Flavoring Butter*

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(Manuscript received July, 1960)

SUMMARY

The feasibility of flavoring Canadian butter with starter distillate and with a commercial preparation of synthetic diacetyl was investigated at various concentrations. Although official graders preferred control samples to flavored samples, one-third of the members of a taste panel had a definite preference for the flavored samples and only one-fifth preferred the control samples. The remaining panel members showed no preference. Starter distillate butters were preferred to "commercial diacetyl" butters, and 0.50 oz of distillate per 100 lb of fat was preferred to 0.25 oz or 1 oz, whereas for "commercial diacetyl" the preference decreased with increasing concentration.

The levels of acetylmethylcarbinol and of diacetyl in the flavored butters were respectively in the range 0.11–0.27 ppm and 0.24–0.64 ppm. These levels did not change even after 1 year storage at −10° F. Peroxide values indicated that the keeping quality of the butter was unaffected by either flavoring.

It is well established that butter made from acid cream has poor keeping quality. The requirements for long storage of butter have led to changes in buttermaking procedures to enhance keeping quality. Some of these changes, such as the use of unripened cream, the standardization of the cream to a low acidity, and possibly the washing of the butter granules, have harmed flavor. Since butter is consumed mainly for its flavor, the question arises whether the bland-flavored butter that results from overemphasis on keeping quality would be preferred by most consumers free to choose a more highly flavored butter.

It is desirable to search for means of enhancing butter flavor without harming keeping quality. Butter flavor can be enhanced by ripening the cream, avoiding excessive neutralization of the cream acidity, eliminating the washing of butter granules, adding starter to cream or butter, or adding flavoring materials such as diacetyl or starter distillate. For keeping purposes, ripened cream butter is out of the question. The standardization of the acidity of butter serum to a pH above 7.0 has never been shown to improve keeping quality. Butter washing has been found (White *et al.* 1956) to be of no help to keeping quality under present Canadian conditions. The presence of starter organisms, even when the factor of acidity is eliminated, has been shown by Wiley *et al.* (1939) to harm the keeping quality of butter. However, recent reports by McDowall *et al.* (1960 a, b) indicated that the use of sweet-cream starter butter does not harm the keeping quality of butter. The use of pure diacetyl has been found to give an unnatural aroma to butter (Babel and Hammer, 1944; Hunziker, 1940; McDowall, 1953; Mohr and Eichstadt, 1935).

Ruehe and Ramsey (1935) improved the aroma of butter by adding starter distillate to either cream or butter. Beck *et al.* (1938) found that this procedure does not affect the keeping quality of butter. Various workers (Babel and Hammer, 1944; McDowall *et al.*, 1960 a, b) have reported that the diacetyl content of butter decreased on storage. No pro-oxidative effect of diacetyl in butter has ever been reported at concentrations below 2 ppm.

Since the manufacture of starter butter requires specialized knowledge, skill, and equipment, it was thought preferable to study more practical means of enhancing butter flavor. The objectives of this study were to determine the stability of some flavor additives in stored butter, their effect on the keeping quality of butter, and whether butter flavored with starter distillate or a commercial diacetyl flavoring would be preferred to conventional butter by a small panel of tasters.

EXPERIMENTAL

Manufacturing. Twenty-seven lots of butter were made from Special Grade cream obtained from the Institute Dairy. They involved three flavoring treatments, three levels of concentration, and three replications. The flavoring treatments were: control, starter distillate, and commercial diacetyl flavoring, and the levels of concentration were 0.25, 0.50, and 1.00 oz per 100 lb of butter. The fat content of the cream was 36%; the acidity, initially 0.11%, was standardized to 0.10% with sodium sesquicarbonate. The cream was pasteurized 10 min at 180° F, cooled to 46° F, and held there until churning. Average churning temperature was 48° F. Just before working, each churning of butter was split into three lots for the following treatments: (a) control, (b) starter distillate added, (c) diacetyl flavoring added. The nine churnings were made at intervals of about three weeks, from May to November, to allow time for the analyses.

The starter distillate and the diacetyl flavoring were both from commercial sources, and both contained 0.11% diacetyl and no measurable quantity of acetylmethylcarbinol. The diacetyl flavoring did not contain any citric, acetic, or propionic acid. Since the loss of diacetyl in the buttermilk is very high (Mohr and Wellm, 1937), the flavoring was added to the butter at the salting-stage. The salt content was standardized at 1.9%, and the moisture content at 16%.

Samples from each split churning were saved for the initial analyses. Some 20 one-pound prints from each split churning were wrapped in parchment paper, boxed, and stored at −10° F until examination.

Methods of analysis. Samples from each split churning were scored for flavor initially, at 6 months, and at 12 months, by three government graders who had no knowledge of the experiment. A study of flavor preference was made with an experienced taste panel of 15 people. This panel is regularly used for the appraisal of flavor, odor, or other organoleptic properties of various dairy products. In addition, a detailed procedure of tasting was given to each panelist, and several blank tasting sessions were conducted to train the panel. To reduce the work of the taste panel it was assumed that the three control lots in each replicate were alike in flavor, leaving seven samples for examination in each replicate.

A ranking procedure was used to establish flavor preference. A balanced incomplete block design was used whereby the seven treatments in each of the three replicates were presented to the panel in 35 blocks of 3 units. Each block of samples was ranked

* Contribution No. 37.

1694

Table 1. Effect of added flavoring on flavor scores of stored butter (3 replications)

Flavoring per 100 lb butter	Storage at −10° F	Control 1	Control 2	Control 3	Starter distillate 1	Starter distillate 2	Starter distillate 3	Diacetyl flavoring 1	Diacetyl flavoring 2	Diacetyl flavoring 3	Totals
0.25 oz	Initial	39	39.5	40	40	39	39	39.5	38	38	352
	6 months	39	39	39	39	39	39	39	39	39	351
	1 year	38.5	40	39	39	39.5	39	39	39	38.5	351.5
0.50 oz	Initial	40	40	39	38.5	39	38	37.5	39	38	349
	6 months	39.5	40	39.5	39.5	39.5	39	38.5	39	39	353.5
	1 year	39.5	39	39	39	38.5	39	39	38	38	349
1.00 oz	Initial	39	40	40	37	39	38	38	38	38	347
	6 months	40	40	39	39	39	39	38.5	39	38	351.5
	1 year	39	39	39	39	38.5	39	39	38	38	348.5
	Totals	353.5	356.5	353.5	350	357	349	348	347	341.5	

from 1 to 3 in order of preference for flavor by each member of the taste panel. Each treatment appeared five times in the same block with each other treatment.

Samples from each lot were analyzed for diacetyl and acetylmethylcarbinol at 0 and 3 days; 1, 2, 3, and 5 weeks; 2 months; and 1 year, according to the method of Prill and Hammer (1938). Assuming 100% purity for reagent grade diacetyl, the diacetyl recovery was 93%. For determination of the combined diacetyl and acetylmethylcarbinol, 20 ml of 10% ferric chloride were added to 100 g of butter before distillation, and the degassing with nitrogen was omitted. Peroxide values were determined by the method of Lips *et al.* (1943).

RESULTS AND DISCUSSION

Flavor score. The flavor scores given by Canadian official graders in Table 1 and analyzed for significance in Table 2 indicate that the graders preferred the control samples to the flavored butters, and the starter distillate butters to the diacetyl flavoring butters. As expected, preference was in the inverse order of concentration. The 6-month samples received a higher average score than the fresh or the 1-year samples. The flavor preference is indicative of the trend in Canada for official graders to favor butter with "bland" flavor. McDowall (1953) reported a similar observation with New Zealand butter containing 0.05 mg diacetyl. The New Zealand graders criticized the butter as harsh and "overfull" in flavor.

Table 2. Analysis of variance of flavor scores

Source of variation	Degrees of freedom	Mean square
Flavoring	2	5.36[a]
Control vs flavoring	1	8.68[a]
Starter dist. vs commercial diacetyl	1	2.05[b]
Concentration	2	0.53
Linear	1	1.01[b]
Quadratic	1	0.02
Storage	2	0.70[b]
Linear	1	0.02
Quadratic	1	1.39[b]
Flavoring × concentration	4	0.47
Flavoring × storage	4	0.58[b]
Concentration × storage	4	0.53
Flavoring × concentration × storage	8	0.15
Replication	2	0.53
Error	52	0.29

[a] Significant at P = 0.01.
[b] Significant at P = 0.05.

Taste panel. Table 3 shows the rank totals for the 15 panelists. The over-all analysis of variance of the normal varieties (Fisher and Yates, 1953) with the treatment totals adjusted according to Cochran and Cox (1957) indicates that the difference between treatments is almost significant at P = 0.05. The adjusted treatment mean square on a unit basis was 2.01, against a P = 0.05 of 2.24. The concordance coefficient (Kendall, 1948) indicated also that the members agreed on a difference between treatments.

Table 3. Flavor ranking of butters flavored with starter distillate or diacetyl flavoring. (Each value[a] is the sum of 15 judgments by taste panel members)

Block no.	Control	Starter distillate (oz) per 100 lb of fat 0.25	0.5	1	Diacetyl flavoring (oz) per 100 lb of fat 0.25	0.5	1
1	27			28			35
2	30		29	31			
3	29			27		33	
4	32	29		29			
5	35			35	30		
6	35		26				29
7	35					25	30
8	28	28					34
9	32				26		32
10	32		26			32	
11	34	27	32				
12	32		31		27		
13	30	26				34	
14	30				32	28	
15	31	29			30		
16			27	30			33
17				31		26	30
18		25		30			35
19				27	30		33
20			29	32		29	
21		26	29	35			
22			29	29	32		
23		30		27		33	
24				30	30	30	
25		28		29	33		
26			27			32	31
27		30	27				33
28			26		28		36
29		29				29	32
30					31	27	29
31		30			27		33
32		30	26			34	
33			28		32	30	
34		27	32		31		
35		31			27	32	
Totals	469	425	424	411	419	451	485
Adjusted totals	457.2	410.6	410.2	447.7	449.6	451.5	463.2

[a] Increasing values = decreasing preference.

Since taste preference is known to vary with individuals, an over-all analysis of variance of preference data could be misleading when there are reverse preferences by substantial portions of the panel. To obtain the full information from the preference data, the members were grouped on the basis of the significance of the chi-square values of their preference. Table 4 indicates that five members preferred the

1694

Table 4. Grouping of panel members according to their flavor preference

Panelists	Treatment B	C	D	E	F	G	Chi-square
	No. times in five the treatment was preferred to the control.						
Preferring flavored butters							
1	5	1	4	1	2	1	12.4
2	4	5	1	4	5	5	20.4
3	4	3	2	1	5	5	14.0
4	3	5	5	4	3	3	12.4
5	5	5	5	5	4	5	26.8
Preferring control sample							
6	0	1	2	0	2	2	12.4
7	0	0	3	1	3	0	12.4
8	0	1	1	0	1	0	20.4
Showing no preference							
9	3	3	2	4	1	2	4.4
10	2	3	1	1	1	3	6.0
11	1	1	1	3	3	1	7.6
12	3	3	0	1	3	3	7.6
13	3	1	3	0	3	2	7.6
14	3	2	2	2	1	1	4.4
15	1	2	3	1	3	2	4.4

flavored samples to the control, three preferred the control to the flavored samples, and seven showed no preference.

For the panelists showing an over-all preference for the flavored butters, the analysis of variance of the normal variates after adjustments for the treatments for incomplete block analysis (Cochrane and Cox, 1957) showed a highly significant difference between treatments. The multiple range test of Duncan (1955) indicated that the members preferred the flavored butters over the control but had no preference among the flavored butters, varying in flavoring type and in flavoring concentration.

For the three members showing an over-all preference for the control over the flavored samples, the analysis of variance showed a significant difference between the adjusted treatments. The multiple range test of Duncan established that these panelists did not distinguish between the control and the low level of starter distillate, but preferred these to all other treatments. Among the flavored samples, the low and medium levels of starter distillate were preferred to the samples with diacetyl flavoring or with high level of starter distillate.

Acetylmethylcarbinol. The average acetylmethylcarbinol content of the control samples was 0.11 ppm. This value is an agreement with the value of 0.16 ppm reported by Mohr and Wellm (1937) for sweet-cream butter. The acetylmethylcarbinol content of the flavorings used was not measurable by the method used. However, the addition of flavorings to butter effected a slight increase in the acetylmethylcarbinol content, more so for commercial diacetyl than for starter distillate. Even at the rate of 1 oz of flavoring per 100 lb of butter the acetylmethylcarbinol content was raised only to 0.17 ppm and 0.21 ppm for starter distillate and for commercial diacetyl, respectively. The variates storage and concentration had no effect on the acetylmethylcarbinol content of the butter.

According to Hedrick and Hammer (1942), starter butter may contain as much as 16 ppm of acetylmethylcarbinol. McDowell *et al.* (1960 a, b) obtain 1.1–3.9 ppm in their sweet-cream starter butter. They found that storage for 8 months at 14° F effected a slight increase (8–15%), significant at $P = 0.01$, in the acetylmethylcarbinol content of the butter. The increase was not significant after 4 months. Presence of starter and relatively high storage temperature may have accounted for this slight increase.

Diacetyl. The diacetyl content of the control samples averaged 0.16 ppm. This value is in the range of those reported by other workers for sweet-cream butter. Cox (1942) found 0.02–0.20, average 0.07 ppm, Barnicoat (1937) 0.05 ppm, and Mohr and Wellm (1937) 0.2 ppm.

Starter butters can have as much as 2 ppm diacetyl, as in Danish butter (Pette, 1948), or have less than 0.5 ppm, as in most French butters (Pien *et al.*, 1937). According to Barnicoat (1937) and Davies (1937), the diacetyl content of mild starter butter is about 0.4 ppm, and that of "high-acid" butter is 1.5 ppm. Davies classified butters on the basis of their diacetyl content as follows: 0.2–0.6 ppm is mild, 0.7–1.5 ppm is full, and 2 ppm is strong. With this classification, the flavored butters in our experiment would fall into the category of mild (Tables 5 and 6).

On adding flavoring to butter, there was a loss of about 50% of diacetyl. This percentage loss increased with the level of added flavoring. McDowall (1953) found a similar loss, which he attributed to loss to the atmosphere and to wash water during churning. Since the flavoring was added to the butter at the

Table 5. Diacetyl content (ppm[a]) of stored butter flavored with starter distillate

Storage at −10° F	Control[b]	Oz of added distillate per 100 lb of butter 0.25	0.50	1.00	Mean
Fresh	0.14	0.26	0.39	0.42	0.28
3 days	0.15	0.25	0.30	0.43	0.28
1 week	0.16	0.26	0.29	0.44	0.29
2 weeks	0.11	0.20	0.28	0.33	0.25
3 weeks	0.17	0.24	0.31	0.19	0.28
5 weeks	0.18	0.26	0.32	0.42	0.29
8 weeks	0.13	0.26	0.31	0.40	0.29
1 year	0.15	0.24	0.29	0.13	0.28
Mean	0.16	0.25	0.30	0.41	0.28

[a] Individual values are averages of three replicates.
[b] Control values are averages of nine churnings.

Table 6. Diacetyl content (ppm[a]) of stored butter flavored with diacetyl flavoring

Storage at −10° F	Control[b]	Oz of added distillate per 100 lb of butter 0.25	0.50	1.00	Mean
Fresh	0.11	0.32	0.41	0.61	0.38
3 days	0.15	0.32	0.45	0.59	0.38
1 week	0.16	0.28	0.42	0.64	0.38
2 weeks	0.11	0.27	0.42	0.58	0.35
3 weeks	0.17	0.28	0.45	0.60	0.38
5 weeks	0.18	0.29	0.43	0.58	0.37
8 weeks	0.18	0.30	0.38	0.57	0.36
1 year	0.15	0.26	0.42	0.55	0.34
Mean	0.16	0.29	0.43	0.60	0.37

[a] Individual values are averages of three replicates.
[b] Control values are averages of nine churnings.

1694

salting stage in our experiment, wash water was not a factor. However, the diacetyl could have disappeared by volatilization and by oxidation or reduction.

Tables 5 and 6 show the diacetyl content of butter for both flavorings at various levels. Table 7 indicates

Table 7. Analysis of variance of diacetyl values

Source of variation	Degrees of freedom	Mean square
Flavoring	1	0.137*
Concentration	2	1.161*
Storage	6	0.005
Flavoring × concentration	2	0.126*
Flavoring × storage	12	0.085
Flavoring × concentration × storage	12	0.098*
Error	81	0.075

* Significant at P = 0.01, using the appropriate significant interaction to test for flavoring, concentration, and storage.

that in spite of the equivalent strength of the flavorings, more diacetyl was imparted to butter by commercial diacetyl than by starter distillate. This is substantiated by the significant interaction between flavoring and concentration.

Table 7 also shows that storage for 2 months or 1 year at −10° F did not change the diacetyl content of the butter. In two published papers, McDowall (1960 a, b) found that storage of sweet-cream starter butter at 14° F decreased the diacetyl content by 14–20% in 4 months. Analysis shows their data were significant at P = 0.01. Prolonged storage for 8 months did not change the diacetyl content. The authors did not mention whether the butter was salted. The presence of microorganisms, along with the relatively high storage temperature and possibly a low salt content, probably accounts for the decrease in diacetyl content during storage. As reported by Babel and Hammer (1944), many other workers have observed that the diacetyl content of butter decreased on storage; this was generally attributed to the reducing action of microorganisms or to oxidation by air, especially in the presence of acid or in the absence of added salt. With salted butter, several authors have reported that the diacetyl was more stable.

The keeping quality of the butter was not affected by the presence of flavorings, as indicated by the absence of any difference in peroxide values between the treated and the control samples after 1 year of storage at −10° F.

Acknowledgment

The authors are indebted to the official graders and to the Institute staff for cooperation in grading and ranking the butters.

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German Translation

Possibilities of Aroma Determination in Bread*

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Source: Brot und Geback 20(10): 189-93, 1966.

"Good bread, an honest life and clear thinking: in fact, this is the same thing."
Justus von Liebig

In the past decades, the concern of European nutritional science about a healthy diet has moved to another plane, namely an excessive supply, rather than a shortage, of available foodstuffs and the related rapid and continuous changes in consumption habits are today the reason for a critical evaluation of the food situation on the part of many people. In this case, attention is mainly directed to a development which must be considered as particularly serious, namely the increasing consumption of fat, which in most European countries has already far exceeded the amount considered as optimal and continues to increase steadily.

For our congress, this observation is of special significance and interest because of the inverse relationship between grain consumption and fat consumption by the population. If we disregard the extreme conditions during the wartime and postwar years, in which a shortage of certain foods caused profound changes in consumption habits, a direct relation between grain and fat consumption can be noted, regardless of the fact that concumption habits vary in individual countries and that, for example, grain

* Report presented at the 4th International Grain and Bread Congress in Vienna, May 1966.

makes up a higher percentage of total consumption in Eastern countries of Central Europe than in Western countries. Table 1 gives a comparison of the grain and fat consumption per head of population, based on statistical data from different countries (1-5).

Table 1

Grain and Fat Consumption in Central Europe

Country Land	Year Jahr	Getreide-verzehr (1) (g pro Kopf der Bevölkerung und Tag)	Fett-verzehr (2) (2a)
(3) Bundesrepublik	1964/65	198	131
(4) Niederlande	1961	211	129
(3) Bundesrepublik	1961/62	212	128
(3) Bundesrepublik	1958/59	232	124
(3) Bundesrepublik	1955/56	265	121
(5) DDR	1963	264	121
(6) Schweiz	1959/60	228	120
(5) DDR	1961	269	119
(7) Deutschland	1935/39	303	112
(5) DDR	1955	333	102
(8) Ungarn	1961	375	102
(9) CSSR	1962	364	100
(10) Polen	1962	405	91

1 -- Grain consumption
2 -- Fat consumption
2a - in g per head of population per day
3 -- West Germany
4 -- Netherlands
5 -- East Germany
6 -- Switzerland
7 -- Germany
8 -- Hungary
9 -- Czechoslovakia
10 -- Poland

Although Table 1 already shows the assumed relation between grain and fat consumption, the picture becomes even clearer on hand of a graphic illustration (Fig. 1). From the individual values used in this graph,

a correlation factor of - 0.98 can be calculated, i.e. an extremely good correlation when error possibilities in the statistical consumption calculations are taken into account.

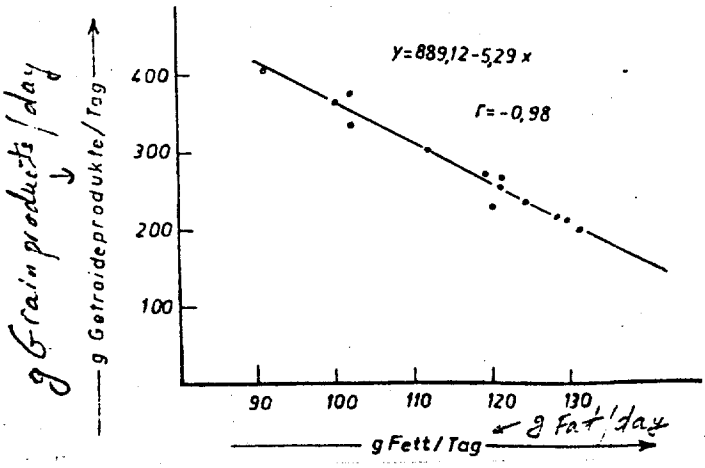

Fig. 1. Relations between consumption of grain products and total fat consumption per head of population.

Consequently, if this relation between grain and fat consumption is used as a base, the concern of nutritional science can only increase, since the decrease in the consumption of grain used for making bread shown in Table 2 derived from figures published in West and East Germany, must necessarily result in an increase of fat consumption. Even if we assume that grain consumption will become stable at a value of about 65 kg per head of population per year, as can be deduced from present development trends in bread-consuming industrial countries, a daily consumption of about 135 g fat per head of population would finally be reached in all countries considered here. This figure is far in excess of the optimal considered normal amount of 80-90 g/day, and causes concern from a health standpoint.

Table 2

Decrease in the Consumption of Grain Used for Making Bread in Germany

(in kg per head of population per year)

Year	West Germany		Germany	
	Jahr	Bundes-republik	Deutsch-land	DDR
1935/39	—	108,0		—
1950/51	96,9	—		—
1955/56	90,9	—		117,3
1956/57	88,5	—		—
1957/58	86,1	—		109,6
1958/59	82,5	—		104,0
1959/60	79,4	—		96,2
1960/61	77,3	—		92,9
1961/62	74,8	—		96,5
1962/63	72,3	—		91,9
1963/64	70,9	—		94,3

East Germany

From such considerations, it follows necessarily that nutrition science must be particularly concerned with maintaining (preserving) and even increasing grain and bread consumption. We must therefore find the causes which might be responsible for the decreased bread consumption. Although there are many different reasons for the corresponding change in our consumption habits, the question which should be at the center of such considerations is whether today's bread corresponds to the ideas of the consumer and whether we should pay any attention to the frequent opinions of older people, who state that the bread used by our ancestors had a better flavor than our present day bread baked in modern installations.

Especially in regard to flavor quality, no exact answer can be given to the above question, due to the absence of a comparable value scale. The organoleptic test is not suitable for recording a slight decrease in the flavor quality of bread which may take place during the course of ration-

alization and improvement measures of the baking process. Thus, is it possible that the overall technical progress, which has introduced radical changes in bread manufacturing during the past decades, could have actually caused a considerable deterioration of the bread's flavor?

We must admit here that today's organoleptic analysis has no longer remained standing in the primitive state of yesterday. The modern and frequently used methods for evaluating the flavor of foods rest primarily on the application of statistical methods aimed at better safeguarding and defining the numerical figures obtained by organoleptic means. These methods, which are based in particular on difference testing, e.g. in the form of the triangular test, or on quality testing, such as in the form of the so-called profile method, have provided a long time ago a well-founded basis to the field of sensory analysis, which earlier has often been considered as unscientific (6-9).

On the other hand, we must always clearly recognize the limits of such testing procedures. These limits are set precisely by the sensitivity and versatility of our sense organs. A 5 or 10 percent decrease in the flavor of any kind of food product, e.g. as a result of storage effects or a certain change in the technology, will not be detected in most cases in the organoleptic test, because the abundance (richness) of individual sensations makes it difficult, if not impossible, to perform an organoleptic detection (perception) of the difference in flavor when these differences are so slight. Therefore, modern methods of sensory analysis are quite capable of detecting without any difficulty definite differences in flavor, but not slight differences.

As a matter of fact, considerations of this kind have provided consider-

able impetus to analytic aroma research on food products during the past 10 years. An objective approach to flavor sensitivity by means of analytic chemical methods appears to be attractive at first glance, since even slight changes in flavor, which are hard to detect by organoleptic means, can hardly escape analytic determination. Research on aromatic components of food products, which has been going on, primarily in the U.S.A., with considerable expenditures of modern equipment and capacity, has already disclosed to us today that such analytical procedures also have their limits. Here also, the evaluation of analytical results is difficult in view of the sensitivity and versatility of our sense organs, because the latter are able to record simultaneously a large number of isolated impressions given by specific substances and to combine these into a definite general impression.

Table 3, showing the number of aromatic products detected so far in bread, clearly illustrates the difficulty in flavor testing by analytical chemical methods. Such a test can be used with certain prospects for success only when it is possible to determine the amounts of individual substances recorded (perceived) by our sense organs. In view of the large number of aromatic products present in bread, such an attempt seems to be almost hopeless from the very beginning, and quite hopeless when we consider the different flavor effectiveness of these aromatic products (26).

In applying such a critical scale, we should keep in mind, however, that the aim of an analytical chemical determination of the flavor of food products cannot be set as far as to expect that the flavor impression obtained during consumption can be represented by a figure or a sum of figures. The analytical determination should rather be considered as an auxiliary product, which can be advantageously used, along with other criteria, in answering

Table 3

Aromatic products found in bread and dough.

1 Stoffgruppe	2 Substanz	3 Zuerst identifiziert von	
a) Alkohole	a) Äthanol	Smith/Coffmann	(1960)
	Propanol	Smith/Coffmann	(1960)
	Isobutanol	Smith/Coffmann	(1960)
	Isoamylalkohol	Wick/Figueiredo/Wallace	(1964)
	2-Methylbutanol	Smith/Coffmann	(1960)
	β-Phenyläthanol	Smith/Coffmann	(1960)
	2,3-Butandiol		
b) Säuren	b) Ameisensäure	Schulz/Drews	(1957)
	Essigsäure	—	
	Propionsäure	—	
	Buttersäure	Wiseblatt	(1960)
	Isovaleriansäure	Wiseblatt	(1960)
	Capronsäure	Wiseblatt	(1960)
	γ-Butyralacton	Smith/Coffmann	(1960)
c) Ester	c) Äthylformiat	Smith/Coffmann	(1960)
	Äthylacetat	Johnson/Miller/Curnutte	(1958)
	Äthyllactat	Smith/Coffmann	(1960)
	Äthylpyruvat	Wiseblatt/Kohn	(1960)
	Äthyllaevulinat	Wiseblatt/Kohn	(1960)
	Propandiol-monoacetat	Smith/Coffmann	(1960)
d) Aldehyde	d) Formaldehyd	Ng/Reed/Pence	(1960)
	Acetaldehyd	Wiseblatt/Kohn	(1960)
	Propanal	Wick/Figueiredo/Wallace	(1964)
	Butanal	Miller/Johnson/Robinson	(1961)
	Isobutanal	Ng/Reed/Pence	(1960)
	Pentanal	Ng/Reed/Pence	(1960)
	2-Methylbutanal	Ng/Reed/Pence	(1960)
	3-Methylbutanal	Rothe	(1960)
	Hexanal	Ng/Reed/Pence	(1960)
	2-Äthylhexanal	Wiseblatt/Kohn	(1960)
	2-Butenal	Wick/Figueiredo/Wallace	(1964)
	Methylglyoxal	Baker/Parker/Fortmann	(1953)
	Crotonaldehyd	Wiseblatt/Kohn	(1960)
	Furfurol	Komm/Lehmann	(1940)
	Oxymethylfurfurol	Kretovic/Tokareva	(1950)
	Benzaldehyd	Kohn/Wiseblatt/Fosdick	(1961)
	Phenylacetaldehyd	Rothe	(1960)
	Zimtaldehyd	Smirnova/Tokareva/Kretovic	(1964)
e) Ketone	e) Aceton	Wiseblatt/Kohn	(1960)
	2-Butanon	Ng/Reed/Pence	(1960)
	2-Pentanon	Wick/Figueiredo/Wallace	(1964)
	2-Hexanon	Wiseblatt/Kohn	(1960)
	3-Heptanon	Wiseblatt/Kohn	(1960)
	Acetoin	Visser't Hooft/de Leeuw	(1935)
	Diacetyl	Visser't Hooft/de Leeuw	(1935)
f) Schwefelverbindungen	f) Methylmercaptan	Rotsch/Dörner	(1957)
	Methional	Rothe	(1960)

1 -- Product group
2 -- Compound
3 -- Identified for the first time by

a) Alcohols

a) Ethanol
Propanol
Isobutanol
Isoamyl alcohol
2-methyl butanol
β-phenylethanol
2,3-butanediol

b) Acids	b) Formic acid Acetic acid Propionic acid Butyric acid Isovaleric acid Caproic acid γ-Butyrolactone	c) Esters	c) Ethyl formate Ethyl acetate Ethyl lactate Ethyl levulinate Propanediol mono-acetate
d) Aldehydes	d) Formaldehyde Acetaldehyde Propanal Butanal Isobutanal Pentanal 2-Methylbutanal 3-Methylbutanal Hexanal 2-Ethylhexanal 2-Butenal Methyl glyoxal Crotonaldehyde Furfural Hydroxy methyl furfural Benzaldehyde Phenyl acetaldehyde Cinnamaldehyde	e) Ketones	e) Acetone 2-Butanone 2-Pentanone 2-Hexanone 3-Heptanone Acetoin Diacetyl
		f) Sulfur Compounds	f) Methylmercaptan Methional

some definite questions of detail. A few examples will demonstrate that, it is possible, in fact, to make statements in that direction.

The flavor quality of bread is known to be influenced by the baking time, because more aromatic compounds are formed in the crust with increasing time of exposure to heat. The optimum baking time of the bread types tested in Table 4 is 60 minutes. It was found that the content of aromatic products increases with increasing baking time, and that apparently there is a relation between the content of the products selected in the table and the flavor intensity.

Table 5 shows, on hand of studies performed on white bread by Linko, Johnson and Miller (27), as well as our own studies on rye bread, that,

Table 4

Aldehyde Formation in the Crust of Rye Bread as A Function of the Baking Time

(mg % of dry substance)

Baking time

	Backzeit (Min.) 30	50	70
Krustenstärke (mm) — Crust strength	1	3	4,5
Geschmack der Kruste — Taste of crust	malzartig mild, wenig Aroma (1)	angenehmer Röstgeschmack, schwach bitter, aromatisch (2)	starker Röstgeschmack, stark bitter, vollaromatisch (3)
Acetaldehyde	0,95	1,56	1,89
Furfurol	0,33	0,74	1,67
Isobutanal	0,02	0,19	0,70
Isopentanal	0,50	2,03	3,60
Methylglyoxal	0,60	1,00	1,30

1 -- mild, malt-like flavor, little aroma
2 -- pleasant roasted flavor, slightly bitter, aromatic
3 -- strong roasted flavor, strongly bitter, full aromatic

parallel to a stale formation of bread, a decrease in the content of certain carbonyl compounds is noted. In the crumb, the content of aromatic compounds increases at first by diffusion from crust areas to the center, and the content also drops here later. However, also in this case, a change in the content of carbonyl compounds apparently runs parallel to certain definite changes in flavor.

Whereas the above examples merely show a relationship between certain definite flavor sensations and the content of individual aromatic products, the following example already leads us to a concrete questioning. It is known that with an increasing proportion of wheat the flavor of mixed rye-wheat bread becomes more neutral and milder, a fact which cannot be attri-

buted only to the decrease in acid content. It was to be determined whether this transition to a more neutral flavor runs parallel to the amount of wheat flour added, or whether there is a limit here between a rye bread flavor and a mixed bread flavor. For this purpose, an organoleptic study was first carried out. Crust samples of rye bread, containing 0, 20, 40 and 60% wheat flour were compared by numerous testers in a triangular test. In this case, the differences shwon in Table were obtained, namely an apparently definite decrease in flavor during transition from a 20 to a 40% wheat flour addition.

Table 5

Changes in the Content of Carbonyl Compounds During Bread Storage (mg/100 g)

Storage days	White bread					Storage days	Rye bread				
	Weißbrot*)						Roggenbrot**)				
Lagertage	Formaldehyde	Aceton	Acetaldehyde	Isovaleraldehyde		Lagertage	Methylglyoxal	Furfurol	Acetaldehyde	Isovaleraldehyde	
		Kruste — Crust						Kruste — Crust			
0	0,91	15,8	2,01	2,62		1	1,01	7,69	2,73	3,59	
3	1,04	13,1	2,14	2,60		3	0,83	3,92	2,02	3,76	
7	0,85	7,0	1,54	2,11		6	0,73	1,64	1,86	1,48	
		Krume — Crumb						Krume — Crumb			
0	0,20	0,88	0,36	0,45		1	0,12	0,24	0,30	0,12	
3	0,29	2,85	0,72	0,95		3	0,13	0,28	0,30	0,20	
7	0,19	0,78	0,49	0,46		6	0,04	0,17	0,22	0,19	

* Excerpts from studies of Linko, Johnson and Miller (27).
** Personal studies.

Table 6

Organoleptically Detectable Differences in Flavor Between Grey Rye Bread Containing Various Admixtures of Wheat Flour

(Trigangular test of crumb bread; + = definite difference; - = uncertain difference; flour types R 1150, W 812 used; baking time, 60 min.).

1 Roggenmehl / Weizenmehl	100/0	80/20	60/40	40/60
100/0	./.	—	+	+
80/20	—	./.	+	+
60/40	+	+	./.	—
40/60	+	+	—	./.

1 -- Rye flour/wheat flour.

As can be seen in Table 7, the analysis of individual aromatic products confirms this fact to a certain extent. Out of the 4 aromatic compounds tested, 3 show a tendency to decrease with increasing wheat flour content of the bread. However, for these 3 compounds, the jump is the greatest in each case when the wheat flour content in the mixed bread lies between 20 and 40%. The question under consideration can therefore be answered with greater assurance from two different sides, namely by means of an organoleptic and chemical test, that the typical rye bread flavor is preserved only up to a wheat flour addition of 20%.

In all examples selected here, the number of aromatic products considered is very small in comparison to the total number of compounds which have so far been detected in bread. This is due to the difficulty of the method which so far has made it possible to separate and quantitatively determine only certain specific compounds with a tolerable effort. It is

Table 7

Changes in the Content of Carbonyl Compounds in Rye Bread Crumb With Increasing Proportions of Wheat Flour*

1 Brotsorte (Mehlrohstoff)	2 Säuregrad	3 – Gehalt (mg-%) an Methylglyoxal	Furfurol	Isovaleraldehyde	Acetaldehyde
100 % R 1150	8,9	0,15	0,16	0,28	0,32
80 % R 1150 + 20 % W 812	8,0	0,13	0,15	0,25	0,34
60 % R 1150 + 40 % W 812	7,0	0,09	0,11	0,07	0,38
40 % R 1150 + 60 % W 812	5,3	0,09	0,10	0,07	0,27

1 -- Bread type (flour raw material)
2 -- Degree of acidity
3 -- Content (mg 5) of

* 1.5 kg breads, 60 min. baking time, crumb samples taken from center of bread after 24 hrs storage.

obvious that considerable error possibilities must be taken into account with such an incomplete analysis. The following final example demonstrates this weak aspect of analytical aroma research performed so far.

Table 8 gives values for the content of 5 aromatic products, recorded during long-term baking in a steam baking chamber. If we consider first the total extinction as a measure of the sum total of volatile aromatic products, then we note a continuous increase of this factor, except for a drop occurring in the initial stage caused by evaporation of low-boiling aromatic products in the dough. Since the flavor intensity also shows a continuous increase with increasing baking time, again there seems to be a relation between the total content of carbonyl compounds and the flavor intensity.

Table 8

Content of Carbonyl Compounds in Pumpernickel Bread As A Function of Baking Time

(data in µg/100 g fresh substance, average values from 2 series of tests)

1 Backzeit (Std.)	2 Gesamt-extinktion*)	Methylglyoxal	Furfurol	Isopentanal + Isobutanal	Diacetyl	Acetaldehyde
0	1,56	144	0	469	40	1668
2	1,36	162	120	218	28	628
4	1,78	233	300	335	33	1111
6	2,27	291	550	243	41	921
8	2,65	380	710	221	—	1118
10	2,83	378	900	335	46	1096
12	3,19	464	1130	395	—	1201
16	3,55	492	1670	—	29	—
20	4,45	444	2800	—	32	—
24	4,82	393	3790	312	32	1020
3 Als Vergleich:						
Graubrotkrume 4		170	190	310	20	310
Weißbrotkrume 5		70	30	240	20	200
Roggenvollkornbrotkrume 6		190	280	350	10	500

1 -- Baking time (hrs)
2 -- Total extinction *)
3 -- As comparison
4 -- Grey bread crumb
5 -- White bread crumb
6 -- Rye whole-meal bread crumb

* Extinction values of dinitrophenylhydrazones in CCl_4-solution, measured in an Elko II colorimeter (Filter S 38).

In the concentration values found for individual products, these parallels are noted only in furfural, which is an aromatic compound of low volatility. In the other products, there is an overlapping (superposition) of novel formation and evaporation of products during the baking process, so that none of these products can be considered as an indicator of flavor impression. A complete and all-around picture could be obtained only under the condition that all aromatic products could be considered and determined quantitatively, a procedure that cannot be realized at the present

time with a tolerable effort. From the increase of the total extinction with increasing baking time we can conclude that the great increase noted with furfural also probably takes place with other aromatic products of low volatility. Consequently, when the analytical study is limited to a few aromatic products, inaccurate or even false conclusions are unavoidable in many cases.

This last example was intended to show that an indicator analysis involving the determination of the flavor of individual aromatic products must always be viewed with a certain amount of criticism. One should be conscious here also of the possibilities and limitations of such methods; if one wants to avoid erroneous conclusions. These restrictions will remain valid at least until it will be possible to define and determine, from the total range of aromatic products, those compounds which are primarily responsible for flavor.

On the other hand, we can be assured that the prospect of a chemical-analytical determination of aromatic compounds in food products will assume an increasing importance. Undoubtedly, we stand today at the starting point of a development which will exert a positive effect not only from a scientific viewpoint, but also in regard to the quality of our bread. In fact, the application of such methods will make it possible to counteract against the further decrease in bread consumption, with its unfavorable consequences for our health. The possibility of an objective evaluation of the flavor intensity can provide us with a valuable auxiliary tool for directing modern bread manufacturing processes to a greater extent towards those properties of bread which most attract consumers, namely flavor and aroma.

1740

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1740

Gutes Brot, rechtes Leben und klares Denken:
es ist eigentlich dasselbe.

Justus von Liebig

1740

Brot. Gebäck 20(10) 189-93 (1966)

Möglichkeiten der Aromabestimmung bei Brot*)

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Table 1

Die Sorge der europäischen Ernährungswissenschaft um eine gesunde Ernährung hat sich in den letzten Jahrzehnten auf eine andere Ebene verlagert: Nicht Mangel an Nahrungsmitteln, sondern deren Überfluß und eine damit verbundene rasche und kontinuierliche Veränderung der Verzehrsgewohnheiten sind heute Anlaß zu vielseitiger kritischer Betrachtung. Dabei liegt das Hauptaugenmerk auf einer Entwicklung, die als besonders bedenklich anzusehen ist, nämlich der ständigen Zunahme im Verzehr an Fett, der in den meisten europäischen Ländern das für optimal angesehene Maß bereits weit überschritten hat und ständig weiter im Zunehmen begriffen ist.

Für einen Brotkongreß ist diese Feststellung deshalb von Bedeutung und Interesse, weil zwischen dem Getreideverzehr und dem Fettverzehr der Bevölkerung eine umgekehrte Beziehung besteht. Wenn man von den extremen Verhältnissen der Kriegs- und Nachkriegsjahre absieht, denen der Mangel an bestimmten Nahrungsmitteln eine tiefgreifende Änderung der Verzehrsgewohnheiten erzwang, läßt sich für die Länder Mitteleuropas eine unmittelbare Abhängigkeit zwischen Getreide- und Fettverzehr ableiten, und zwar unabhängig davon, daß die Verzehrsgewohnheiten in den einzelnen Ländern variieren und zum Beispiel im östlichen Mitteleuropa der Getreideanteil einen höheren Prozentsatz des Gesamtverzehrs ausmacht als in den weiter westlich gelegenen Ländern. Tabelle 1 vermittelt einen aus statistischen Unterlagen verschiedener Länder entnommenen Vergleich zwischen Pro-Kopf-Verbrauch der Bevölkerung an Getreide und Fett (1-5).

Tabelle 1
Getreide- und Fettverzehr in Mitteleuropa

Country Land	Year Jahr	Getreideverzehr (1) (g pro Kopf der Bevölkerung und Tag)	Fettverzehr (2)
(3) Bundesrepublik	1964/65	198	131
(4) Niederlande	1961	211	129
(3) Bundesrepublik	1961/62	212	128
(3) Bundesrepublik	1958/59	232	124
(3) Bundesrepublik	1955/56	265	121
(5) DDR	1963	264	121
(6) Schweiz	1959/60	228	120
(5) DDR	1961	269	119
(7) Deutschland	1935/39	303	112
(5) DDR	1955	333	102
(8) Ungarn	1961	375	102
(9) CSSR	1962	364	100
(10) Polen	1962	405	91

Läßt bereits Tabelle 1 die behauptete Beziehung zwischen Getreide- und Fettverzehr erkennen, so wird das Bild noch eindeutiger, wenn eine graphische Darstellung gewählt wird (Abb. 1). Aus den hier benutzten 13 Einzelwerten errechnet sich ein Korrelationskoeffizient von −0,98, d. h. eine bei Berücksichtigung der Fehlermöglichkeiten der statistischen Verbrauchsberechnung außerordentlich gute Korrelation.

Legt man diese Beziehung zwischen Getreide- und Fettverzehr folglich als gegeben zugrunde, so werden sich die

*) Vortrag anl. des 4. Internat. Getreide- u. Brotkongresses, Wien, Mai 1966

1740

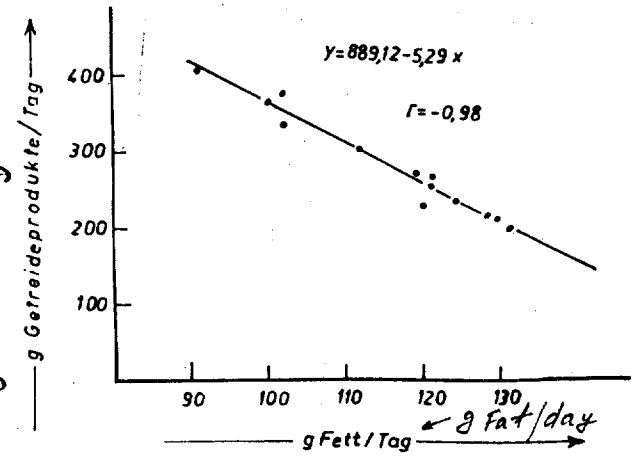

Abb. 1
Beziehungen zwischen dem Pro-Kopf-Verzehr an Getreideprodukten und an Gesamtfett

West Germany Table 2

Tabelle 2
Rückgang des Brotgetreideverzehrs in Deutschland
(kg pro Kopf der Bevölkerung und Jahr) Germany

Year

Jahr	Bundesrepublik	Deutschland	DDR
1935/39	—	108,0	—
1950/51	96,9	—	—
1955/56	90,9	—	117,3
1956/57	88,5	—	—
1957/58	86,1	—	109,6
1958/59	82,5	—	104,0
1959/60	79,4	—	96,2
1960/61	77,3	—	92,9
1961/62	74,8	—	96,5
1962/63	72,3	—	91,9
1963/64	70,9	—	94,3

East Germany

Bedenken der Ernährungswissenschaft nur verstärken, denn der aus Tabelle 2 für das Beispiel der Bundesrepublik und der DDR abzuleitende Rückgang im Verbrauch an Brotgetreideerzeugnissen muß zwangsläufig eine weitere Erhöhung des Fettkonsums nach sich ziehen. Selbst wenn man voraussetzt, daß sich der Getreideverzehr schließlich auf einen Wert von ca. 65 kg pro Kopf der Bevölkerung und Jahr stabilisiert, wie dies aus dem Trend der jetzigen Entwicklung in den brotverzehrenden Industrieländern abzuleiten ist, so würde schließlich in allen hier zur Diskussion stehenden Ländern ein täglicher Konsum von etwa 135 g Fett pro Kopf der Bevölkerung erreicht werden. Dies ist ein im Vergleich zu der für optimal gehaltenen Norm von 80 bis 90 g pro Tag weit überhöhter und zu gesundheitlichen Bedenken Anlaß gebender Wert.

Aus solchen Überlegungen folgt zwangsläufig, daß es für die Ernährungswissenschaft ein besonderes Anliegen darstellt, den Getreide- und Brotkonsum zu erhalten oder gar zu steigern. Es gilt daher, nach den Ursachen zu forschen, welche für den Rückgang des Brotverzehrs verantwortlich sein dürften. Obwohl die Gründe für eine entsprechende Wandlung unserer Verzehrsgewohnheiten vielschichtig sind, steht doch im Mittelpunkt solcher Überlegungen die Frage, ob das Brot von heute den Vorstellungen des Verbrauchers entspricht oder ob man der häufigen Behauptung älterer Leute Gewicht beimessen darf, die meinen, das Brot unserer Vorfahren habe besser geschmeckt als unser heutiges, auf modernen Anlagen erzeugtes Brot.

Speziell im Hinblick auf die Geschmacksqualität ist diese Frage bisher nicht exakt zu beantworten, weil es an einem vergleichbaren Wertmaßstab fehlt. Der organoleptische Test ist nicht geeignet, einen etwa im Zuge von Rationalisierungsmaßnahmen zu verzeichnenden geringen Abfall der Geschmacksqualität von Brot zu registrieren. Kann so nicht aber die Summe des technischen Fortschritts, welcher die Brotherstellung in den letzten Jahrzehnten von Grund auf verändert hat, tatsächlich einen wesentlichen Geschmacksabfall herbeigeführt haben?

Dabei ist anzuerkennen, daß auch die organoleptische Analyse heute nicht mehr auf dem primitiven Stand von gestern stehengeblieben ist. Die bereits vielfach eingesetzten modernen Verfahren zur Geschmacksbewertung von Lebensmitteln stützen sich in erster Linie auf den Einsatz statistischer Methoden zur besseren Absicherung und Definition von auf organoleptischem Wege gewonnenem Zahlenmaterial. Diese Methoden, welche sich insbesondere auf Unterschiedsprüfungen, z. B. in Form des Dreieckstests, oder auf Qualitätsprüfungen, etwa in Form der sogenannten Profilmethode, stützen, haben auch dem früher häufig als unwissenschaftlich betrachteten Wissensgebiet der sensorischen Analyse längst zu einer begründeten Basis verholfen (6—9).

Auf der anderen Seite muß man sich aber über die Grenzen derartiger Testverfahren jederzeit im klaren sein. Diese Grenzen werden gerade durch die Empfindlichkeit und Vielseitigkeit unserer Sinnesorgane gesteckt. Ein Geschmacksabfall eines beliebigen Lebensmittels um 5 oder 10 %, z. B. als Folge von Lagereinflüssen oder einer bestimmten Veränderung einer Technologie, wird sich der Feststellung durch den organoleptischen Test in den meisten Fällen entziehen, weil die Fülle der Einzelempfindungen die organoleptische Wahrnehmung des Geschmacksunterschiedes bei solchen geringen Differenzen erschwert, wenn nicht unmöglich macht. Die modernen Verfahren der sensorischen Analyse sind folglich wohl geeignet, deutliche Geschmacksunterschiede einwandfrei zu erfassen, nicht aber solche geringerer Intensität.

Im Grunde genommen sind es Überlegungen dieser Art gewesen, welche der analytischen Aromaforschung bei Lebensmitteln im letzten Jahrzehnt zu einem beachtenswerten Aufschwung verholfen haben. Eine Objektivierung der Geschmacksempfindung auf dem Wege über chemisch-analytische Verfahren erscheint auf den ersten Blick bestechend, denn der analytischen Feststellung können sich auch geringe, organoleptisch schwer nachweisbare Veränderungen kaum entziehen. Die mit erheblichem Aufwand an modernen Geräten und Kapazität vor allem in den USA vorangetriebene Forschung auf dem Gebiet der Aromastoffe von Lebensmitteln hat aber schon heute auch die Grenzen dieser Verfahrensweise aufgezeigt. Auch hier erschwert die Empfindlichkeit und Vielseitigkeit unserer Sinnesorgane die Auswertung analytischer Ergebnisse, weil diese Organe in der Lage sind, eine Vielzahl von, durch bestimmte Substanzen ausgelösten, Einzelausdrücken gleichzeitig zu registrieren und zu einem bestimmten Gesamteindruck zu kombinieren.

Tabelle 3 macht allein an Hand der Zahl der bis heute im Brot nachgewiesenen Aromastoffe die Schwierigkeit eines chemisch-analytischen Geschmackstestes deutlich. Ein solcher Test kann ja nur dann mit einiger Aussicht auf Erfolg zum Einsatz kommen, wenn es gelingt, die von unseren Sinnesorganen registrierten Einzelsubstanzen mengenmäßig zu bestimmen. Bei der vorhandenen Vielzahl von Aromastoffen im Brot erscheint ein solches Vorhaben aber von vornherein als fast aussichtslos, und erst recht dann, wenn man zusätzlich die unterschiedliche Geschmackswirksamkeit der Aromastoffe in die Betrachtung einbezieht (26).

Tabelle 3

Tabelle 3
Im Brot und Teig vorkommende flüchtige Aromastoffe
(vgl. 10—25)

Stoffgruppe	Substanz	Zuerst identifiziert von	
Alkohole a)	Äthanol		
	Propanol	Smith/Coffmann	(1960)
	Isobutanol	Smith/Coffmann	(1960)
	Isoamylalkohol	Smith/Coffmann	(1960)
	2-Methylbutanol	Wick/Figueiredo/Wallace	(1964)
	β-Phenyläthanol	Smith/Coffmann	(1960)
	2,3-Butandiol	Smith/Coffmann	(1960)
Säuren b)	Ameisensäure	Schulz/Drews	(1957)
	Essigsäure	—	
	Propionsäure	—	
	Buttersäure	Wiseblatt	(1960)
	Isovaleriansäure	Wiseblatt	(1960)
	Capronsäure	Wiseblatt	(1960)
	γ-Butyralacton	Smith/Coffmann	(1960)
Ester c)	Äthylformiat	Smith/Coffmann	(1960)
	Äthylacetat	Johnson/Miller/Curnutte	(1958)
	Äthyllactat	Smith/Coffmann	(1960)
	Äthylpyruvat	Wiseblatt/Kohn	(1960)
	Äthyllaevulinat	Wiseblatt/Kohn	(1960)
	Propandiol-monoacetat	Smith/Coffmann	(1960)
Aldehyde d)	Formaldehyd	Ng/Reed/Pence	(1960)
	Acetaldehyd	Wiseblatt/Kohn	(1960)
	Propanal	Wick/Figueiredo/Wallace	(1964)
	Butanal	Miller/Johnson/Robinson	(1961)
	Isobutanal	Ng/Reed/Pence	(1960)
	Pentanal	Ng/Reed/Pence	(1960)
	2-Methylbutanal	Ng/Reed/Pence	(1960)
	3-Methylbutanal	Rothe	(1960)
	Hexanal	Ng/Reed/Pence	(1960)
	2-Äthylhexanal	Wiseblatt/Kohn	(1960)
	2-Butenal	Wick/Figueiredo/Wallace	(1964)
	Methylglyoxal	Baker/Parker/Fortmann	(1953)
	Crotonaldehyd	Wiseblatt/Kohn	(1960)
	Furfurol	Komm/Lehmann	(1940)
	Oxymethylfurfurol	Kretovic/Tokareva	(1950)
	Benzaldehyd	Kohn/Wiseblatt/Fosdick	(1961)
	Phenylacetaldehyd	Rothe	(1960)
	Zimtaldehyd	Smirnova/Tokareva/Kretovic	(1964)
Ketone e)	Aceton	Wiseblatt/Kohn	(1960)
	2-Butanon	Ng/Reed/Pence	(1960)
	2-Pentanon	Wick/Figueiredo/Wallace	(1964)
	2-Hexanon	Wiseblatt/Kohn	(1960)
	3-Heptanon	Wiseblatt/Kohn	(1960)
	Acetoin	Visser't Hooft/de Leeuw	(1935)
	Diacetyl	Visser't Hooft/de Leeuw	(1935)
Schwefelverbindungen f)	Methylmercaptan	Rotsch/Dörner	(1957)
	Methional	Rothe	(1960)

Bei Anlegen eines derart kritischen Maßstabes darf man jedoch nicht außer acht lassen, daß das Ziel der chemisch-analytischen Erfassung des Geschmackes von Lebensmitteln nicht so weit gesteckt sein kann, daß etwa der beim Verzehr zu verzeichnende Geschmackseindruck in eine Zahl oder eine Summe von Zahlen gefaßt werden soll. Die analytische Bestimmung kann vielmehr nur als ein Hilfsmittel verstanden werden, welches bei der Beantwortung bestimmter Detailfragen vorteilhaft neben anderen Kriterien herangezogen werden kann. Daß in dieser Richtung liegende Aussagen grundsätzlich möglich sind, mag an Hand einiger weniger Beispiele demonstriert werden.

Tabelle 4

Tabelle 4
Aldehydbildung in der Kruste von Roggenbrot in Abhängigkeit von der Backzeit
(mg-% der Trockensubstanz)

	Backzeit (Min.) 30	50	70
Krustenstärke (mm)	1	3	4,5
Geschmack der Kruste	malzartig mild, wenig Aroma	angenehmer Röstgeschmack, schwach bitter, aromatisch	starker Röstgeschmack, stark bitter, vollaromatisch
Acetaldehyde	0,95	1,56	1,89
Furfurol	0,33	0,74	1,67
Isobutanal	0,02	0,19	0,70
Isopentanal	0,50	2,03	3,60
Methylglyoxal	0,60	1,00	1,30

Die Geschmacksqualität von Brot wird bekanntlich durch die Backzeit beeinflußt, weil mit zunehmender Dauer der Hitzeeinwirkung in der Kruste mehr Aromastoffe gebildet werden. Die optimale Backzeit der in Tabelle 4 untersuchten Brotsorte liegt bei 60 Minuten. Es zeigt sich, daß mit zunehmender Backzeit der Gehalt an Aromastoffen ansteigt, daß also zwischen den Gehaltswerten der hier ausgewählten Substanzen und der Geschmacksintensität offensichtlich eine Beziehung besteht.

Tabelle 5 demonstriert an Hand von Untersuchungen von Linko, Johnson und Miller an Weißbrot (27) sowie eigenen Untersuchungen an Roggenbrot, daß parallel zum Altbackenwerden von Brot ein Rückgang im Gehalt an bestimmten Carbonylverbindungen zu beobachten ist. In der Krume erfolgt anfangs eine Zunahme durch Diffusion von Aromastoffen aus den Krustenbezirken zum Zentrum, später fällt auch hier der Gehaltswert ab. Bestimmten geschmacklichen Veränderungen läuft jedoch offensichtlich auch in diesem Falle eine Veränderung im Gehalt an Carbonylverbindungen parallel.

Sollten die vorangegangenen Beispiele lediglich den Zusammenhang zwischen bestimmten Geschmacksempfindungen und dem Gehalt an einzelnen Aromastoffen aufzeigen, so führt uns das folgende Beispiel bereits zu einer konkreten Fragestellung. Mit zunehmendem Weizenanteil wird bei Roggen-Weizen-Mischbrot bekanntlich der Geschmack neutraler und milder, ein Umstand, der nicht nur auf den sinkenden Säuregehalt zurückzuführen ist. Es sollte nun festgestellt werden, ob dieser Übergang zum neutraleren Geschmack der Zusatzhöhe an Weizenmehl parallel läuft

Tabelle 5

Tabelle 5
Änderungen im Gehalt an Carbonylverbindungen während der Brotlagerung
(mg/100 g)

Weißbrot*) Lagertage	Formaldehyde	Acetone	Acetaldehyde	Isovaleraldehyde	Roggenbrot**) Lagertage	Methylglyoxal	Furfurol	Acetaldehyde	Isovaleraldehyde
		Kruste					Kruste		
0	0,91	15,8	2,01	2,62	1	1,01	7,69	2,73	3,59
3	1,04	13,1	2,14	2,60	3	0,83	3,92	2,02	3,76
7	0,85	7,0	1,54	2,11	6	0,73	1,64	1,86	1,48
		Krume					Krume		
0	0,20	0,88	0,36	0,45	1	0,12	0,24	0,30	0,12
3	0,29	2,85	0,72	0,95	3	0,13	0,28	0,30	0,20
7	0,19	0,78	0,49	0,46	6	0,04	0,17	0,22	0,19

*) Auszüge aus Untersuchungen von Linko, Johnson und Miller (27).
**) Eigene Untersuchungen

oder aber, ob hier etwa eine Grenze zwischen roggenbrotartigem Geschmack und Mischbrotgeschmack existiert. Diesem Ziel diente zunächst eine organoleptische Untersuchung. Krumenproben von Roggenbrot mit 0, 20, 40 und 60 % Weizenmehlanteil wurden von zahlreichen Testpersonen im Dreieckstest verglichen. Dabei ergaben sich die in Tabelle 6 dargestellten Unterschiede, nämlich ein offensichtlich deutlicher Geschmacksabfall beim Übergang von 20 auf 40 % Weizenmehlzusatz.

Tabelle 6

Tabelle 6
Organoleptisch wahrnehmbare Geschmacksunterschiede zwischen Roggengraubroten mit Weizenmehlbeimischung

(Dreieckstest von Krumenbroten; + = eindeutiger Unterschied, — = Unterschied nicht gesichert; verwendete Mehltypen R 1150, W 812; Backzeit jeweils 60 Min.)

(1) Roggenmehl / Weizenmehl	100/0	80/20	60/40	40/60
100/0	./.	—	+	+
80/20	—	./.	+	+
60/40	+	+	./.	—
40/60	+	+	—	./.

Nach Tabelle 7 liefert die Analyse einzelner Aromastoffe hierfür eine gewisse Bestätigung. Von den vier getesteten Aromastoffen zeigen nämlich drei die Tendenz einer Abnahme mit zunehmendem Weizenmehlanteil des Brotes. Bei diesen drei Stoffen ist jedoch in jedem Fall der Sprung zwischen 20 und 40 % Weizenmehlanteil im Mischbrot am größten. Die vorliegende Fragestellung läßt sich folglich von zwei Seiten, nämlich durch den organoleptischen und den chemischen Test, und damit viel sicherer dahingehend beantworten, daß der typische Roggenbrotgeschmack nur bis zu einem Weizenmehlzusatz von 20 % erhalten bleibt.

Bei allen hier ausgewählten Beispielen ist die Zahl der in die Betrachtung einbezogenen Aromastoffe sehr klein gewesen im Vergleich zur Gesamtzahl der bisher im Brot nachgewiesenen Stoffe. Dies ist durch die Schwierigkeit der Methodik bedingt, welche bisher nur bestimmte Substanzen mit tragbarem Aufwand abzutrennen und quantitativ zu bestimmen gestattet. Es ist aber selbstverständlich, daß man bei einer derart unvollständigen Analyse erhebliche Fehlermöglichkeiten in Rechnung zu stellen hat. Das letzte Beispiel soll diese schwache Seite der bisherigen analytischen Aromaforschung demonstrieren.

In Tabelle 8 sind die Gehaltswerte an fünf Aromastoffen zusammengefaßt, wie sie beim langdauernden Backprozeß in der Dampfbackkammer zu verzeichnen sind. Betrachtet man zunächst die Gesamtextinktion als ein Maß für die Summe an flüchtigen Aromastoffen, so ist — abgesehen von dem im Anfangsstadium erfolgenden, auf Verflüchtigung niedrig siedender Teig-Aromastoffe zurückzuführenden Rückgang — eine kontinuierliche Zunahme zu vermerken. Da auch die Geschmacksintensität mit steigender Backzeit laufend zunimmt, scheint wiederum eine Beziehung zwischen dem Gesamtgehalt an Carbonylverbindungen und der Geschmacksintensität zu bestehen.

Bei den für die Einzelsubstanzen gefundenen Gehaltswerten findet man diese Parallele aber nur beim Furfurol wieder, das zu den schwerflüchtigen Aromastoffen zählt. Bei den übrigen Substanzen überlagern sich offensichtlich Neubildung und Verflüchtigung im Verlauf des Backvorganges, so daß keiner dieser Stoffe als Indikator für den

Tabelle 7

Tabelle 7
Änderungen im Gehalt von Roggenbrotkrume an Carbonylverbindungen bei zunehmendem Weizenmehlanteil*)

(1) Brotsorte (Mehlrohstoff)	(2) Säuregrad	(3) Gehalt (mg-%) an Methylglyoxal	Furfurol	Isovaleraldehyde	Acetaldehyde
100 % R 1150	8,9	0,15	0,16	0,28	0,32
80 % R 1150 + 20 % W 812	8,0	0,13	0,15	0,25	0,34
60 % R 1150 + 40 % W 812	7,0	0,09	0,11	0,07	0,38
40 % R 1150 + 60 % W 812	5,3	0,09	0,10	0,07	0,27

*) 1,5-kg-Brote mit 60 Min. Backzeit, Entnahme von Krumenproben aus dem Brotzentrum nach 24 Std. Ablagerung.

Tabelle 8

Tabelle 8
Gehalt an Carbonylverbindungen in Pumpernickel in Abhängigkeit von der Backzeit
(Angaben in µg/100 g Frischsubstanz, Mittelwerte von 2 Versuchsreihen)

(1) Backzeit (Std.)	(2) Gesamtextinktion*)	Methylglyoxal	Furfurol	Isopentanal + Isobutanal	Diacetyl	Acetaldehyde
0	1,56	144	0	469	40	1668
2	1,36	162	120	218	28	628
4	1,78	233	300	335	33	1111
6	2,27	291	550	243	41	921
8	2,65	380	710	221	—	1118
10	2,83	378	900	335	46	1096
12	3,19	464	1130	395	—	1201
16	3,55	492	1670	—	29	—
20	4,45	444	2800	—	32	—
24	4,82	393	3790	312	32	1020
(3) **Als Vergleich:**						
Graubrotkrume (4)		170	190	310	20	310
Weißbrotkrume (5)		70	30	240	20	200
Roggenvollkornbrotkrume (6)		190	280	350	10	500

*) Extinktionswert der Dinitrophenylhydrazone in CCl_4-Lösung bei Messung im Elko II-Colorimeter (Filter S 38).

1740

Geschmackseindruck in Frage kommt. Ein vollständiges und abgerundetes Bild könnte sich erst unter der Voraussetzung ergeben, daß sämtliche Aromastoffe in die Betrachtung einbezogen und quantitativ bestimmt würden, ein Vorhaben, das gegenwärtig nicht mit tragbarem Aufwand zu realisieren ist. Aus der Zunahme der Gesamtextinktion mit steigender Backzeit ist zu schließen, daß die beim Furfurol beobachtete starke Zunahme sehr wahrscheinlich auch für andere schwerflüchtige Aromastoffe zutreffen dürfte. Wenn man folglich die analytische Untersuchung auf einige wenige Aromastoffe beschränkt, so werden ungenaue oder gar falsche Schlußfolgerungen in manchen Fällen unvermeidlich.

Dieses letzte Beispiel sollte zeigen, daß einer Indikatoranalyse zur Geschmacksbestimmung auf dem Wege über einzelne Aromastoffe stets mit einer gewissen Kritik begegnet werden muß. Man muß sich auch hier der Möglichkeiten und der Grenzen derartiger Verfahren bewußt sein, wenn Fehlschlüsse vermieden werden sollen. Diese Einschränkung wird zumindest solange ihre Gültigkeit bewahren, bis es gelingt, aus dem Gesamtspektrum der Aromastoffe die für den Geschmack maßgebenden zu definieren und zu bestimmen.

Auf der anderen Seite darf man wohl sicher sein, daß der Perspektive der chemisch-analytischen Feststellung von Aromastoffen in Lebensmitteln steigende Bedeutung zukommen wird. Zweifellos stehen wir heute erst am Anfang einer Entwicklung, die positive Auswirkungen nicht nur in wissenschaftlicher Hinsicht, sondern auch in bezug auf die Qualität unseres Brotes mit sich bringen dürfte. Im Grunde genommen wird damit der Einsatz solcher Verfahren geeignet sein, einem weiteren Rückgang im Brotverzehr mit seinen nachteiligen Folgen für unsere Gesundheit entgegenzuwirken. Die Möglichkeit einer objektiven Erfassung der Geschmacksintensität kann uns ein wertvolles Hilfsmittel in die Hand geben, um die modernen Verfahren der Brotherstellung stärker im Hinblick auf diejenigen Eigenschaften auszurichten, welche den Verbraucher beim Brot am meisten ansprechen, nämlich auf Geschmack und Aroma.

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1780

[Agr. Biol. Chem., Vol. 32, No. 4, p. 405~411, 1968]

(1780)

Chemical Studies on Smelling Compounds in Hen's Egg

Part I. Volatile Carbonyl and Basic Compounds in Egg White

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Received September 20, 1967

Volatile smelling compounds of freezed egg white, freshness of which was kept by freezed storage, were collected by steam-distillation. After DNP-hydrazones of volatile carbonyl compounds were separated into four classes by column chromatography, DNP-hydrazones contained in each class were separated by thin layer chromatography. *Rf* and melting point of recrystalized compounds were compared with those of authentic compounds. Volatile basic compounds were collected as hydrochlorides and detected by paper and thin layer chromatography.

Acetone, acetaldehyde, formaldehyde, 2-pentanone, 2-butanone, diacetyl, except two unknown compounds as volatile carbonyl compounds, and ammonia, methylamine, dimethylamine and putrescine as volatile basic compounds were tentatively identified.

Correlations between these compounds and smell of freezed egg white were discussed.

It is generally accepted that smell of egg yolk is influenced by feeds,[1~2] but it is not clear whether smell of egg white is directly influenced by them. Egg white has a special smell (rather nasty smell) even without the influence of feeds. Ammonia has only been reported as a smelling component in fresh egg white,[3] but other smelling components have not been found yet.

The aim of this study is to separate and identify some volatile carbonyl and basic compounds in egg white.

EXPERIMENTAL

1) Egg white

Egg white which was kept its freshness by freezing at −20°C within two days after laying and storing at the same temperature, was taken out within one month of storage and used after thawing at about 4°C.

2) Collection of volatile smelling components

Ten kilogram of the thawed egg white was dialysed to 10 times its volume of 4°C distilled water with thymol for 3~4 days using visking tube. Outer solution in dialysis was regarded as the sources of smelling compounds.

3) Isolation of smelling compounds

a) Volatile carbonyl compounds. Details of isolation procedure, mainly according to the method of Pippen *et al.*,[4] are shown in Table I.

By this procedure, monocarbonyl 2,4-dinitrophenylhydrazones fraction and poly-carbonyl 2,4-dinitrophenylhydrazones fraction were isolated from the outer solution of dialysis of egg white. Moreover, this procedure was also applied to solutions which were collected after a part of outer solution for dialysis of egg white was passed through Amberlite IR-120 or Amberlite IR for the isolation of volatile basic components or volatile carbonyl components. Both hydrazones from outer solution [illegible]

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TABLE I. ISOLATION PROCEDURE OF VOLATILE CARBONYL COMPOUNDS

Outer sol. in dialysis

Steam distilled to DNP-hydrazine sol (2 g/1 liter 2N HCl) after adjusting outer sol's pH to 2 with phosphoric acid

Distilled sol. with DNP-hydrazone

Keeping at 40 C for 3 hr, standing still at 4 C for 2 days, and then filtered

DNP-hydrazones (A)

Washed with water and dried *in vacuo* at 60 C

Extract with chloroform

Extracted sol.

Dried up by distillation under reduced press.

Dry matter

(Monocarbonyl-DNP-hydrozones)

Res.

Ext. with hot nitrobenzene

Extracted sol.

Concentrated by distillation under reduced press., then cooled and filtered.

Res.

Sol.

Ppt.

(Polycarbonyl-DNP-hydrazones)

TABLE II. ISOLATION PROCEDURE OF VOLATILE BASIC COMPOUNDS

Outer sol. in dialysis

Pass through Amberlite IR-120 (H-form)

Passed sol.

Elute with 4N HCl

Eluate

Concentrated under reduced press.

Concentrate

Steam distill. in alkaline condition (pH 10–11) to dil. HCl solution

Distillate

Dried up by distillation under reduced press.

Dry matter

(Basic compounds mixture)

from the solution treated with ion exchanger were used for analysis.

b) Volatile basic compounds. Details of isolation procedure are shown in Table II.

4) Separation and identification of volatile carbonyl or basic compounds

a) Column chromatography. Class separation of volatile monocarbonyl compounds as 2,4-dinitrophenylhydrazones (DNP-hydrazones) was accomplished by column with silicic acid and Celite.[9] Silicic acid and Celite heated at 140 C for 20 hr were mixed in the ratio of 2:1 and a mixture of which with petroleum ether (less than 60 C) was poured into the column. The volume of adsorbent in the column is 10 cm in length and 3.7 cm in diameter. Hydrazones fraction taken up in small amounts of petroleum ether were poured into adsorbent. Unadsorbed parts were eluted by petroleum ether. Separated hydrazones were taken out with adsorbent from the column individually, and eluted by petroleum ether or alcohol.

b) Thin layer chromatography. Adsorbent mixture for thin layer chromatography of each class of

DNP-hydrazone was prepared by adding 15 g of Kiselguhr-G (Merk) to 30 ml of aqueous solution containing 5.25 g of carbowax-400.[5] Adsorbent mixture for volatile basic compounds was prepared by adding 30 g of Kiselguhr-G to 60 ml of water. Thin layer of 275 u thickness was made with adsorbent mixture on the plate, 20×20 cm, through applicator, and was heated at 105 C for 30 min after standing still for 2 hr at room temperature. Accomplished thin layer was kept in desiccator.

Separated solution of monocarbonyl-DNP-hydrazones or volatile bases hydrochlorides were concentrated or dissolved in small amounts of water respectively. Each prepared sample solution was applied to thin layer chromatography. As developing solvent, petroleum ether was used for carbonyl compounds derivatives[6] and the mixture of phenol, water and acetic acid (75 : 25 : 4, g/v/v)[6] or the mixture of water and acetic acid (8 : 2, v/v)[7] for volatile bases hydrochlorides. Dragendorff reagent or 0.1 % ninhydrin chloroform solution was sprayed on the thin layer to identify third amines or ammonia and other amine hydrochlorides respectively. In that case, spot colors were revealed completely at 110 C for 10 min.

c) Paper chromatography. Aqueous solution of volatile bases hydrochlorides was put on Toyo-filterpaper No. 50 and was developed with upper layer obtained from an equilibrated mixture of *n*-butanol-water-acetic acid (50 : 1 : 49, v/v) in one dimentional ascending method.[8] Spray reagents were the same as in thin layer chromatography. Ammonia and amine hydrochlorides spots were revealed completely at 100 C for 10 min.

d) Melting point. Each DNP-hydrazone extracted from thin layer chromatogram by alcohol was recrystalized by 90% alcohol and dried thoroughly over cupric sulfate under vaccum. Melting point was measured by electric heating using slight amounts of hydrazone crystal.

e) Wave length in adsorption maximum. The measurement was carried out by Hitachi Perkinelmer Spectrophotometer Model 139 using chloroform as solvent of DNP-hydrazone.

5) H. T. Badings and J. G. Wassinki, *Neth. Milk and Dairy J.*, **17**, 132 (1963).
6) G. R. Mizuno, E. McMeans and J. R. Chipault, *J. Agr. Food. Chem.*, **14**, 229 (1966).
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RESULTS AND DISCUSSION

1. Class separation of DNP-hydrazones mixture by column chromatography

The result of column chromatographic separation of monocarbonyl-DNP-hydrazones mixture is shown in Fig. 1. Each fraction, class 1, 2, 3 or 4 in Fig. 1 was of different color respectively. Column chromatography of polycarbonyl-DNP-hydrazones was not carried out, because this derivatives could be recrystalized by hot alcohol and crystals of which melt above 300 C. Therefore, it was tentatively suggested that polycarbonyl-DNP-hydrazone was identical with diacetyl-DNP-hydrazone.

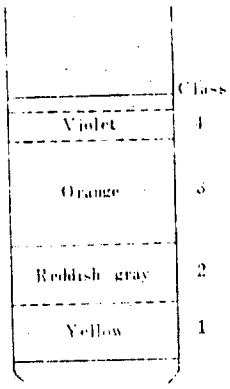

FIG. 1. Class Separation of Monocarbonyl-DNP-hydrazones Mixture by Column Chromatography.

2. Separation of monocarbonyl-DNP-hydrazones in each class by thin layer chromatography

Thin layer chromatogram of monocarbonyl-DNP-hydrazones mixture is shown in Fig. 2. There were no differences of numbers of spot in chromatogram of monocarbonyl-DNP-hydrazones with various concentration, and seven spots were separated clearly.

Eluted monocarbonyl-DNP-hydrazones solution by petroleum or alcohol from each separated class was concentrated under reduced pressure. Concentrated sample solution was put on thin layer and was developed by petroleum ether at room temperature. From

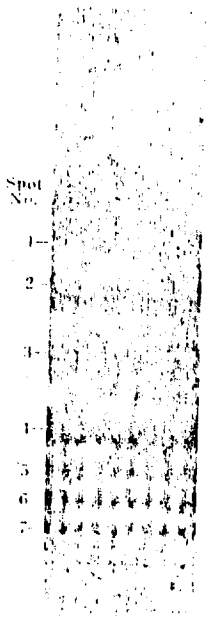

FIG. 2. Thin Layer Chromatogram of Monocarbonyl-DNP-hydrazones Mixture Obtained from Egg White.

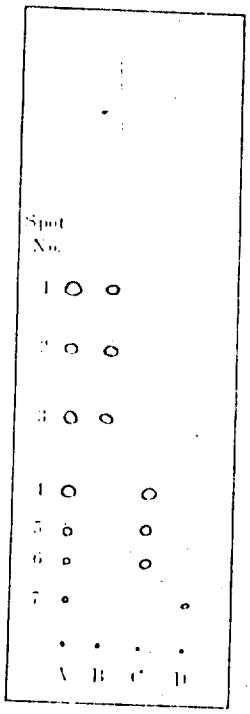

FIG. 3. Thin Layer Chromatogram of Monocarbonyl-DNP-hydrazones Separated in Each Class.

A, Monocarbonyl-DNP-hydrazones mixture; B, Class 2 in CC; C, Class 3 in CC; D, Class 4 in CC; CC: Column Chromatography.

thin layer chromatogram of each concentrated elute by column chromatography, it was elucidated that spot No. 1, 2 and 3 were contained in class 2, No. 4, 5 and 6 in class 3, No. 7 in class 4. (Fig. 3)

Typical thin layer chromatograms, obtained in development of authentic samples and monocarbonyl-DNP-hydrazones mixture, are shown in Fig. 4 and 5, and *Rf* values obtained from these chromatogram were summerized in Table III, together with those of authentic compounds. *Rf* of compounds shown in spot No. 7 of class 4 and spot No. 4 of class 3 were not identical with *Rf* of any authentic compounds used. Compounds in class 1 was not developed.

3. Melting point and wave length of maximum adsorption of separated monocarbonyl-DNP-hydrazone by thin layer chromatography

Each spot appeared on thin layer chromatogram was cut off together with adsorbent mixture and extracted with ether or petroleum ether.

Each separated compound was purified by repeating recrystalization. Crystals obtained were used for measurement of melting point and wave length of maximum adsorption. Results obtained are shown in Table III together with the values of authentic compounds.

Crystals of compounds correspond to spot No. 7 in class 4 and spot No. 6 in class 3 could not be obtained probably because of their trace amounts.

The results summerized in Table III tentatively suggested that volatile carbonyl compounds collected by steam-distillation contain 2-

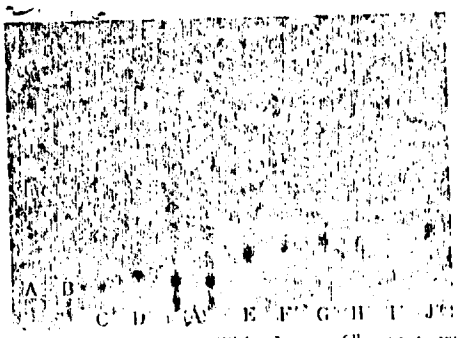

FIG. 4. Comparison of Thin Layer Chromatogram of Monocarbonyl-DNP-hydrazones Mixture and Authentic Samples (some saturated aldehydes and crotonaldehyde).

A′, formaldehyde; B′, acetaldehyde; C′, propionaldehyde; D′, butyraldehyde; E′, hexanal; F′, heptanal; G′, octanal; H′, nonanal; I′, crotonaldehyde; J′, dodecanal; Ⓐ, monocarbonyl-DNP-hydrazones mixture.

FIG. 5. Comparison of Thin Layer Chromatogram of Monocarbonyl-DNP-hydrazones Mixture and Authentic Samples (some ketones and unsaturated aldehydes).

A, acetone; B, 2-butanone; C, 2-pentanone; D, 2-heptanone; E, 2-octanone; F, 2-nonanone; G, hepta-2-enal; H, octa-2-enal; I, nona-2-enal; J, dodeca-2-enal; Ⓐ, monocarbonyl-DNP-hydrazones mixture.

TABLE III. DNP-HYDRAZONES OF CARBONYL COMPOUNDS ISOLATED FROM EGG WHITE

Spot No. in TLC	Carbonyl Compounds tentatively identified	mp (°C)		*Rf* in TLC		λmax	
		I.S.	A.S.	I.S.	A.S.	I.S.	A.S.
1	2-Pentanone	141	138~140	0.41	0.41	366.0	366.5
2	2-Butanone	113	112~113	0.32	0.33	365.0	364.5
3	Acetone*	122	123	0.21	0.22	364.0	364.0
4	--	--		0.16		366.0	
5	Acetaldehyde	167	166	0.07	0.08	354.0	354.0
6	Formaldehyde	--	181	0.04	0.04	--	344.0
7	-	-		0.02		--	
Polycarbonyl	Diacetyl**	300<	300<	—	-		

I.S., Isolated Samples; A.S., Authentic Samples; TLC, Thin Layer Chromatogram

* Elemental analysis shows almost the same nitrogen percentage (23.47%) as that (23.52%) of acetone.

** Shows a violet color in alcoholic KOH.

pentanone, 2-butanone, acetone, acetaldehyde, formaldehyde, diacetyl and two unknown compounds.

4. Chromatographic identification of volatile bases hydrochloride

Results of separation of volatile bases hydrochlorides mixture by paper chromatography and thin layer chromatography are shown in Fig. 6. *Rf* values and spot colors exhibited in chromatogram were listed up in Table IV together with *Rf* values of reference basic compounds. These results show that volatile basic compounds may contain ammonia, putrescin, monomethylamine and dimethylamine.

Weurman and Rooij[9] reported that traces of ethyl and trimethylamine as well as recognizable amounts of methyl and dimethylamine were found in 50 g of whole egg by

9) C. Weurman and Carry De Rooij, *J. Food Sci.*, **26**, 239 (1961).

TABLE IV. VOLATILE BASIC COMPOUNDS FOUND IN EGG WHITE

	Authentic Sample						Isolated Sample			
	a	b	c	d	e	f	1	2	3	4
PC	0.09	0.14	0.21	0.27	0.30	0.45	0.26	0.21	0.10	—
TLC-1	0.26	-	0.20	0.51	0.65	—	0.48	0.20	0.26	—
TLC-2	0.51	0.67	0.82	0.86	0.65	0.59	0.88	0.81	0.52	0.65
Color	P	P	Y	P	P	P	P	Y	P	P
					Identify	—	—	d	c	a	e

a putrescine; b cadaverine; c ammonia; d methylamine; e dimethylamine; f trimethylamine

Color

P, Pink; Y, Yellow

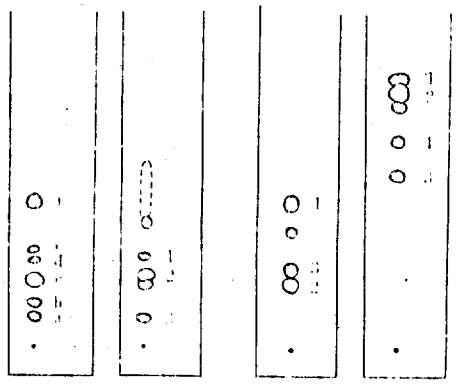

FIG. 6. Paper Chromatogram and Thin Layer Chromatogram of Basic Compounds.

a putrescine; b cadaverine; c ammonia; d methylamine; e dimethylamine; d trimethylamine.
PC (Paper Chromatogram): Solvent-Butanol : Acetic Acid : Water = 50 : 1 : 49.
TLC (Thin Layer Chromatogram-1; Solvent-Phenol : Water : Acetic Acid = 75 : 25 : 4.
TLC-2; Solvent-Water : Acetic Acid = 8 : 2.

distillation at pH 8 and chromatographic analysis, although no amine were found in 30 g of egg white. The problem is whether or not amines found out in our experiment were moved from yolk through egg yolk membrane. Further study will reveal this point.

5. On the influence of heating on volatile carbonyl compounds

It should be important to consider formation of secondary products in steam distillation, for instance, formation of aldehyde due to Strecker reaction. So attempts were made to collect volatile carbonyl compounds without steam-heating. 2,4-Dinitrophenylhydrazine-

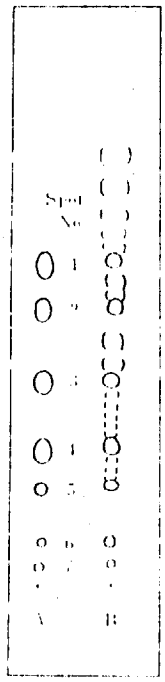

FIG. 7. Thin Layer Chromatogram of Monocarbonyl-DNP-hydrazones Mixture Obtained with and without Steam-distillation.

A: With steam-distillation
B: Without steam-distillation

1780

2N HCl-solution was added to ether-extracted solution of egg white. After removal of ether DNP-hydrazone was extracted by ethyl acetate and ethyl acetate solution was dried by distillation under reduced pressure. Dry compounds mixture was dissolved in petroleum ether and the solution was examined by thin layer chromatography in the same procedure. The chromatogram is shown in Fig. 7.

It is presumed that all spots appeard in chromatogram through steam-distillation were contained in those through ether-extraction. Accordingly it may be considered that secondary volatile compounds, if any, could not be so much collected. It should rather be noted that some of traces compounds might have been missed unaboidably during the experimental procedure.

As secondary formations of volatile amines during steam-distillation have not been accepted,[9] this problem was neglected.

6. On the total contents of volatile carbonyl and basic compounds in egg white

Total amounts of volatile carbonyl compounds were calculated as acetone from 2N HCl-washed and dried weight of their DNP-hydrazones. Total amounts of volatile bases were determined by back titration of N/50 HCl solution in which basic components were distilled, and calculated as ammonia. These determination were carried out on 300 g of fresh egg white layed in winter (January), as well as in summer (August), those of which were not freezed. Results obtained are shown in Table V.

TABLE V. TOTAL AMOUNTS OF VOLATILE CARBONYL AND BASIC COMPOUNDS

	Carbonyl compound	Basic compound
a	10 mg	0.7 mg
b	11 mg	0.6 mg

a, Egg white in summer; b, Egg white in winter
Amount of carbonyl and basic compounds calculated as acetone and ammonia, respectively, in 100 g egg white.

Strictly speaking, it should be noted that amounts of carbonyl compounds might be rather smaller than real amounts because of possibility of loss in washing dry mixture by 2N HCl solution.

7. Correlation between carbonyl or basic compounds and smell of egg white

Amines found in this experiment might be related to fishylike or semenlike odor.

Bacteriologically speaking, putrescin can be produced by bacterial decarboxylation of arginine or ornithine. Contribution of this action to egg white, used in this experiment, however, may well be denied in consideration of the preservation history of sample egg and the presence of trace amounts of free arginine or ornithine. The presence of putrescin may be reasonable[10] on the presence of spermidine and spermine in egg white, and also of Fischer and Bohn's report[11] on the presence of putrescin in various animal organ. Lindsay[12] reported that when the ratio of acetaldehyde and diacetyl is much more than 1:3.2, green flavor may occur in butter. In this experiment we found both acetaldehyde and diacetyl in egg white qualitatively, but not quantitatively. Quantitative determination of these compounds should be attempted in further study. Also other smelling compounds, for instance, acidic components and sulfur containing compounds will be investigated in further study.

10) H. W. Dudley, M. G. Rosenheim and O. Rosenheim, *Biochem J.*, **18**, 1263 (1924) [*C.A.*, **19** 1147 (1925)].

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1784

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J. Food Sci. 33(4): 440-41, (1968) 511

Quantitative Determination of Diacetyl by Electron Capture

INTRODUCTION

THE IMPORTANCE OF DIACETYL as a flavor compound in foods is widely recognized. In some existing methods for the quantitative determination of diacetyl in foods, the diacetyl is purged from the sample with nitrogen gas, trapped as dimethylglyoxime in buffered hydroxylamine and converted to a colored complex by reaction with ferrous sulfate (Owades *et al.*, 1963; Pack *et al.*, 1964). The colored complex is then measured spectrophotometrically. A polarographic method for the determination of diacetyl in buttermilk has been described (Ferren *et al.*, 1967). These methods are suitable for measuring diacetyl in the parts per million range but not in lesser amounts. Since the average flavor threshold for diacetyl in milk has been found to be 10–20 parts per billion (ppb) (Bennett *et al.*, 1965; Hempenius *et al.*, 1966; Scanlan, 1967), a method capable of determining diacetyl in this range would be useful in flavor work.

A gas entrainment, on-column trapping procedure for gas chromatographic analysis of flavor volatiles designed by Morgan and Day (1965) has been used for the quantitative determination of volatile compounds in foods (Bills *et al.*, 1966; Langler *et al.*, 1967). As originally described, a flame ionization detector was employed for the detection of compounds. However, a recent report indicated that the electron capture detector is 1,200 times more sensitive to diacetyl than the flame ionization detector (Shilman *et al.*, 1966). This communication describes the quantitative determination of diacetyl in the parts per billion range using the gas entrainment, on-column trapping procedure with an electron capture detector.

EXPERIMENTAL

KNOWN AMOUNTS OF DIACETYL were added to samples of whole milk. Ten-ml samples of milk were pipetted into screw-capped vials (Kimble No. 60957, size 1) containing approximately 1 mg of tetradecanol to control foaming during collection of volatiles. The vials were sealed by

means of screw-caps fitted with silicone rubber liners and shaken vigorously to mix the milk and the tetradecanol.

The operating parameters for the analyses were:

Entrainment apparatus

Purge time and rate	10 min at 10 ml/min of N_2
Water bath temperature	37°C ± 2°C

Gas chromatographic conditions

Instrument	F & M Model 810
Detector temperature	200°C
Detector pulse interval	150 microseconds
Detector purge rate	140 ml/min of 5% methane in argon
Column	20% 1,2,3-*Tris*(2-cyanoethoxy) propane on 80–100 mesh Celite 545
Column dimensions	13.5 ft × ⅛-in. O.D.
Column temperature	isothermal 70°C
Flow rate	24 ml/min of 5% methane in argon

RESULTS AND DISCUSSION

Peak heights obtained for the various concentrations of diacetyl added to milk were plotted to obtain the standard curve shown in Fig. 1. Each point on the curve represents the mean of two determinations. Although not shown on the curve in Fig. 1, samples containing 80 and 160 ppb were also analyzed and the recorder response was also linear throughout this range.

Five replicate samples of heated milk were analyzed for diacetyl. The mean for these samples was 30.3 ppb, the range 29.0–31.5 ppb and the standard deviation 1.04.

The procedure described herein is a sensitive, rapid method for the quantitative analysis of diacetyl. Concentrations of diacetyl at the average flavor threshold for milk can easily be determined by this procedure. In our laboratory samples of milk containing as little as 2–3 ppb of diacetyl have been successfully analyzed. Although the procedure has been used only on milk, undoubtedly a variety of food products could be analyzed for diacetyl using this procedure.

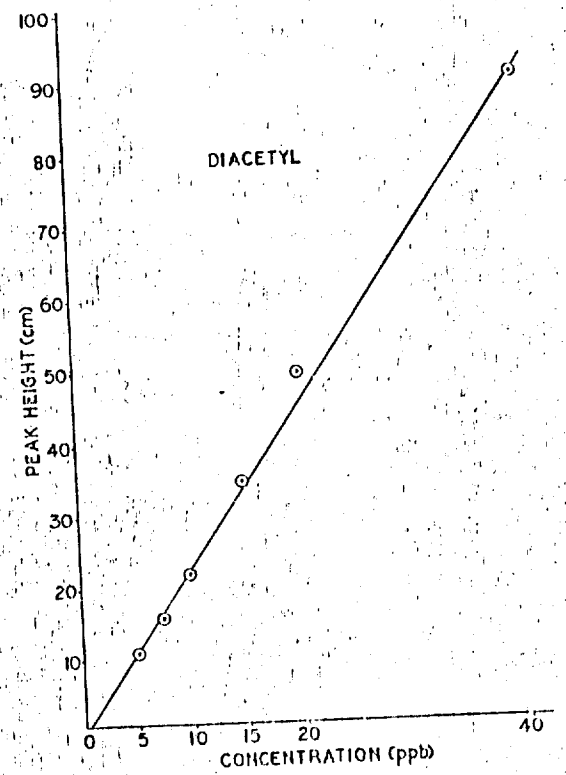

Fig. 1. Recorder response obtained with various concentrations of diacetyl in milk.

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Supported in part by Public Health Service grant No. UI-142 from the National Center for Urban and Industrial Health.

1785

...at-Induced Volatile Compounds in Milk [1]

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J. Dairy Sci. 51(7) 1001-1007, (1968)

Abstract

... preheated at 82 C for 30 min, was ... to 146 C for four seconds and cooled ... in a tubular heat exchanger. Immediately after heat treatment, the milk was ... distilled at 30 C in a semicontinuous ... reduced pressure, glass apparatus. Raw ... was distilled in a similar manner for control. Continuous liquid-liquid ethyl ... extractions were employed to recover compounds from the aqueous distillates. Chromatography, mass spectrometry, and spectrophotometry, and odor concentration were used to characterize the compounds in the ethereal concentrates.

The following compounds were found to be heat induced: The $C_{3,4,5,7,8,9,10,11,13}$ n-methyl ketones, the $C_{8,10,12}$ delta-lactones, ... aldehyde, furfural, phenylacetaldehyde, ... oct-1-en-3-ol, n-heptanol, 2-butoxy-... maltol, acetophenone, benzonitrile, ... azole, and diacetyl. Other food ... could yield these heat-induced compounds provided the basic materials from which they originate were present ... the heat treatment. The following compounds were found in both heated and raw milk: A dichlorobenzene, a trichlorobenzene, methyl iodide, and diacetyl. ... nitrile, dichlorobenzene, trichloro-..., and methyl iodide were present in ... ethereal concentrates in trace amounts. The concentration of diacetyl in heated raw milk was determined by a modified entrainment, on-column trapping GLC ... The amount of diacetyl in raw ... was 5 ppb, while the amount in the ... milk was 38 ppb, which is above the ... flavor threshold for diacetyl in ... It is suggested that diacetyl contributes to the rich or heated note in the flavor ... heated milk.

... for publication February 2, 1968.
... Paper no. 2423, Oregon Agricultural Experiment Station. This investigation was ... in part by Public Health Service Research Grant no. UI 00142-05 from the National ... for Urban and Industrial Health.
... present address: Research and Development, ... Flavors & Fragrances, Inc., 521 ... 57th Street, New York, New York 10019.

It is generally recognized that heat alters the flavor of milk. The kind and intensity of the flavor change depends on the time and temperature of the heat treatment. Cooked, heated or rich, and caramelized flavors develop in milk as the heat treatment becomes progressively severe.

Numerous compounds have been identified in heated milk. Patton and co-workers studied heated milk systems in reference to nonenzymatic browning reactions and most of this work is summarized in a review by Patton (23). The heat production of methyl ketones and lactones has received a great deal of attention over the past few years (21, 28). The role of methyl ketones in the flavor of sterile concentrated milk recently has been studied by Arnold and Lindsay (3). In spite of these advances, the identity and significance of a number of compounds in heated milk remain unknown.

The purpose of this investigation was to study the changes which occur in the volatile fraction of milk immediately after heat treatment.

Experimental Procedure

Heat treatment. All of the heated milk studied in this investigation was forewarmed at 82 C for 30 min. It was then heated to 146 C, held for 4 seconds, and subsequently cooled to 4 C. The UHT treatment and cooling were accomplished in a modified laboratory model Spiratherm (a tubular heat exchange unit).

Organoleptic evaluations. Milk was heated and held at 4 C for six days. After six days, another batch of milk was heated. On the following day both milks were subjected to organoleptic evaluation by nine people experienced in ascertaining flavor defects of milk. A score sheet which permitted separate intensity rating for the sulfurous and heated or rich notes was used. The purpose of the experiment was to evaluate these two notes in one-day- and seven-day-old heated milk.

Recovery of volatiles. Twenty gallons each of heated and control raw milk were steam-distilled in a low-temperature, reduced-pressure, distillation apparatus previously described by Lindsay et al. (18). The time interval between the heat treatment and the distillation was 4 to 24 hr. The only variation from the procedure described by Lindsay et al. was that hot water instead of steam was used in the heat exchanger. The

product temperature during distillation was 25-30 C. Approximately 10% of the volume of milk was removed and collected as distillate. Ethyl ether (peroxide-free) was used as the extracting solvent for the aqueous distillates. The aqueous distillates were extracted in a conventional continuous liquid-liquid extracting device designed for a solvent less dense than water. For each extraction, approximately two liters of aqueous distillate were saturated with reagent grade NaCl, and the solution extracted continuously for 24 hr with 300 ml of ethyl ether. The ethereal solutions were dried over excess Na_2SO_4, then fractionally distilled with a 1- by 60-cm column packed with glass helices to remove the bulk of the ethyl ether.

Analysis of flavor concentrates. Six gas-liquid chromatography (GLC) runs were conducted to separate the compounds present in the ethereal concentrates. Gas chromatographic conditions for Runs One to Five are summarized in Table 1. In Runs Three to Six, the effluent from the chromatographic columns was subjected to rapid-scan mass spectrometric analysis. An Atlas CH-4 Nier-type mass spectrometer (9-ft. 60-degree sector, single focusing instrument) modified for direct analysis of GLC effluents was used. Spectra were recorded with a Honeywell Model 1508 Visicorder.

In Run Six, five fractions from the heated milk ethereal concentrate (1H–5H) and five corresponding fractions from the raw milk ethereal concentrate (1R–5R) were trapped from a 20% Apiezon M packed column and transferred to a 91.4- by 0.254-mm id Carbowax 20 M capillary column for subsequent mass spectral analysis according to the procedure described by Scanlan et al. (24). The gas chromatographic conditions for the 20% Apiezon M packed column were the same as for Run 1. The operating conditions for the mass spectral analyses of Traps 1H-5H and 1R-5R were:

GLC instrument:	F & M Model 810 with modified sample inlet system (24).	
	Traps	Isothermal (C)
Column temperature:	1H and 1R	85
	2H and 2R	98
	3H and 3R	118
	4H and 4R	140
	5H and 5R	160
Flow rate:	1 ml of He/min	

Infrared spectroscopy was used to help characterize a number of the compounds present in

TABLE 1. Gas chromatographic conditions.

	Packed Apiezon M (Run 1)	Packed Carbowax 20 M (Run 2)	Packed Apiezon M (Run 3)	Capillary butanediol succinate (Run 4)	Capillary Carbowax 20 M (Run 5)
Column length	3.66 M	3.66 M	3.66 M	91.4 M	91.4 M
Column diameter	0.32 cm od	0.32 cm od	0.32 cm od	0.254 mm id	0.254 mm id
Solid support	80/100 mesh acid-alkali treated Celite 545	80/100 mesh DMCS[a] treated Chromosorb G	80/100 mesh acid-alkali treated Celite 545	.....	.....
Stationary phase, %	20	2.5	20	.....	.....
Carrier gas flow ml/min	30 of N_2 at 100 C	35 of N_2 at 70 C	35 of He at 100 C	1 of He at 80 C	1 of He at 70 C
Initial oven temp	100 C for 20 min	70 C for 10 min	100 C for 20 min	80 C for 10 min	70 C no hold
Temp program rate	4 C/min	4 C/min	3 C/min	6 C/min	2 C/min
Final oven temp	200 C	210 C	200 C	200 C	175 C
GLC instrument	Aerograph Model 204	Aerograph Model 204	Barber-Colman Model 5000	F & M Model 810	F & M Model 810

[a] Dimethyldichlorosilane treated.

1785

...ilk ethereal concentrate. The tech-...ed by Stevens et al. (26) was used ...pose.

...determination of diacetyl. The ...nt, on-column trapping technique ...by Morgan and Day (19) provides a ...determining concentrations of vola-...ds without extraction or distillation. ...d Lindsay's (25) modification of this ...in which an electron capture detec-...ployed instead of a flame ionization ...was used to determine the amount of ...heated and raw milk.

...flavor threshold determination for ...The average flavor threshold for di-...raw whole milk was determined accord-...ethod reported by Wasserman (27). ...milk, with diacetyl concentrations ...from 5 to 40 parts per billion (ppb), ...pared in glass-stoppered bottles and ...equilibrate at 4 C for 2 hr. Imme-...serving to the 20-member taste ...samples were tempered to 19 C. The ...flavor threshold was determined by ...per cent of panelists having thresh-...concentration level versus the con-...the test solutions and locating ...ation at the 50% response.

...ts and Discussion

...of the organoleptic evaluation of ...en-day-old heated milk are shown ...The results indicate that the sulfur-...sulfide note decreased consider-...the first and seventh day of stor-...In contrast, the heated or rich note ...ghtly over the same period of time. ...ary of the compounds identified in ...and raw milk ethereal concentrates is ...Tables 3 and 4. The reference and ...mass spectral data for a number ...pounds identified are shown in Ta-...ough not shown in Tables 3 and 4, ...sulfide and acetaldehyde were observed ...heated milk by the gas-entrainment, on-column trapping technique of Morgan and Day (19). It has been demonstrated that the concentration of both of these compounds increases upon heating milk (14, 20).

The methyl ketones and lactones, found in substantially larger concentration in the heated ethereal concentrate, have been well documented as heat-induced compounds in milk. Chromatographic evidence also suggested that benzaldehyde occurred in larger concentration in the heated than in the raw milk. The precursor of benzaldehyde in heated milk is not known, but its formation during the caramelization of sugars has been reported by Hodge (10).

The identification of phenylacetaldehyde in heated milk was considered tentative, as it was based on relative retention times and odor confirmation. Phenylacetaldehyde could result from the Strecker degradation of phenylalanine.

The identification of vanillin, a very flavorful compound, was also based on relative retention time and odor confirmation. It seems likely that vanillin might contribute to the rich, heated note in the flavor of heated milk. As Cobb et al. (6) suggested, vanillin in heated milk probably originates from plant lignin. Perhaps rumen microorganisms break down lignin to coniferyl alcohol which passes into the milk and is oxidized to vanillin when the milk is heated. Coniferin, believed to be a precursor of lignin in plants (22), also offers the possibility of serving as a vanillin precursor in milk.

Oct-1-en-3-ol has been identified in oxidized dairy products by Forss and Stark (7). A pathway for its formation from methyl linoleate was postulated by Hoffmann (12). It seems unlikely that oct-1-en-3-ol resulted from autoxidation in this study, as the compound was found in heated but not in the raw milk. Our results suggest that oct-1-en-3-ol can result from heat as well as autoxidation.

Benzonitrile was produced in the heated milk. It is difficult to speculate on the source of this compound. Cyanogenetic glycosides have been found in forage plants, such as sorghum, millet, and in several legumes (17). A material such as this, or fragment therefrom, could possibly pass from the forage into the milk and decompose upon heating. Regardless of its source, benzonitrile is a highly toxic compound and its significance should be investigated.

A di- and a trichlorobenzene were identified in heated as well as raw milk. It was not possible to determine which positional isomers were present. At least two different routes are possible for the entry of these compounds into milk. They could originate from chlorinated pesticides or from chlorine containing bactericidal agents.

...Mean intensity scores of the sulfide and ...notes in one- and seven-day-old heated milk.

...sulfide, ...sulfide	Heated, rich	
7-day milk	1-day milk	7-day milk
2.2	3.1	3.7

...point intensity scale where 1 = none and ...

TABLE 3. Compounds identified in ethereal concentrate from heated milk.

Compound	Run no.	Trap no.	t_R/t_R Extract	t_R/t_R Authentic	MS ref.	IR ident.	Odor confirmation	Litmus
Acetone	3		0.0855	0.0860[a]	1			
	4		0.573	0.583[d]				
Butanone	3		0.158	0.149[a]	1			
	5		1.05	1.03[e]				
2-Pentanone	4		0.703	0.680[b]	1		+	
2-Heptanone	1,2,3 4,5		1.00	1.00[a]	1	+	+	
2-Octanone	6	3H	0.653	0.630[g]	1			
2-Nonanone	1		2.48	2.58[a]	1	+	+	
	4		1.62	1.63[d]			+	
2-Decanone[m]	6	4H	1.08	1.09[h]	1			
2-Undecanone	1		1.33	1.33[i]	1	+	+	
	4		2.28	2.26[d]				
2-Tridecanone	1		1.78	1.78[i]	1	+		
	4		2.81	2.79[d]				
Delta-octalactone	6	5H	1.34	1.42[i]	2		+	
Delta-decalactone	1		1.85	1.90[i]	2	+	+	
	5		1.50	1.49[i]				
Delta-dodecalactone	2		5.82	6.10[h]	2		+	
	5		1.93	1.96[j]				
Hexanal	4		0.816	0.819[d]	1			
Benzaldehyde	3		2.00	2.03[a]	1			
	4		2.04	2.09[d]				
Furfural	3		0.814	0.797[a]	1			
Phenylacetaldehyde	1		2.45	2.38[a]			+	
	2		3.44	3.36[h]				
Vanillin	1		1.75	1.66[i]				
	2		6.35	6.65[h]				
Hexanoic acid	1		2.44	2.34[a]			+	+
Octanoic acid	1		1.22	1.20[i]		+	+	+
Decanoic acid	1		1.52	1.51[i]		+	+	+
Ethanol	5		1.12	1.04[e]	1			
Oct-1-en-3-ol	6	3H	0.762	0.736[g]	11			
n-Heptanol	6	3H	0.855	0.872[g]	1			
2-Butoxyethanol	6	2H	1.75	1.78[f]	1			
Diacetyl	6	1H	0.340	0.340[b]	16		+	
			Sample enrichment on 1,2,3-*tris*(Cyanoethoxy)propane[c]					
Maltol	1		1.37	1.42[a]			+	
Acetophenone[m]	3		2.71	2.89[a]	1			
Ethyl acetate	4		0.636	0.635[d]	1			
Benzothiazole	3		4.32	4.10[a]	1		+	
	2		4.27	4.16[b]				
Toluene	3		0.717	0.668[a]	1			
Naphthalene	3		4.10	4.25[a]	1			
Dichlorobenzene	3		2.78	2.88[a]	1			
Trichlorobenzene	3		4.07	4.00[a]	1			
Methyl iodide	5		0.967	0.961[e]	1			
Benzonitrile	6	3H	1.19	1.14[g]	1			
Chloroform	5		1.12	1.14[e]	1			

[a] Apiezon M—relative to 2-heptanone.
[b] Carbowax 20 M packed—relative to 2-heptanone.
[c] GLC conditions same as for quantitative determination of diacetyl.
[d] BDS capillary—relative to 2-heptanone.
[e] Carbowax 20 M capillary—relative to ethyl acetate.
[f] Carbowax 20 M capillary—relative to 2-heptanone.
[g] Carbowax 20 M capillary—relative to benzaldehyde.
[h] Carbowax 20 M capillary—relative to 2-nonanone.
[i] Apiezon M—relative to 2-nonanone.
[j] Carbowax 20 M capillary—relative to 2-tridecanone.
[k] Apiezon M—relative to octanoic acid.
[l] Mass spectrometry was used for the identification of all compounds which show a mass spectral reference.
[m] Mass spectral identification was tentative.

TABLE 4. Compounds identified in ethereal concentrate from raw milk.

Compound	Run no.	t_R/t_R Extract	t_R/t_R Authentic	MS ref.	IR ident.	Odor confirmation	Litmus
[illegible]	3	0.091[a]	0.086	1			
[illegible]	3	0.157[a]	0.145	1		+	
[illegible]	5	1.05[e]	1.03				
[illegible]one	4	0.698[d]	0.680	1		+	
[illegible]one	4	1.00[d]	1.00	1		+	
[illegible]one	4	1.62[d]	1.63	1			
[illegible]lactone	1	1.55[k]	1.59	2		+	
[illegible]	5	1.49[l]	1.49				
[illegible]decalactone[m]	5	1.94[l]	1.96	2			
[illegible]al	4	0.813[d]	0.819	1			
[illegible]	5	1.43[e]	1.35				
[illegible]aldehyde	4	2.05[d]	2.09	1			
[illegible]oic acid	1	0.801[k]	0.705			+	+
[illegible]ic acid	1	1.00[k]	1.00			+	+
[illegible]oic acid	1	1.26[k]	1.25				
[illegible]	3	0.072[a]	0.068	1			
[illegible]	4	0.570[d]	0.577				
[illegible]yl		Sample enrichment on 1,2,3-*tris*-(2 Cyanoethoxy)propane[c]				+	
[illegible]tate	4	0.635[d]	0.635	1			
	5	1.00[e]	1.00				
[illegible]palmitate	4	4.00[d]	4.10	2			
[illegible]benzene	3	2.72[a]	2.88	1			
	4	1.66[d]	1.70				
[illegible]benzene	3	4.19[a]	4.00	1			
[illegible]lide	5	0.926[e]	0.961	1			

[illegible] 3 for footnotes.

[illegible] iodide identified in both the heated [illegible] milk could come from a similar source. [illegible]s are commonly used sanitizing agents [illegible] account for the presence of methyl [illegible] milk.

[illegible] all practical purposes most of the di-[illegible]ound in heated milk is heat induced.

[illegible] Reference and experimental mass spectra data.[a]

	135	108	69	63	45	82
[illegible]						
[illegible]	100	35	27	13	12	12
[illegible]ental	100	43	25	10	10	10
	180	182	74	184	181	183
[illegible]						
[illegible] (1[illegible])	100	95	32	28	11	10
[illegible]ental	100	80	35	32	10	12
	103	76	50	51	75	104
[illegible] (1[illegible])	100	32	17	10	8	7
[illegible]ental	100	38	20	15	15	10
	43	15	86	42		
[illegible] 14	100	28	14	11		
[illegible]ental	100	28	13	13		
	142	127	141	15	139	140
[illegible]						
[illegible] 1[illegible]	100	38	14	13	5	4
[illegible]ental	100	38	21		7	7
	57	43	29	72	27	41
[illegible]ol (11)	100	23	21	19	17	16
[illegible]ental	100	26	20	20	14	13

[illegible]a are in terms of relative abundance.
[illegible] for 1,2,4-trichlorobenzene.

The diacetyl content of raw, forewarmed, and forewarmed plus UHT treated milk is shown in Table 6. The data indicate that the amount of diacetyl increased as the heat treatment increased. To evaluate the significance of the increase in diacetyl concentration, the average flavor threshold for diacetyl in raw milk was determined and found to be 12 ppb. This agrees very well with reports for the average flavor thresholds of 10 ppb (4) and 19 ppb (8) for diacetyl in milk. It seems, therefore, that the heat treatment used in this study increased the diacetyl content from a 5-ppb subthreshold level to 38 ppb, which is well above the average flavor threshold in milk. On this basis, one would expect diacetyl to contribute to the flavor of heated milk. It was felt by members of the organoleptic taste panel, that diacetyl contrib-

TABLE 6. Amount of diacetyl in raw, forewarmed, ultrahigh-temperature–treated milk.

Sample	Amount of diacetyl (ppb)
Raw milk	5
Forewarmed[a]	22
UHT-treated[b]	38

[a] Heated to 82 C for 30 min.
[b] Heated to 82 C for 30 min and then to 146 C for 4 sec.

utes to the rich, heated note in the flavor of heated milk.

Diacetyl is known to be a nonenzymatic browning reaction product (10), and it is most probably produced in this way in heated milk. In a report dealing with nonenzymatic browning reactions in model systems, Hodge et al. (11) proposed a route for diacetyl formation. They suggested that diacetyl is formed from a methyl alpha-dicarbonyl intermediate which is formed in one of the major pathways of nonenzymatic browning reactions (10). More recently Heynes, Sage, and Paulsen (9) proposed a scheme for diacetyl formation in nonenzymatic browning systems in which glycine, or a derivative of glycine, and methylglyoxal react to produce diacetyl. This might be pertinent to the heat-induced production of diacetyl in milk, as Jenness and Patton (13) indicate that methylglyoxal is probably produced by heating lactose, and trace amounts of free amino acids are known to exist in milk.

It is significant that a small amount of diacetyl (3–5 ppb) was observed in all raw milk samples examined. Three samples of raw milk were analyzed within 20 min after being drawn from the cow in an effort to eliminate post-milking bacterial action as a cause. The diacetyl content of these samples was 5, 3, and 3 ppb. A distilled water control did not produce a peak for diacetyl. These facts indicate that raw milk contains diacetyl. The diacetyl in raw milk is probably transmitted to the milk from forage eaten by the cow.

Acknowledgment

The authors are grateful to the Cherry-Burrell Corporation for contributing the laboratory model Spiratherm used in this study.

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Effect of Albumin on the Antibacterial Activity of Diketones [1]

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Received April 27, 1951

Introduction

In a previous report from this laboratory (1) it was shown that 5,5′-dibromo-2,2′-dihydroxy benzil (dibromosalicil) lost much of its antibacterial activity when serum protein was added to the culture medium. In the medium II by Schmidt and Moyer (2) a dibromosalicil concentration of 0.03 mg./100 ml. was sufficient to suppress the growth of *Micrococcus pyogenes* var. *aureus* by 50% during an incubation time of 18 hr. In the presence of 1 g. beef albumin/100 ml. medium, more than 160 times as high a concentration (4.9 mg./100 ml.) was required to produce the same degree of inhibition. This observation suggested that dibromosalicil would not be effective in the systemic treatment of infectious diseases. Clinical trials (1) showed accordingly no beneficial results on oral or intravenous administration of this compound.

It seemed of interest to study the antibacterial activity of related compounds and in particular to investigate whether the inhibition of antibacterial effectiveness by albumin was characteristic for diketones, indicating a competitive distribution of the antibacterial agent between bacterial and other proteins. This report describes the results obtained when a total of 32 diketones and 4 related compounds were tested for activity against *M. pyogenes* var. *aureus*, *Escherichia coli*, and *Streptococcus Lancefield* in the presence and absence of albumin.

[1] A preliminary report was presented at the 112th Meeting of the American Chemical Society, New York City, September, 1947.

Experimental

Determination of Antibacterial Activity

The bacteriostatic activity of the various diketones was measured turbidimetrically as described earlier (1). The organisms used were *Micrococcus pyogenes* var. *aureus* (ATCC 6538), *Escherichia coli* (ATCC 9637), and *Streptococcus Lancefield* H69-D5 (a nonpathogenic, group D hemolytic streptococcus). In the experiments with albumin, 1 ml. sterile (Seitz-filtered) 12% bovine albumin solution (Armour, fraction V) in 2% NaCl solution was added to 10 ml. medium, followed by 1 ml. of the diketone solution to be tested.

Preparation of Diketones

Of the 36 compounds tested, 10 were available commercially, as indicated by (*C*) in the tables or when the name of the substance is mentioned for the first time. Two diphenylmethane derivatives were obtained through the courtesy of Dr. Paul B. Marsh, Beltsville, Md. The remaining 24 compounds were synthesized in this laboratory, according to the procedures listed as references in the tables. The synthesis of three benzil derivatives (marked *S* in the tables) is described below.

Synthesis of 2,2′,4,4′-Tetrahydroxybenzil

2,4-Dimethoxybenzaldehyde does not undergo the benzoin condensation (3) and resorcil is therefore not obtainable by the customary route employed to obtain other hydroxybenzils. Karrer and Ferla (4) described a synthesis starting with the condensation of cyanogen, HCl, and resorcinol, followed by hydrolysis of the diimide. In a search of the literature it was noticed that a product described in two earlier reports (5,6) on the reaction of resorcinol with ethyl oxalate had a melting point agreeing with that reported by Karrer and Ferla for 2,2′,4,4′-tetrahydroxybenzil. The type formula $C_{14}H_{10}O_6$ obtained by Michael (5) for his yellow crystalline material was that of a tetrahydroxybenzil, but the structural formulas proposed by this author did not include that of a benzil. The procedure of Tingle and O'Byrne (6) differed slightly from that of Michael, but the product obtained was apparently the same.

For the formation of a benzil, the molecular proportions should be 2 resorcinol: 1 ester: 2 metallic sodium, instead of the equimolecular quantities used by Tingle and O'Byrne. The following procedure was developed: 4.8 g. sodium wire was suspended in 200 ml. anhydrous ether, and 15 g. ethyl oxalate was added in one portion, followed by a solution of 22 g. resorcinol in 100 ml. of dry ether. The reaction was started and maintained by the addition of a total of 15 ml. alcohol in 5-ml. portions during a period of 3 hr. During that time the mixture was heated on a steam bath and was then kept at room temperature (protected from moisture) for an additional period of 20–24 hr. Water was then added to bring solids in solution. The ether layer was separated and found to contain unchanged resorcinol. The aqueous layer was acidified with 7 *N* sulfuric acid and extracted repeatedly with ether. The ether extracts, combined and dried over sodium sulfate, yielded, on removal of the solvent, a tan crystalline product, m.p. 255–256° (from alcohol). Karrer and Ferla (4) reported a m.p. of 256° for resorcil. The phenylhydrazone (pale yellow, crystalline) prepared from the product according to the procedure described by Karrer and Ferla had a m.p. of

1788

262–264° (dec.); Karrer reported 263°. The tetramethyl ether, prepared from the product and methyl sulfate, melted at 129–131° (Karrer found 125–126°). Since the conclusion of this work, Schrauffstätter (7) gave independently additional evidence to establish that the reaction product between resorcinol and ethyl oxalate was identical with 2,2′,4,4′-tetrahydroxybenzil prepared according to Karrer and Ferla.

Synthesis of 3,3′,4,4′-Tetrahydroxybenzil

The tetramethyl ether of this compound was obtained in the usual manner by the condensation of 3,4-dimethoxybenzaldehyde to tetramethoxybenzoin, followed by oxidation with Fehling solution. Tetramethoxybenzil (0.5 g.) was dissolved in a mixture of 25 ml. glacial acetic acid and 25 ml. HBr (48%) and heated to 125–135° for 5 hr. The dark reaction mixture was extracted with ether and the tetrahydroxybenzil was transferred into aqueous solution by washing the ether extract with 2 *N* Na_2CO_3 solution. After acidification with 7 *N* H_2SO_4 (color change from deep orange to yellow, no precipitate) the aqueous solution was extracted with ether and the ether extract was dried over Na_2SO_4. Removal of the solvent left a yellow oil which solidified on addition of a small amount of water. The reaction product was dried at 105° for 5 hr. and recrystallized from water. In three different experiments the yield varied from 0.14 to 0.22 g. (34–53%); m.p. 233–236°; Barger and Ewins (8) gave 236° as the m.p. of veratril prepared in a different manner.

Synthesis of 2,2′-Dihydroxy-3,3′-dimethoxybenzil

Five grams 2,2′,3,3′-tetramethoxybenzil (prepared in the usual manner from 2,3-dimethoxybenzaldehyde) was added to a solution of 50 g. anhydrous $AlCl_3$ in 250 ml. nitrobenzene. The mixture was kept at 55° for 7 hr., then poured on ice, stirred vigorously for 1 hr., and kept at 5° for an additional 16 hr. The material was then centrifuged, the water layer was siphoned off and discarded, and the nitrobenzene layer was mixed with 500 ml. 2 *N* NaOH and stirred for 30 min. The alkaline aqueous layer yielded on acidification with 2 *N* H_2SO_4 a yellow precipitate which was filtered off and dried in vacuum over $CaCl_2$. Obtained (after recryst. from 70% CH_3COOH and from water) 3.33 g. (73%), m.p. 165.5–166.5°. The analysis of this product revealed that only two of the four methoxy groups had been changed into hydroxy groups:

Anal. Found: C, 63.49%; H, 4.70%; OCH_3, 20.52%. Calcd. (dihydroxydimethoxybenzil): C, 63.57%; H, 4.67%; OCH_3, 20.53%. Calcd. (tetrahydroxybenzil): C, 61.32%; H, 3.68%; OCH_3, 0.

Identification of the structure of this compound as 2,2′-dihydroxy-3,3′-dimethoxybenzil is based on the following observations: Attempts to demethylate 3,3′-dimethoxybenzil with the procedure outlined above failed, but demethylation proceeded smoothly when 2,2′-dimethoxybenzil was subjected to the same conditions. This indicated that a methoxy group in position 3 was more resistant to $AlCl_3$ than a methoxy group in position 2. Oxidation of the new compound with hydrogen peroxide in alkaline solution under conditions which formed benzoic acid from benzil, gave an acid with a m.p. 150–151°. The m.p. of 2-hydroxy-3-methoxybenozic acid is given in the literature (9) as 150°. Bromination of the oxidation product gave a compound with a m.p. 208°; Lit. m.p. of 5-bromo-2-hydroxy-3-methoxybenzoic acid is 211° (9). The addition of $FeCl_3$ solution to the oxidation product of the new compound produced a purple color, similar to that obtained with 2-hydroxybenzoic acid. A solution of 2-methoxybenzoic acid remained colorless on addition of $FeCl_3$, and the same was observed on addition of $FeCl_3$ to 3-hydroxybenzoic acid (10) and to 3-hydroxy-4-methoxybenzoic acid (10).

Results and Discussion

Unsubstituted benzil (*C*) in the presence or absence of albumin had no inhibitory effect on the growth of all three test organisms in concentrations up to 30 mg./100 ml. Several hydroxybenzils were considerably less effective than dibromosalicil (see Table I) and showed a decrease in activity when albumin was added to the culture medium. Pulvinic acid was selected as an example for compounds with an increased distance between the two aromatic rings (as compared to the benzils), but here again albumin lowered the effectiveness of the substance.

Replacement of one aromatic ring in benzil by a methyl group (acetylbenzoyl) increased antibacterial activity above that of the hydroxybenzils, but did not abolish the effect of albumin. When both benzene rings were replaced by methyl groups (diacetyl) or hydrogen atoms (glyoxal) there was a marked increase in antibacterial activity and the degree of inhibition by a given concentration of these compounds was not significantly altered on addition of albumin.

Several α-diketones with longer carbon chains were less active than glyoxal and their effectiveness decreased further on addition of albumin, with the exception of 2,6-dimethyl-4,5-octanedione, which was not significantly influenced by albumin (see Table I). The monoketone amyl methyl ketone (*C*) was tested for purposes of comparison and found to be ineffective in a concentration of 80 mg./100 ml. The cyclic α-diketone, 3-methyl-1,2-cyclohexanedione (16), and the γ-diketone, 2,5-hexanedione (*C*) were both inactive in concentrations up to 125 mg./100 ml. Two halogenated diphenylmethane derivatives were similar in their activities (also on addition of albumin) to dibromosalicil, although the keto groups had been replaced by a methylene group.

Some of the results obtained with β-diketones are shown in Table II. Dibenzoylmethane, the compound resembling the inactive benzil in structure, was found to be second in activity only to dibromosalicil and the halogenated diphenylmethane derivatives. It differed from glyoxal, which was slightly less active, by the marked decrease in its

1722

TABLE I

Antibacterial Activity of α-Diketones and Related Compounds in the Presence and Absence of Albumin[a]

Compound	Ref.	Concentration of inhibitor (*mg./100 ml.*) and degree of growth					
		M. pyogenes var. *aureus*		*E. coli*		*S. Lancefield*	
		Medium II	Medium II + albumin	Medium II	Medium II + albumin	Medium II	Medium II + albumin
2,2′-Dihydroxy-5,5′-dibromobenzil	11	*0.03*	*4.9*	*2.7*	10 *F*	*0.05*	*7*
2,2′-Dihydroxybenzil	11	*26*	60 *G*	48 *F*	48 *F*	60 *F*	60 *F*
4,4′-Dihydroxybenzil	12	*23*	*43*	*47*	60 *G*	60 *G*	60 *G*
2,2′,4,4′-Tetrahydroxybenzil	*S*	*15*	*28*	100 *M*	100 *G*	*100*	100 *M*
3,3′,4,4′-Tetrahydroxybenzil	*S*	*45*	*45*	*48*	40 *G*	40 *F*	40 *F*
2,2′-Dihydroxy-3,3′-dimethoxybenzil	*S*	*22*	30 *F*	30 *F*	30 *G*	30 *F*	30 *G*
Pulvinic acid	13	*17*	*39*	100 *G*	—	100 *F*	—
Acetylbenzoyl	*C*	*17*	*30*	*19*	*35*	*62*	*86*
Diacetyl	*C*	*23*	*23*	*7*	*7*	*41*	*45*
Glyoxal	*C*	*4*	*3.6*	*11*	*11*	*8.9*	*11.5*
3,4-Hexanedione	14	95 *F*	95 *G*	95 *G*	95 *M*	95 *G*	95 *G*
3,4-Octanedione	14	*49*	*62*	99 *G*	99 *G*	99 *M*	99 *M*
5,6-Decanedione	14,15	*77*	90 *F*	*64*	*72*	90 *M*	90 *M*
2,6-Dimethyl-4,5-octanedione	14,15	*20*	*18*	*104*	*100*	94 *G*	94 *M*
2,3-Octanedione	*C*	*19*	*32*	*40*	49 *M*	*33*	49 *F*
2,2′-Dihydroxy-5,5′-dichlorodiphenylmethane	*M*	*0.07*	*5.1*	*1.7*	*14*	*0.2*	*13*
2,2′-Dihydroxy-5,5′-dibromodiphenylmethane	*M*	*0.04*	*4.1*	*1.6*	*23*	*0.1*	*18*

[a] Numbers in *italics* are concentrations required for 50% inhibition of growth. Numbers followed by a letter give highest concentrations tested, not sufficient for 50% inhibition. The degree of growth is indicated by the letters: *F* (full growth) 85–100% growth, *G* (good growth) 65–85% growth, and *M* (medium growth) 55–65% growth.

effectiveness on addition of albumin. Introduction of a hydroxy group into the aromatic ring or replacement of one ring by a methyl group lowered the activity against all three test organisms (see Table II).

The following cyclic β-diketones were prepared and tested: 2-ethyl-4-*n*-propyl-1,3-cyclopentadione (23), 5,5-dimethyl-1,3-cyclohexanedione

TABLE II

Antibacterial Activity of β-Diketones in the Presence and Absence of Albumin[a]

Compound	Ref.	Concentration of inhibitor (*mg./100 ml.*) and degree of growth					
		M. pyogenes var. *aureus*		*E. coli*		*S. Lancefield*	
		Medium II	Medium II + albumin	Medium II	Medium II + albumin	Medium II	Medium II + albumin
Dibenzoylmethane	*C*	*1.0*	*6.2*	*3*	*13*	*2.8*	13 *G*
2-Hydroxy-2′-methoxydibenzoylmethane	17	*2.8*	*6.8*	18 *G*	18 *M*	*8.7*	18 *F*
Benzoylacetone	*C*	*30*	*19*	*14*	*15.5*	*46*	*25*
2,4-Pentanedione	*C*	*55*	*27*	*114*	*109*	127 *G*	*30*
2,4-Hexanedione	18,19	104 *F*	*48*	*105*	*99*	104 *G*	*49*
5-Methyl-2,4-hexanedione	20	100 *F*	*100*	*84*	*88*	100 *F*	100 *F*
2,4-Heptanedione	19,21	*42*	*63*	*62*	*66*	*60*	*83*
3,5-Heptanedione	20	100 *F*	*73*	*108*	*108*	100 *G*	100 *M*
6-Methyl-2,4-heptanedione	20	*54*	*35*	*48*	*54*	75 *F*	*57*
2,4-Octanedione	19,22	109 *G*	109 *F*	*43*	*43*	109 *G*	109 *G*
2,4-Nonanedione	22	*35*	*38*	*24*	*34*	*50*	*73*

[a] Numbers in *italics* are concentrations required for 50% inhibition of growth. Numbers followed by a letter give highest concentrations tested, not sufficient for 50% inhibition. The degree of growth is indicated by the letters: *F* = 85–100% growth, *G* = 65–85% growth, and *M* = 55–65% growth.

(24), 2-bromo-5,5-dimethyl-1,3-cyclohexanedione (25), and 2,4-dibromo-5,5-dimethyl-1,3-cyclohexanedione (26). None of these four compounds showed more than traces of activity in concentrations up to 100 mg./100 ml.

Particularly interesting are the results obtained with eight aliphatic β-diketones. Of the compounds with the carbonyl groups in positions

17088

2 and 4, those with an odd number of carbon atoms (in the straight chain) were considerably more active than the homologs with an even number of carbon atoms. Furthermore, five of these substances were found to be more active in the presence of rather than in the absence of albumin against one (5-methyl-2,4-hexanedione; 3,5-heptanedione), two (6-methyl-2,4-heptanedione), or all three of the test organisms (2,4-pentanedione; 2,4-hexanedione).

The decrease in antibacterial activity of certain diketones in the presence of albumin is understandable if one assumes that the carbonyl groups react with amino groups of the protein molecule and are thus prevented from interference with bacterial metabolism. The reverse, *i.e.* an increase in activity in the presence of albumin, is perhaps also due to the formation of a β-diketone-albumin complex, but no explanation for the increased activity of such a complex can be given at present. The fact that a simple structural change, such as the introduction of a methylene group between two carbonyl groups, can significantly alter the influence of protein on an antibacterial agent is not only of theoretical interest. It may find practical applications in efforts to develop new antibacterial agents which remain effective in the blood stream in the presence of plasma proteins.

Summary

1. Thirty-two diketones and four related compounds were tested for antibacterial activity in the presence and absence of albumin.

2. Among aliphatic α-dicarbonyl compounds, diacetyl and glyoxal were the most active and their effectiveness was not altered by the addition of albumin.

3. Aromatic α-diketones (benzil, hydroxybenzils) were not as active as dibromosalicil and usually became even less active in the presence of albumin. Cyclic diketones showed very little antibacterial activity.

4. Aliphatic β-diketones and benzoylacetone were unique in that most members of this group were more potent antibacterial agents in the presence of rather than in the absence of albumin. Aromatic β-diketones lost some activity on addition of albumin.

5. Procedures for the synthesis of 2,2′,4,4′-tetrahydroxybenzil, 3,3′,-4,4′-tetrahydroxybenzil, and 2,2′-dihydroxy-3,3′-dimethoxybenzil are given.

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1728

Rev. Internat. des Tabacs 25:89, 1950

Diacetyl A Component of the Aroma of Tobacco Smoke

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Diacetyl is often found as a component of odor and taste in food products. In its pure state, it is a yellow oil, slightly liquid with a strong characteristic odor.

Diacetyl has its origin, under the influence of heat, in the decomposition of tartaric acid, sugars, carbohydrates and cellulose. The same effect is brought about by leavens. Apart from these kinds of ideas, we discovered that diacetyl can also be formed as product of the elaboration of leavens in lactic acid, and at the same time, we realized that it produced the natural odor of butter. We also found it in fresh butter.

Since that time, diacetyl has acquired great economic importance as an aroma, especially in the production of margarine.

We have looked for and found diacetyl in many other food products, as well as in those discovered through the effect of microorganisms in food in which carbohydrates decompose with heat. We found it in caramel, roasted coffee and cocoa beans, beer and honey. But diacetyl is not found only in microorganisms and in bees, but also in the blood and urine of mammals, especially in man.

Transformed into nickel dioxime, hard to dissolve and of scarlet color, diacetyl can be made visible in the liquid to be analyzed, and it can be extracted as solid dioxime and identified with the help of the melting point.

We also examined tobacco smoke in relation to this research. We carefully avoided the tobacco consumption as we did also with cellulose as well as paper and wood. This is why we used for a flame instead of a match, a lamp-post and in place of clay pipes, pipes of ordinary wood, and we chose instead of cigarettes, pure tobacco or different kinds of cigars, among other cigars, low in nicotine.

With the aid of the lamp-post, we lit 3 gr. of tobacco in clay pipes. Each pipe was connected to two large washing bottles each containing 3 cm. of 10% sulphuric acid. A water jet pump blows across the pipes and the bottles a light current of air, the bubbles being able to be counted at the time when they leave the 0.5 cm. diameter tube. By using a stronger current of air, we would have basic interference in the smoke.

We added to the liquids in the bottles 0.33 ccm. of an aqueous solution of 20% nitric hydroxylamine, 0.2 ccm. of a 1.27% aqueous soltion of nickel sulfate and a few drops of 25% ammonia, as much as necessary to make the solution basic. We cooled the still basic solution under running water after having boiled it for one minute. After decanting the impure nickel dioxime present in the form of black grease, we put it in contact with 96% alcohol. Scarlet crystals are thus formed. These are filtered and washed successively with water, alcohol and ether. After allowing them to dry, we put the red nickel salt under a pressure of 4 mm. at 260° and purified it. After decomposing it with sulfuric acid, we mixed ether with the dioxime which was formed in compressing the ether. Pure white dioxime remains with a melting point of 234°. In mixing it with the pure artificial dioxime, it always showed the same melting point, characteristic for diacetyl dioxime.

From 1 kg. of tobacco, we got 0.2640 gr. of red nickel dioxime which

corresponds to 0.1573 gr of diacetyl. The following table shows tobacco smoke compared with food products containing a lot of diacetyl.

1 kg of tobacco	. . .	0.2640 gr. nickel dioxime
1 kg of coffee	. . .	0.0184 gr. nickel dioxime
1 kg of cocoa	. . .	0.0012 gr. nickel dioxime
1 kg of butter	. . .	0.0006 gr. nickel dioxime
1 kg of beer	. . .	0.0003 gr. nickel dioxime
1 kg of honey	. . .	0.0001 gr. nickel dioxime

Although the quantity of diacetyl in the above mentioned food products is very small, it does nevertheless preserve the odor, which is confirmed by observations made on the ressemblance between the odors of some of the above-mentioned food products.

We also did experiments in order to improve the aroma of tobacco smoke by adding diacetyl to it. At present, we are engaged in research with the aim of discovering the stage which precedes diacetyl in tobacco smoke.

DIACÉTYLE, un composant de l'arome de la fumée du tabac

par M. le Prof. Dr. Hans SCHMALFUSS
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Rev. intern. tabacs 25 : 89 (1950)

Le diacétyle se trouve souvent, en tant que composant de l'odeur et du goût, dans les produits alimentaires. A l'état pur il est une huile jaune, légèrement liquide, d'une forte odeur caractéristique.

Le diacétyle tire son origine, sous l'influence de la chaleur, de la décomposition de l'acide tartrique, des sucres, des hydrates de carbone et de la cellulose. Le même effet produisent les ferments. Partant de cet ordre d'idées nous avons découvert que le diacétyle peut se former même comme produit d'élaboration des ferments de l'acide lactique, et nous l'avons en même temps reconnu comme produisant l'odeur naturelle du beurre. Nous l'avons même trouvé dans du beurre frais.

Depuis lors le diacétyle a acquis une grande importance économique comme arome, surtout dans la production de la margarine.

Nous avons cherché et trouvé le diacétyle dans maints autres produits alimentaires, aussi bien dans ceux exposés à l'action de microrganismes que dans les aliments dont les hydrates de carbone se décomposent par la chaleur. Nous l'avons tiré du caramel, du café et du cacao torréfié, de la bière et du miel. Mais le diacétyle ne se trouve pas seulement dans les microrganismes et dans les abeilles, mais aussi dans le sang et l'urine des mammifères, surtout de l'homme.

Transformé dans le dioximine de nickel, difficilement soluble et de couleur écarlate, le diacétyle peut être rendu visible dans le liquide que l'on veut analyser, et il peut être extrait comme dioxime solide, en le caractérisant à l'aide du point de fusion.

Nous avons également examiné la fumée du tabac en rapport avec ces recherches. Nous avons évité soigneusement qu'avec le tabac ne soient consommés également la cellulose ainsi que le papier et le bois. C'est pourquoi nous avons employé pour l'allumage, au lieu d'une allumette, le bec de gaz et au lieu des pipes de terre, les pipes de bois communes, et avons choisi au lieu des cigarettes, du tabac pur ou des cigares d'origine différente, entre autres des cigares pauvres en nicotine.

A l'aide du bec de gaz nous avons allumé 3 gr. de tabac dans les pipes de terre. Chaque pipe était reliée à 2 bouteilles de lavage contenant chacune 3 cm. d'acide sulfurique au 10 %. Une pompe à jet d'eau aspirait à travers les pipes et les bouteilles, un faible courant d'air, les bulles pouvaient être encore comptées au moment où elles sortaient du tube d'un diamètre de 0,5 cm. En nous servant d'un courant d'air plus fort nous aurions eu des bases gênantes dans la fumée.

Nous avons ajouté aux liquides contenus dans les bouteilles 0,33 ccm d'une solution aqueuse au 20 % d'hydroxylamine nitrique, 0,2 ccm d'une solution aqueuse à 1,27 de sulfate de nickel et des gouttes d'ammoniaque au 25 %, autant qu'il fallait pour rendre la solution basique. Nous avons rafraîchi la solution encore basique sous de l'eau courante après l'avoir bouillie pendant une minute. Après avoir décanté le dioximine de nickel impur se présentant sous forme d'une graisse noirâtre, nous l'avons frotté avec de l'esprit-de-vin à 96 %. Ainsi se sont formées les aiguilles écarlates. Celles-ci ont été filtrées et lavées, l'une après l'autre, avec de l'eau, de l'alcool et de l'éther. Après les avoir laissées sécher, nous avons fait monter le sel de nickel rouge à une pression de 4 mm et à 260° et l'avons purifié. Après l'avoir décomposé avec de l'acide sulfurique, nous avons assimilé avec de l'éther le dioxime qui s'était formé, en refoulant l'éther. Il en est resté du dioxime pur, blanc, au point de fusion de 231°. En le mélangeant avec du dioxime artificiel, pur, il a donné toujours le même point de fusion caractéristique pour le diacétyle-dioxime.

De 1 kg. de tabac nous avons tiré 0,2640 gr. de dioximine rouge de nickel, ce qui correspond à 0,1573 gr. de diacétyle. Le tableau suivant montre que la fumée de tabac en comparaison avec d'autres produits alimentaires, donne beaucoup de diacétyle.

1 kg de tabac ...	0,2640 gr.	de dioximine	de nickel
1 kg de café	0,0184 gr.	»	»
1 kg de cacao ...	0,0012 gr.	»	»
1 kg de beurre ..	0,0006 gr.	»	»
1 kg de bière ...	0,0003 gr.	»	»
1 kg de miel ...	0,0001 gr.	»	»

Bien que la quantité de diacétyle dans les produits alimentaires sus-mentionnés soit exiguë, ils en conservent néanmoins l'odeur, ce qui est confirmé par des observations faites sur la ressemblance entre les odeurs d'aucuns des produits alimentaires sus-indiqués.

Nous avons fait aussi des essais pour amender l'arome de la fumée du tabac en y ajoutant du diacétyle. A présent nous nous livrons à des recherches en vue de découvrir la phase qui précède le diacétyle dans la fumée du tabac.

Diacetyl as the Buttery Flavor Component in Soybean Oil[1,2]

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J. Amer. Oil Chem. Soc. 43(8) 401-402, (1966)

Abstract

Soybean oil often exhibits a buttery flavor in the early stages of autoxidation. Molecular distillates from "buttery" soybean oil consisted of aqueous and oily layers. The aqueous layer contained the buttery flavor. During gas chromatography, the buttery flavor compound had a retention time similar to diacetyl on both polar and nonpolar columns. Diacetyl-bis-2,4-dinitrophenylhydrazone was isolated from the aqueous layer of the distillate. Diacetyl added to fresh soybean oil faithfully reproduced the buttery flavor.

Introduction

BUTTERY FLAVOR is known to be associated with reverted flavor in autoxidized soybean oil. Daubert and O'Connell (1) described buttery flavor as the initial change in fresh autoxidizing soybean oil. Chang et al. (2) noted a buttery fraction among components distilled from autoxidized soybean oil. Moser et al. (3) in their study of linolenate-containing oils, found that buttery flavor occurred in the initial stages of oxidation in all oils studied.

In this study, buttery flavor in soybean oil has been accounted for by the isolation of diacetyl from soybean oil that exhibited a strong buttery flavor.

Methods

SOYBEAN OIL WAS OBTAINED from Swift and Company and had been refined by their usual procedures and stabilized with 0.005% citric acid. Buttery flavor could be induced in this oil in one to three days by contamination with metal ions, either by the addition of traces of copper palmitate or by passing the oil through a molecular still with metal parts. Volatile materials were obtained from the oil by high vacuum distillation in a Rota-Film still at room temperature (4). Gas chromatography was carried out as described previously (5) except 1/4 in. columns 7 ft long were used. Columns packed with butanediol succinate (30% on 45/60 mesh Chromosorb P) were operated at 145C; Apiezon L (20% on 45/60 mesh Chromosorb P) columns at 135C.

Organoleptic evaluations were made by a panel of judges experienced in tasting soybean oil and milk fat. Flavor distillates were examined by adding portions of the distillate to fresh, good-quality corn oil. The amounts of distillate and oil were chosen to give a concentration of the flavor compounds in the tasting oil about twice that in the oil from which they were isolated. This was done to make up for losses of the volatile material, overcome the background flavor of the corn oil and facilitate the detection of compounds present near their flavor thresholds. The aqueous layer of the flavor distillate was emulsified into the corn oil by shaking before organoleptic evaluation. Fractions from the gas chromatograph were examined by bubbling the gas from the tail pipe of the instrument through fresh oil. The amount of distillate injected was chosen so it would give a concentration of the flavor in the tasting oil two or three times higher than in the original oil if there were no losses. Diacetyl and pentanal and vinylethyl ketone were purified by gas chromatography prior to organoleptic examinations. The flavor threshold for diacetyl was determined by presenting 4 ml samples of deodorized mineral oil containing tenfold increments of diacetyl to a panel of judges. The samples were presented in random order and could be retasted or compared with a control oil at will. The frequency of detection of a difference from the control was corrected for the frequency with which a negative control sample was identified as different. The level at which 50% of the panel detected a difference from the control was defined as the threshold.

2,4-Dinitrophenylhydrazine derivatives (DNPH) were prepared with a saturated solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid. Alkali spectra were taken immediately after treating the DNPH derivative with 0.5 N potassium hydroxide in ethanol (6).

Thin layer chromatography of the DNPH derivatives was carried out on SeasorbSilica Gel G plates developed in chloroform-tetrahydrofuran-methanol, 15:4:1, according to Cobb (7).

Petroleum ether (Skellysolve B) was purified by the method of Hornstein and Crowe (8).

Results and Discussion

The volatile material obtained by high vacuum distillation of buttery soybean oil gave intense buttery odors. The volatile material consisted of an aqueous layer and an oily material soluble in light petroleum ether. Organoleptic evaluation of appropriate amounts of each layer in fresh corn oil showed that the buttery fraction resided in the aqueous layer. The oily material had no buttery flavor.

Portions of the aqueous layer were reacted with 0.1 N hydrochloric acid, 0.1 N sodium hydroxide and an aqueous solution of Girard's T reagent. The buttery odor disappeared from the tubes containing sodium hydroxide and Girard's T reagent, but remained in the control and the hydrochloric acid. This indicated that the buttery flavor component was a carbonyl compound.

Fractionation of the aqueous layer by gas chromatography, with organoleptic evaluation of the fractions, showed that the buttery flavor had a retention time close to n-butanal on both butanediol succinate and Apiezon L. Diacetyl, a compound well known for its buttery flavor, was tested and emerged just prior to butanal on the nonpolar column and just after butanal on the polar column. Organoleptic evaluation showed that diacetyl in fresh corn oil or soybean oil at 0.1 ppm reproduced the buttery flavor with fidelity.

The presence of diacetyl in the flavor distillate was demonstrated by the isolation of its DNPH derivative. The aqueous layer of the flavor distillate was reacted with 2,4-dinitrophenylhydrazine, the precipitate was collected by centrifugation, and the aqueous acid layer was decanted. The crystals were washed twice with distilled water and extracted exhaustively with purified petroleum ether to remove the monocarbonyl

[1] Journal Paper No. J-4407 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1517.
[2] Presented at the AOCS Meeting, Houston, April 1965.

derivatives. The petroleum ether-insoluble material was crystallized from ethyl acetate. The absorption spectrum of the crystals in chloroform revealed a peak at 393 mμ, with a shoulder at 430 mμ, which agreed precisely with the spectrum of authentic diacetyl-bis-DNPH. This spectrum is characteristic of dialkylglyoxals and distinguishes them from other carbonyl compounds (9). Near infrared spectra, taken with a Beckman DK instrument with chloroform as the solvent, showed that the material crystallized from ethyl acetate had a spectrum identical to authentic diacetyl-bis-DNPH, except that the former had an absorption band at 2,250 mμ, which was missing from the known. This band probably represents an impurity. When treated with ethanolic potassium hydroxide, both the isolated and authentic derivatives gave identical spectra with an absorption maximum at 550 mμ.

Thin-layer chromatography of the isolated crystalline compound showed that it migrated with the diacetyl-bis-DNPH derivative rather than with those of glyoxal, methyl glyoxal, or 2-ketobutanal.

Previous results from this laboratory (10) have indicated that vinylethyl ketone plays an important role in the flavor of autoxidized soybean oil and that a mixture of vinylethyl ketone and pentanal at about 10 ppb in fresh oil gives a flavor similar to, but not identical with, the flavor of soybean oil in the early stages of autoxidation. The addition of about 6 ppb of diacetyl to this mixture greatly increases the fidelity with which it reproduces the early stages of autoxidized flavor in soybean oil. The flavor threshold of diacetyl in freshly deodorized mineral oil is about 1 ppb.

Diacetyl is a well-known flavor component of fermented dairy products. Pipen et al. (11) isolated diacetyl from heated chicken, but it was not established that the diacetyl arose from the autoxidation of the chicken fat. Hammond and Bird (9) isolated diacetyl as well as glyoxal, methyl glyoxal, and 2-ketobutanal from autoxidized milk fat, but since they found some diacetyl in the milk fat at the beginning of their experiment, it is not certain that it was a product of autoxidation. The precursor of diacetyl in soybean oil is not known. Citric acid is known as a precursor of diacetyl in fermentations (12), and the degradation of the citric acid synergist in the soybean oil during deodorization might give rise to compounds that could later be oxidized to diacetyl. Moser et al. (3), however, reported that a mixture of oils containing linolenic acid produced buttery flavors, with and without citric acid as an additive. This points to linolenic acid as a precursor of diacetyl, but it is difficult to account for this with the fatty acyl hydroperoxide decomposition mechanisms that have been proposed. Any route that might be proposed would seem to require multiple oxygen attack and the accumulation of oxidized intermediates, and this seems inconsistent with the appearance of buttery flavor in the very early stages of autoxidation of the oils. Commercial vegetable oils always contain small amounts of polymers that are known to decrease the stability of the oil (13). It may be that the autoxidation of these polymers initiates autoxidation in deodorized oils and that this reaction also gives rise to diacetyl.

ACKNOWLEDGEMENT

Supported in part by a PHS research grant EF-00081-03 from the Division of Environmental Engineering and Food Protection, Public Health Service.

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[Received April 30, 1965]

1832

(1832)

DISTRIBUTION OF DIACETYL REDUCTASE AMONG BACTERIA[1]

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J. of Dairy Sci. 46(3): 186-9 (1963)

SUMMARY

This work was undertaken to determine comparative amounts of the enzyme diacetyl reductase in several strains of lactic streptococci and *Leuconostoc* species. The level of enzyme in cultures of *Pseudomonas, Alcaligenes,* and coliform bacteria also was examined. Among the lactic streptococci, *Streptococcus diacetilactis* strains were the most active in reducing diacetyl; one strain contained 100 units of diacetyl reductase per milligram of enzyme protein. Strains of *Streptococcus lactis, Streptococcus cremoris, Leuconostoc citrovorum,* and *L. dextranicum* tested were comparatively inactive in diacetyl reduction; these bacteria contained from zero to eight units of enzyme per milligram of protein. From this, the importance of selecting strains containing little or no diacetyl reductase for use in maximum flavor-producing mixed-strain lactic streptococcus starter cultures was indicated. With the exception of one strain each of *Escherichia coli* and *Pseudomonas viscosa,* the coliform, *Pseudomonas* and *Alcaligenes* organisms showed considerable diacetyl reductase activity; *Aerobacter aerogenes* 8724 contained 345 units per milligram of enzyme protein. These results emphasized the consequences of contamination of dairy products by diacetyl reductase-containing bacteria in terms of flavor loss. Possible uses of the enzyme for removal of diacetyl from fermented beverages and citrus juices are mentioned.

Diacetyl has long been regarded as one of the outstanding flavor compounds found in such cultured dairy products as Cottage cheese, cultured buttermilk, cultured sour cream, and ripened cream butter. Knowledge of factors contributing to the loss of diacetyl, therefore, becomes important to insure desirable flavor and aroma in these fermented foods. In a preliminary study (11), cell-free extracts of *Streptococcus diacetilactis* 18-16 were shown to possess the enzyme diacetyl reductase which catalyzed the irreversible reduction of diacetyl to acetoin. The present investigation was made to determine the amount of this enzyme found among organisms used in the manufacture of cultured dairy products. The level of diacetyl reductase present in psychrophilic and coliform organisms that may contaminate dairy products also was examined.

Diacetyl destruction was shown by Elliker (3) to be common to several species of bacteria. Organisms of the *Bacillus, Pseudomonas, Escherichia, Aerobacter, Proteus,* and *Alcaligenes* genera were active in this regard; only *S. lactis* revealed little ability to destroy diacetyl. Galesloot and Hassing (4) reported that *Leuconostoc (Betacoccus cremoris)* was capable of reducing diacetyl and acetoin to 2,3-butanediol, whereas *S. diacetilactis* lacked this ability.

The disappearance of diacetyl has, in some instances, been correlated with the appearance of nonaromatic compounds. Parker and Elliker (8) showed that *Pseudomonas fragi* and *P. viscosa* converted diacetyl to acetoin and possibly 2,3-butanediol, resulting in aroma loss in Cottage cheese. Green et al. (5) reported that pigeon-breast muscle contained a highly specific diacetyl mutase which converted two molecules of diacetyl to two molecules of acetate and one of acetoin. Aubert and Millet (1) obtained a purified extract from *Neisseria winogradsky* which attacked diacetyl but not acetoin. This enzyme also converted two molecules of diacetyl to two of acetate and one of acetoin. Strecker and Harary (12) reported the presence of diacetyl reductase in *S. aureus*: the enzyme required reduced diphosphopyridine nucleotide (DPNH) for activity in cata-

Received for publication October 13, 1962.
[1] Technical paper no. 1605, Oregon Agricultural Experiment Station. Supported in part by a grant from the American Dairy Association.
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lyzing the irreversible reduction of diacetyl to acetoin.

EXPERIMENTAL PROCEDURES

All the organisms used in this work were available from the stock culture collection in the Department of Microbiology, Oregon State University. The cultures were maintained by weekly transfer in sterile nonfat milk as well as a broth designed to support the growth of fastidious *Leuconostoc* species. The broth made with distilled water contained 1.0% yeast extract, 1.0% tryptone, 0.2% Whey-lac,[a] 0.15% sodium actate, 0.5% sodium citrate, 1.0% glucose, 0.07% ascorbic acid, 3.3% tap water, 0.2% sodium chloride, 0.2% monobasic potassium phosphate, and 0.2% anhydrous magnesium sulfate. The organisms were incubated for 18 hr at the optimum growth temperature for each species used.

Diacetyl determinations on milk cultures of *S. diacetilactis* were made as described previously (9).

Cell-free extracts were prepared as follows: Freshly washed cells from one liter of broth medium were suspended in 40 ml of 0.1 M potassium phosphate buffer, pH 7.2, and disrupted in a Raytheon 10KC sonic oscillator for 15 min. Cell debris was removed by centrifugation at 15,000 rpm for 25 min. The supernatant obtained was dialyzed for 24 hr against two changes (2,000 ml) of 0.1 M potassium phosphate buffer at pH 7.0. The resulting cell-free extracts were stored frozen at −20 C until used. Protein determinations were made by the method of Lowry et al. (6).

Diacetyl reductase activity was measured with a Beckman Model DU spectrophotometer by following the decrease in absorbancy at 340 mμ caused by the oxidation of DPNH. Conditions for the assay are indicated in the Results section.

RESULTS

Results on the synthesis and degradation of diacetyl at 30 C by *S. diacetilactis* 18-16 in nonfat milk supplemented with 2.0% citrate are shown in Figure 1. Rapid destruction of diacetyl occurred after 24 hr. In unsupplemented nonfat milk found to contain 0.2% citrate, diacetyl destruction was apparent after about 12 hr of incubation. The cause for this dramatic loss of accumulated diacetyl in milk cultures led to a search for diacetyl reductase in *S. diacetilactis*; spectrophotometric studies

[a] Deionized, spray-dried whey, Consolidated Dairy Products, 635 Elliott Avenue, Seattle 99, Washington.

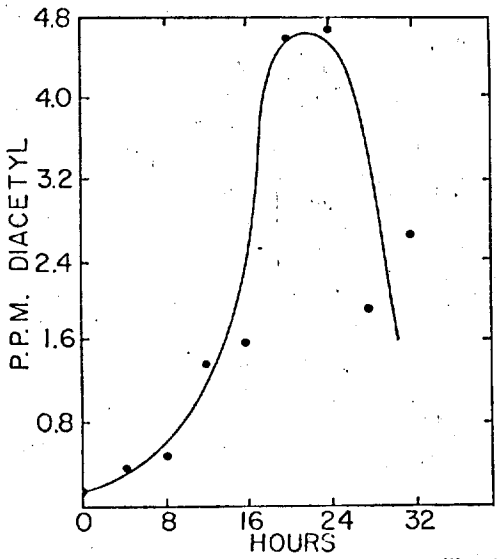

FIG. 1. Diacetyl produced in nonfat milk at 21 C by *S. diacetilactis* 18-16.

using cell-free extracts showed that diacetyl was enzymatically reduced, presumably to AMC. The reaction was not reversible and DPNH was required as the coenzyme. Divalent metal activators were not required by diacetyl reductase; EDTA and dialysis did not inactivate the enzyme. Figure 2 shows typical spectrophotometric data obtained in testing cell-free extracts for diacetyl reductase.

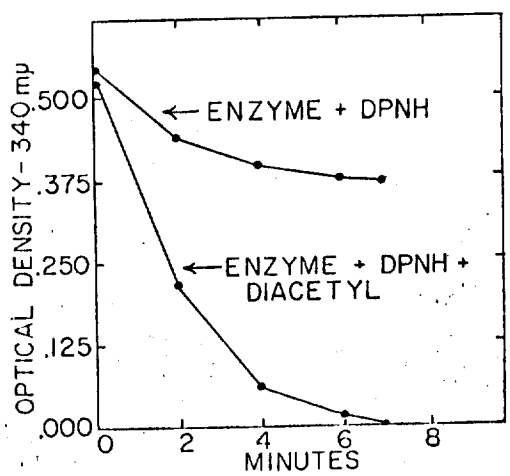

FIG. 2. Oxidation of DPNH by diacetyl reductase in crude enzyme preparation from *S. diacetilactis* 18-16. The complete system contained 10 μmoles of diacetyl, 2.0 mg of DPNH, 2.0 mg of enzyme protein, and sufficient 0.1 M potassium phosphate at pH 7.2 to provide a volume of 3.0 ml.

The amount of this enzyme found in *S. diacetilactis*, *S. lactis*, and *S. cremoris* is shown in Table 1. It may be seen that the specific activity varied from three to 100 for *S. diacetilactis* and zero to eight for both *S. lactis* and *S. cremoris*. The levels found in *Leuconostoc* (Table 1) were uniformly low, varying from zero to eight units per milligram enzyme protein.

TABLE 1

Diacetyl reductase found in lactic streptococci, *Leuconostoc*, psychrophile, and coliform bacteria

Organism	Specific activity[a]	Organism	Specific activity[a]
S. diacetilactis		*L. citrovorum*	
18-16	100	LcF	6
26-2	3	LcF8	0
DRC-1	16	91404	6
DRC-2	8	8081	0
DRC-3	20	82	6
RM-1	20	*L. dextranicum*	
S. lactis		LdF	8
E	4	688	7
27	0	8358	0
C2	8	629	7
C10	2	2-5	0
7962	0	8359	6
11454	4	*Aerobacter aerogenes* 8724	345
S. cremoris		*Escherichia coli* OSU	5
1	8	*Pseudomonas putrefaciens* OSU	64
W	7	*Alcaligenes metalcaligenes* OSU	55
C3	5	*Pseudomonas fragi* OSU	44
C13	3	*Pseudomonas fluorescens* OSU	19
E8	0	*Pseudomonas viscosa* OSU	3

[a] Expressed as units per milligram of enzyme protein, where one unit was defined as the amount of enzyme necessary to cause a 0.001 change in absorbancy at 340 mμ during the second 30-sec time interval after beginning the reaction by adding enzyme.

Examination of psychrophiles and coliform organisms for diacetyl reductase provided the findings also recorded in Table 1. Among the psychrophiles, only *P. viscosa* was relatively inactive; *A. aerogenes* was about 70 times as active as *E. coli*.

DISCUSSION

The exclusive use of *S. diacetilactis* as aroma bacteria in mixed-strain starter cultures has recently been criticized by Badings and Galesloot (2); such cultures were high in acetaldehyde and consequently imparted a yogurt-like flavor to fermented products. From Table 1, still another reason may be seen for exercise of caution when compounding starters containing only *S. diacetilactis* as aroma bacteria; some strains of this organism, such as 18-16, may produce sufficient diacetyl reductase to prevent the accumulation of diacetyl. Even in mixed-strain cultures containing both *Leuconostoc* and *S. diacetilactis*, diacetyl produced by *Leuconostoc* may be rapidly destroyed, especially since Figure 1 suggests diacetyl reductase may be active outside the cell.

The ability of *S. diacetilactis* to reduce diacetyl as found in the present work appears contrary to the report of Galesloot and Hassing (4). However, these latter authors measured the disappearance of diacetyl plus acetoin as an index of diacetyl reduction. Also, it is now known that *S. diacetilactis* possesses a reversible 2,3-butanediol dehydrogenase (11), so that some acetoin would always be present in cultures of this organism.

Selection of strains low in diacetyl reductase for designing mixed-strain starter cultures may have some merit. Strain 26-2, for example, has been used in the manufacture of superior-flavored ripened cream butter (10). The success of the recently patented aroma process of Lundstedt (7), using *S. diacetilactis* 26-2, is perhaps partly accounted for by the low level of diacetyl reductase which this strain possesses.

Extracts of *S. diacetilactis* possessing 100 units of diacetyl reductase per milligram of enzyme protein have been found capable of completely reducing at least 9.0 ppm of diacetyl within 10 min (11). Since the level of diacetyl in cultured dairy products seldom exceeds this amount, even the presence of six to eight units of enzyme, which some *S. lactis*,

S. cremoris, and *Leuconostoc* possess (Table 1), may be significant. However, since optimum temperature and pH conditions for diacetyl reductase are as yet undetermined, such reasoning is only speculative. Nevertheless, because this study revealed that all lactic streptococci and *Leuconostoc* exhibit strains that show little or no evidence of diacetyl reductase, use of such strains in mixed-strain starter cultures would seem warranted.

Results in Table 1 re-emphasize the hazards of product contamination by coliform and psychrophilic organisms. Even slight contamination by an organism as high in diacetyl reductase as *A. aerogenes* would undoubtedly yield a final product lacking flavor and aroma.

The irreversible nature of diacetyl reductase seems unfortunate, since this would even prevent equilibrium stabilization of low amounts of diacetyl. However, since the enzyme is irreversible, it may be used to advantage in removal of diacetyl from fermented alcoholic beverages, such as beer, and also from citrus juices, or, as will be reported in a future paper, in the measurement of diacetyl. It may be presumptive to assume that all diacetyl which is enzymatically destroyed in starter cultures is due to diacetyl reductase. Diacetyl mutase may also be involved.

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Acta Med. Okayama 10 : 1-5 (1956)

DIACETYL AS ACETYL DONOR

By

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Received for publication on 31 August 1955.

Introdnction

Since diacetyl mutase was found in pigeon breast muscle[1], some proposed mechanisms for this reaction have been presented[2)3)4)]. A hypothetical C_2-intermediate generated from diacetyl is believed to be an acetaldehyde-enzyme complex which is similar in behavior to the C_2 unit of acetaldehyde level derived from pyruvate by thiamine enzyme. It has been suggested that the synthesis of acetyl coenzyme A from acetaldehde enzyme complex generated from pyruvate involves the following steps[5]:

a) pyruvate + DPT-enzyme $\longrightarrow$ [acetaldehyde-DPT] + CO_2

b) [acetaldehyde-DPT] + $\begin{matrix} S \\ S \end{matrix}\!\!>$ Lipoic acid $\longrightarrow$ $\begin{matrix} \text{acetyl-S} \\ \text{HS} \end{matrix}\!\!>$ Lipoic acid + DPT-enzyme

c) $\begin{matrix} \text{acetyl-S} \\ \text{HS} \end{matrix}\!\!>$ Lipoic acid + CoA $\longrightarrow$ acetyl-CoA + $\begin{matrix} \text{HS} \\ \text{HS} \end{matrix}\!\!>$ Lipoic acid

d) $\begin{matrix} \text{HS} \\ \text{HS} \end{matrix}\!\!>$ Lipoic acid + DPN^+ $\longrightarrow$ $\begin{matrix} S \\ S \end{matrix}\!\!>$ Lipoic acid + DPNH + H^+

It should appear, therefore, that diacetyl can serve as an acetylating agent. *Doisy & Westerfeld*[6] reported that acetoin could act as an acetyl donor, although a large proportion of acetoin were utilized to form acetoacetic acid[7]. The present paper is concerned with the formation of citrate and the acetylation of SAM by means of diacetyl in the dog heart homogenates.

The following abbreviations are used: DPT, diphosphothiamine; CoA, coenzyme A; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; SAM, sulfanylamide; OAA, oxaloacetic acid.

Experimental

Materials: Diacetyl was prepared by the method of *Pechmann*[8]. Oxaloacetic acid was synthesized by the method of *Wislicenus*[9] and of *Simon*[10]. Coenzyme A was prepared from a hog liver by the method of *Lipmann*[11].

Na-ATP, DPN, DPT were kindly furnished by Dr. P. Handler of Duke University (U. S. A.). D. L-α-Lipoic acid was a generous gift from Dr. L. J. Reed of Texas University (U. S. A.).

Methods: 1 ml of 20% dog heart homogenate prepared in an alkaline isotonic KCl was pipetted into each Warburg flasks containing potassium phosphate buffer, $MgCl_2$, cofactors, and acetyl-acceptors. 0.2 ml of 5% KOH was placed in the center well. Air was used as a gas phase. After equilibrium (10 min., 37°C) diacetyl was introduced into main chamber from the side arm. The reaction was stopped by the addition of 10% Trichloroacetic acid.

Citrate in the filtrate was determind by the method of *Krebs & Eggleston*[12]. SAM was estimated by the method of *Tsuda*[13]. Diacetyl determination was carried out by the method of *White et al.*[14].

Results

a) Acetylation of Sulfanilamide

The date of Table 1 show that the complete system can acetylate about 25 per cent of added SAM. although the contribution of diacetyl to the acetylation of SAM is small. Both DPT and CoA were indispensable for this acetylation reaction. Oxygen consumption was very small in all Warburg flasks. In the presence of ATP and DPN, the acetylation of SAM was not accelerated, but the oxygen consumption was increased. α-Lipoic acid added in this system had no effect on the acetylation of SAM.

b) Citrate Formation.

The results are shown Table 2. The citrate formation from

Table 1. Acetylation of Sulfanilamide

The complete system contained 20% dog heart homogenate - 1 ml. potassium phosphate buffer (pH 7) - 100 μM, $MgCl_2$ - 10 μM, Cystein - 10 μM, CoA - about 10 units, DPT - 100 γ, SAM - 50 γ, Diacetyl - 50 γ. Final volume 3.0 ml., one hour incubation at 37°C.

	SAM acetylated	Diacetyl disappeared
Complete system	12 — 13 γ	18 — 24 γ
〃 minus DPT	5 — 6 γ	10 — 13 γ
〃 minus CoA	6 — 7 γ	negligible
〃 minus DPT & CoA	negligible	negligible

Table 2. Citrate formation

The complete system contained 20% homogenate - 1 ml., potassium phosphate buffer (pH 7) - 100 μM., $MgCl_2$ - 10 μM. Cystein - 10 μM. CoA - about 10 units. DPT - 100 γ. Diacetyl - 5 μM~10 μM. OAA - 10~20 μM. Final volume 3.0 ml., 15~20 min. incubation at 37°C.

Experiment 1. diacetyl - 5 μM, oxaloacetate - 10 μM. 15 min. incubation

	Citrate formed	Diacetyl disappeared
Complete system	205 γ	110 γ
〃 minus DPT	120 γ	30 γ
〃 minus CoA	110 γ	60 γ
〃 minus CoA & DPT	90 γ	40 γ

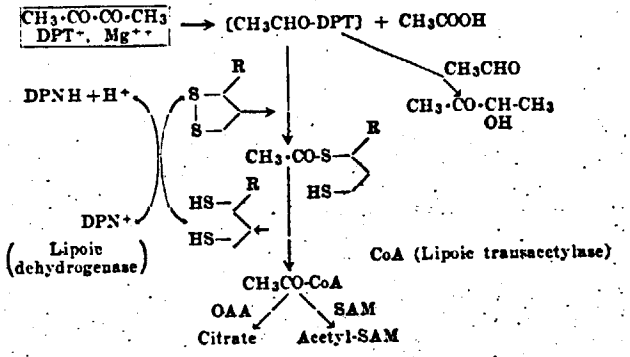
Experiment 2. diacetyl - 10 μM. oxaloacetate - 20 μM. 20 min. incubation

	Citrate formed	Diacetyl disappeared
Complete system	386 γ	250 γ
〃 minus Diacetyl	330 γ	—
〃 minus OAA	100 γ	220 γ
〃 minus Diacetyl & OAA	50 γ	—

diacetyl and oxaloacetate also requires both DPT and CoA. Although the contribution of diacetyl to the citrate formation is also small in the experimental condition used, it appears to be certain that some portion of citrate formed is derived from diacetyl. Oxygen consumption was negligible. Malonate, ATP and DPN had not any measurable effect on the formation of citrate.

Discussion

From the data presented, it is apparent that the diacetyl can act as an acetyl donor in the dog heart homogenate. The acetylation of SAM and the formation of citrate in this experiments may be carried out follows:



Although the fate of acetic acid generated from diacetyl at the first step cleavage by thiamine enzyme is not clear, in this dog heart system acetic acid acts as an acetyl donor only in the presence of ATP. This fact is the point different from C_2-unit generated from diacetyl. Lipoic acid and DPN may be present enough in this system.

Summary

Diacetyl can serve as an acetyl donor for the formation of citrate and the acetylation of sulfanilamide in the dog heart homogenate. Diphosphothiamine and coenzyme A are essential for these reactions.

Grateful acknowledgement is made to *Y. Takeuchi* for technical assistance, to Dr. *S. Mizuhara* for valuable advice, and to the Department of Education for a grant.

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1861

Bull. Soc. Pharm. Nancy 19: 22-30, 1968

Simple and Sensitive Colorimetric Method for the Determination of Diacetyl in Urea

by G. Siest and Y. Landry

Numerous methods have been put forth for the identification and quantitative determination of diacetyl in lactic leavens, butter, wine, beer and fruit juices:

-- Transformation into dimethylglyoxime, after the addition of an excess of hydroxylamine

— According to Englis(1), this dioxime can be determined by a direct measure of the absorption at 226 nm.

— In the presence of Ni++ dimethylglyoxime, in a slightly alkaline milieu, quantitatively gives an insoluble pink precipitate evalulated by gravimetry or by colorimetry after dissolving in chloroform or pyridine (2).

-- In the presence of Ni^{4+} in an alkaline milieu, dimethylglyoxime forms a soluble red compound. These operations are long and delicate (2).

-- In the presence of Fe++ using Prill's and Hammer's method (3), in an ammoniacal milieu, dimethylglyoxime shows a red coloration.

-- Color reactions of diacetyl

— The methods derived from the Voges Proskauer (6) reaction are based on the red color obtained with guanidic derivatives in a basic milieu. Several variations of this method have been proposed (4), (5).

-- Pien and Colleagues (6) condensed diacetyl with diaminobenzinidine in an acid milieu: the quinoxaline formed is measured by colorimetry.

-- The colored compound formed with urea in an acid milieu was used by White and Colleagues (7). But this method is not very precise nor

sensitive because the little studied colored reaction is unstable and difficult to reproduce.

-- Different oxydimetric methods have also been proposed (8) and coming from these, a polarographic technique (9).

Finally, let us note the determination of diacetyl by gas chromatography, a modern method recommended by Shilman (10).

After studying the determination of carbamide compounds with alpha-diacetones (11,12), it seemed to us useful to restate the problem by reversing it: the determination of diacetyl-alpha-diacetone -- which is found in biologic milieux -- with a carbamide derivative.

Choice of Reagents

In the course of our studies on the color reactions of carbamide derivatives with alpha-diacetones, we verified in several rapid experiments, that a strong concentration of urea enabled us to reveal small quantities of diacetyl; as in the reverse operation, a large amount of diacetonic reagent was necessary for the determination of small quantities of urea.

A detailed study of the influence of acid reagents, oxidants and various adjuvants enabled us to define the optimum conditions for the determination of carbamide derivatives. Learning from this experience, we wanted to apply to the determination of diacetyl, the principal improvements by limiting ourselves to the most important reagents:

-- Acid reagents: H_3PO_4 and H_2SO_4

-- Oxidizing compounds: ferric salts in a phosphoric milieu and arsenic acid in a sulfuric milieu have a significant effect on the kinetics of the color reactions.

-- Various adjuvants: essentially, we used carbazone which remarkably

intensified the color reaction while at the same time, stabilizing it. Since our study, Hunninghake and Grisolá (13) have proposed semidine (N-phenyl-p-phenylenediamine) in order to improve the determination by using diacetylmonoxime. We studied this operating method for the determination of diacetyl. The sensitivity obtained is identical to that of the reaction in the presence of carbazone. But the relative stability of the semidine solution and the necessity of an additional manipulation in order to introduce the ferric reagents after warming are sufficient reasons not to prefer such a reagent, difficult to add and hard to come by.

I. Method in an Arsenic-Sulfuric Milieu

Reagents

I. Freshly distilled diacetyl eliminating the first and last quart. solution 0.1 M.

II. Arsenic-Sulfuric reagent.

H_2SO_4 R.P. ..... 50 ml

Distilled water ..... 50 ml

Mix carefully. Let it cool partially and add:

H_3AsO_4 ..... 10 g

Decant if necessary.

III. Carbazone 0.1 M.

IV. Urea 0.1 M.

Determination

Pipette successively into a test-tube:

A quantity of the solution to be measured so that the amount of diacetyl comprises approximately between 2 and 600 nanomoles in a maximum of 3 ml of solution.

Arsenic-Sulfuric Reagent ..... 1.5 ml

Carbazone ..... 0.5 ml

Urea ..... 1.5 ml

Distilled water .....q.s.p. 6 ml

Shake. Put it into a double-boiler and boil for 15 minutes. Cool 10 minutes. Spectrophotometric reading at 460 nm.

-- Daily samples: Aside from the stock solution of diacetyl, prepare dilutions in such a way so as to establish two points on the scale between 2 and 600 nanomoles.

-- Standardization scale: Done as the samples, the curve set out is linear, therefore an extinction co-efficient may be used.

Reagent

I. -- Stock polution of diacetyl M/10 identical to the first method.

II. Phospho-ferric reagent

H_3PO_4 R.P. ..... 90 ml.

$FeCl_2$ 0.2 M ..... 15 ml

III. Carbazone 0.1 M.

IV. Urea 0.1 M.

Determination

Pipette successively into a test-tube:

A quantity of solution so that 2 to 600 nm of diacetyl is contained in a volume of 1 ml at the maximum.

Phospho-ferric reagent ..... 3.5 ml

Carbazone 0.1 M ..... 0.5 ml

Urea 0.1 M ..... 1 ml

Distilled water .....q.s.p. 6 ml

Shake. Place into a double-boiler and boil for 30 minutes. Cool 10 minutes. Spectrophotometric reading at 460 nm.

The standardization scale and the samples are prepared as in the first method. The extinction coefficient is sensitively different.

DISCUSSION

JUSTIFICATION OF METHODS

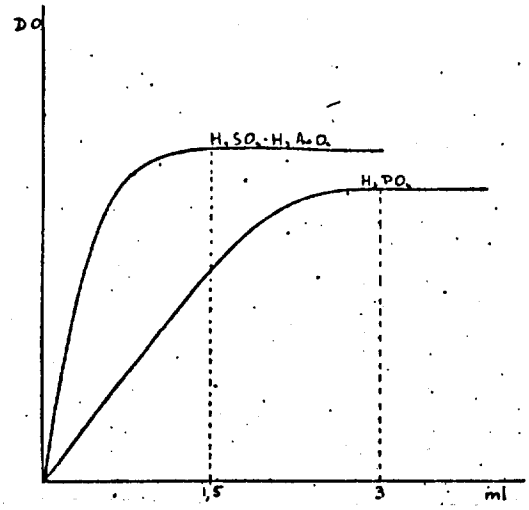
1. -- Acid reagents and oxidants

Arsenic-sulfuric reagent

The optimum for 6 ml of final reacting volume was reached from 1 ml (graph 1). We retained a volume of 1.5 ml.

The most intense color reaction takes place with 3 ml H_3PO_4 (graph 1).

The optimum quantity of Fe^{3+} is introduced by 0.5 ml of M/5 solution. These results enable us to propose a phosphoferric reagent previously described and a volume of 3.5 ml.



Graph 1. Concentrations of acid reagents

2. -- Urea solution

1 ml of 0.1 M urea is the optimum quantity (graph 2) for the two

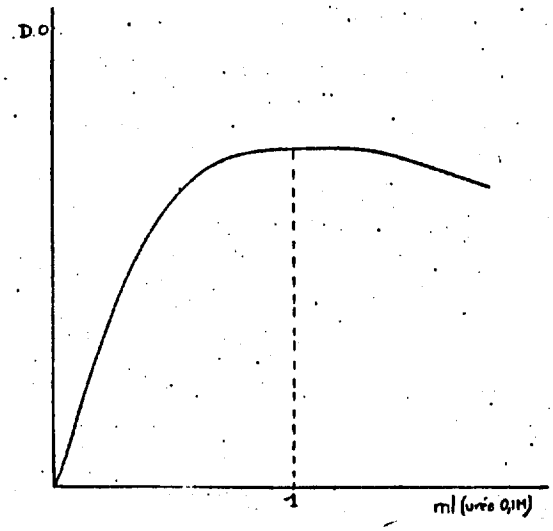
acid reagents.

3. -- Carbazone

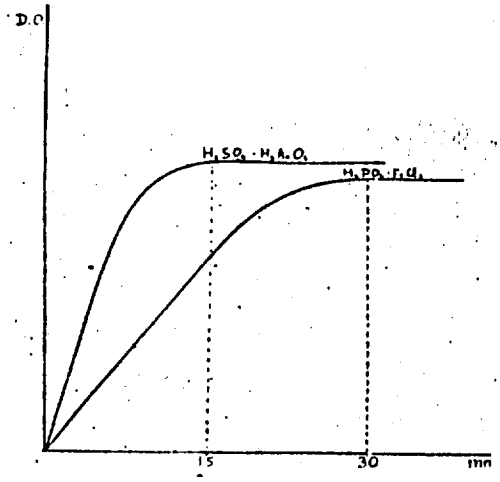
The optimum quantity of carbazone is introduced by 0.5 ml of 0.1 M solution.

4. -- Kinetics of the color reaction

Graph 3 shows that with the chosen reagent proportions, the color reaction takes place completely after 15 mn in an arsenic-sulfuric-carbazone milieu and only after 30 mn in a phosphoferric-carbazone milieu.



Graph 2. Concentration of urea

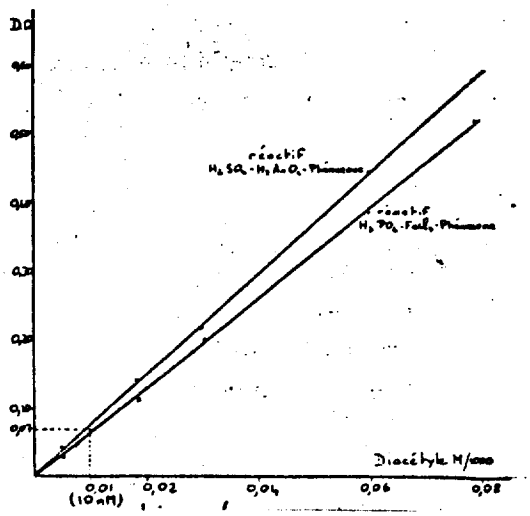


Graph 3. Kinetics of the color reactions taking place at 100 °C

6. -- Sensitivity

The sensitivity of the two methods is identical: 10 nm of diacetyl gives an optical density of 0.07.

The tracing of the readings are linear (graph 4), enabling us to use the extinction coefficient.



Graph 4. Curves of the readings

If we divide all the reagents in half, and therefore the total reacting volume totals 3 ml, this method of determination allows us to measure 2 nM of diacetyl.

REACTION OF ACETOIN WITH THE RECOMMENDED REAGENTS

Acetoin or acetylmethylcarbinol is considered as the boilogical precursor of diacetyl.

CH_3-CO-CO-CH_3	CH_3-CHOH-CO-CH_3
Diacetyl	Acetoin

For this reason, acetoin is often present with diacetyl. In certain cases, it is interesting to determine both substances simultaneously (3) or separately after passing them over ion-exchange resins (14). Therefore,

we tested the reactivity of acetoin under the conditions previously described.

At equal concentrations, acetoin gives colorations 10 times less intense than diacetyl with the arsenic-sulfuric reagent.

In the presence of a low percentage of acetoin, we can therefore ignore its interference.

In a phosphoferric milieu, acetoin is completely oxidized in diacetyl, and this method allows us to measure the sum of diacetyl and acetoin.

Taking into account these results, we prevented the oxidation of acetoin by the suppression of ferric chloride. In a phosphoric milieu and in the presence of carbazone as the only intensifying substance, acetoin no longer interferes in the determination of diacetyl. This last method while slightly less sensitive than those recommended above, seems significant to us because of its specificity from the first assays which we did. We actually did our reasearch with this in mind.

In summary, the methods described offer various possibilities:

-- Determination of the diacetyl-acetoin total.

-- Determination of diacetyl and acetoin after separation.

-- Specific determination of diacetyl in the presence of acetoin.

Méthode colorimétrique simple et sensible de dosage du diacetyle par l'urée

SIEST G. et LANDRY Y.

117

De nombreuses méthodes ont été proposées pour l'identification e le dosage du diacétyle dans les levains lactiques, les beurres, les vins, l bières et les jus de fruits :

— *Transformation en diméthylglyoxime*, après addition d'un excès d'h droxylamine

— Cette dioxime peut être dosée selon ENGLIS (1) par mesure d recte de l'absorption à 226 nm.

— En présence de Ni^{++} la diméthylglyoxime donne quantitativ ment, en milieu faiblement alcalin, un précipité rose insoluble évalué p gravimétrie ou par colorimétrie après dissolution dans le chloroforme e la pyridine (2).

— En présence de Ni^{++} en milieu alcalin la diméthylglyoxime form un complexe rouge soluble. Ces techniques sont longues et délicates (2

— En présence de Fe^{++} selon la technique de Prill et Hammer (3 la diméthylglyoxime donne en milieu ammoniacal une coloration roug

— *Réactions colorées du diacétyle.*

— Les méthodes dérivant de la réaction de Voges Proskauer ((sont basées sur la coloration rouge obtenue avec les dérivés guanidiqu en milieu basique. De nombreuses variantes de cette méthode ont é proposées (4), (5).

— PIEN et coll. (6) condensent le diacétyle avec la diaminobenzin dine en milieu acide : la quinoxaline jaune formée est estimée par col rimétrie.

— Le complexe coloré formé avec l'urée en milieu acide a été utilisé par WHITE et coll. (7). Mais cette méthode était peu précise et peu sensible car la réaction colorée mal étudiée était instable et difficilement reproductible.

— Différentes *méthodes oxydimétriques* ont également été proposées (8) et en découlant, *une technique polarographique* (9).

Enfin, notons le dosage du diacétyle par *chromatographie en phase gazeuse*, méthode moderne préconisée par SHILMAN (10).

Après avoir étudié le dosage des dérivés carbamidés par des alpha-dicétones (11, 12), il nous a paru utile de refaire une mise au point du problème inverse : dosage du diacétyle - alpha-dicétone que l'on trouve dans les milieux biologiques — par un dérivé carbamidé.

CHOIX DES REACTIFS

Au cours de notre étude des réactions colorées des dérivés carbamidés avec des alpha-dicétones, nous avions vérifié en quelques essais rapides, qu'une forte concentration en urée permettait de déceler de faibles quantités de diacétyle, comme à l'inverse un excès important de réactif dicétonique était nécessaire pour le dosage de faibles quantités d'urée.

L'étude détaillée de l'influence des réactifs acides, oxydants et des différents adjuvants nous a permis de définir les conditions optima pour e dosage des dérivés carbamidés. Profitant de cette expérience, nous avons voulu appliquer au dosage du diacétyle, les principales améliorations trouvées en nous limitant aux réactifs les plus intéressants :

— Réactifs acides : H_3PO_4 et H_2SO_4.

— Composés oxydants : les sels ferriques en milieu phosphorique et l'acide arsénique en milieu sulfurique ont une action importante sur la cinétique des réactions colorées.

— Adjuvants divers : nous avions retenu essentiellement la phénazone qui intensifiait remarquablement la réaction colorée tout en la stabilisant. Depuis notre étude, HUNNINGHAKE et GRISOLA (13) ont proposé a semidine (N-phényl-p phénylènediamine) pour améliorer le dosage de l'urée par la diacétylmonoxime. Nous avons étudié cette modalité opératoire pour le dosage du diacétyle. La sensibilité obtenue est identique à celle de la réaction en présence de phénazone. Mais la stabilité relative de la solution de semidine et la nécessité d'une manipulation supplémentaire pour l'introduction d'un réactif ferrique après le chauffage sont des raisons suffisantes pour ne pas préconiser un tel réactif, de surcroît onéreux et peu courant.

1875

METHODES PROPOSEES

I. — METHODE EN MILIEU ARSENICO-SULFURIQUE

Réactifs

I. — Diacétyle fraîchement distillé en éliminant le premier et le dernier quart.
Solution stock 0,1 M.

II. — Réactif-arsénico-sulfurique.

H_2SO_4 R.P.	50 ml
Eau distillée	50 ml

Mélanger avec précaution. Laisser refroidir partiellement et ajouter :

$H_3As.O_4$	10 g

Décanter si nécessaire.

III. — Phénazone 0,1 M.

IV. — Urée 0,1 M.

Dosage

Pipetter successivement dans un tube à essai :

Une quantité de solution à doser telle que la quantité de diacétyle soit comprise approximativement entre 2 et 600 nanomoles dans un maximum de 3 ml de solution.

Réactif arsénico-sulfurique		1,5 ml
Phénazone 0,1 M		0,5 ml
Urée 0,1 m		1,5 ml
Eau distillée	q.s.p.	6 ml

Agiter. Placer au bain-marie bouillant pendant 15 minutes. Refroidir 10 minutes. Lecture au spectrophotomètre à 460 nm.

— Témoins journaliers : A partir de la solution stock de diacétyle, préparer des dilutions de manière à établir deux points de gamme entre 2 et 600 nanomoles.

— Gamme d'étalonnage : Effectuée comme les témoins, le tracé obtenu est linéaire, on pourra donc utiliser un coefficient d'extinction.

II. — METHODE EN MILIEU PHOSPHOFERRIQUE

Réactifs

I. — Solution stock de diacétyle M/10 identique à la première méthode.

II. — Réactif phosphoferrique

H_3PO_4 R.P.	90 ml
$FeCl_3$ 0,2 M	15 ml

III. — Phénazone 0,1 M.

IV. — Urée 0,1 M.

Dosage

Pipetter successivement dans un tube à essai :

Une quantité de solution telle que 2 à 600 nanomoles de diacétyle soit contenu dans un volume de 1 ml au maximum.

Réactif phosphoferrique		3,5 ml
Phénazone 0,1 M		0,5 ml
Urée 0,1 M		1 ml
Eau distillée	q.s.p.	6 ml

Agiter. Placer au bain-marie bouillant 30 minutes. Refroidir 10 mn. Lire au spectrophotomètre à 460 nm.

La gamme d'étalonnage et les témoins sont préparés comme dans la première méthode. Le coefficient d'extinction est sensiblement différent.

DISCUSSION

JUSTIFICATION DES METHODES

. — *Réactifs acides et oxydants*

Réactif arsénicosulfurique :

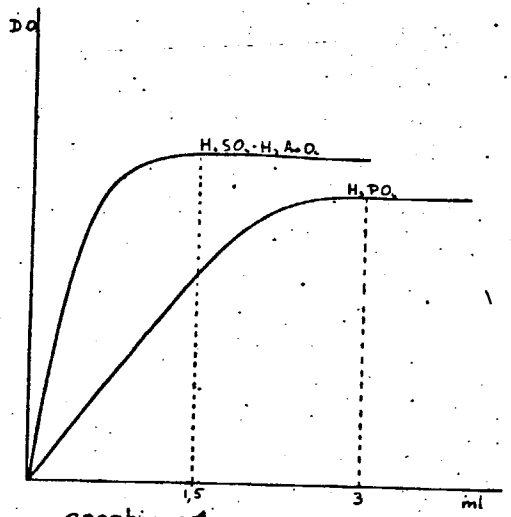
La concentration optima pour 6 ml de volume réactionnel final est atteinte dès 1 ml (graphique 1). Nous avons retenu un volume de 1,5 ml.

18715

Réactif phosphoferrique :

La réaction colorée la plus intense se développe avec 3 ml de H_3PO_4 (graphique 1).

La quantité optima de Fe^{3+} est introduite par 0.5 ml de solution M/5.Ces résultats nous permettent de proposer le réactif phosphoferrique décrit précédemment et un volume de 3,5 ml.



graphique 1

Concentration en réactifs acides

2. — *Solution d'urée*

1 ml d'urée 0.1 M est la quantité optima (graphique 2) avec les deux réactifs acides.

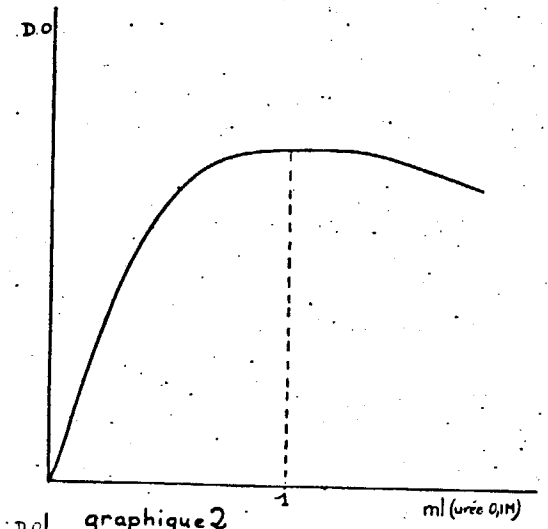
3. — *Phénazone*

La quantité optima de phénazone est introduite par 0.5 ml de solution 0,1 M.

4. — *Cinétique de la réaction colorée*

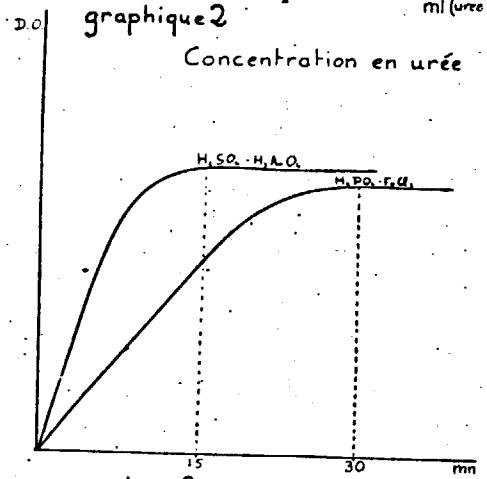
Le graphique 3 met en évidence qu'avec les proportions de réactifs

choisis la réaction colorée est entièrement développée après 15 mn en milieu arsénicosulfurique-phénazone et seulement après 30 mn en milieu phosphoferrique-phénazone.



graphique 2

Concentration en urée



graphique 3

Cinétiques des réactions développées à 100°C

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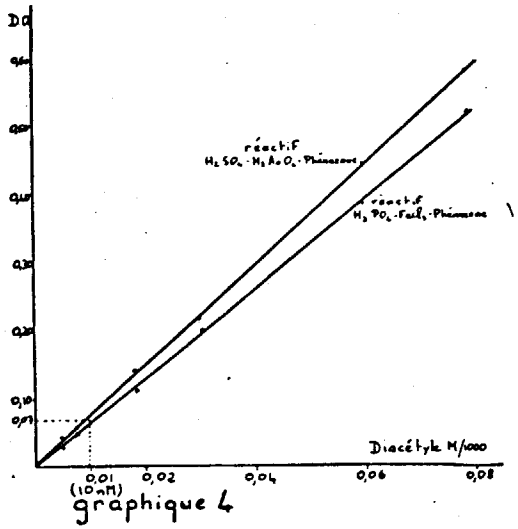
5. — *Stabilité*

Dans les conditions précédemment décrites, les colorations sont stables 48 heures. Elles sont très peu photosensibles.

6. — *Sensibilité*

La sensibilité des deux méthodes est identique : 10 nanomoles de diacétyle donnent une D.O. de 0,07.

Le tracé d'étalonnage est linéaire (graphique 4), ce qui permet d'utiliser le coefficient d'extinction.



graphique 4

Courbes d'étalonnage

Si nous réduisons tous les réactifs de moitié et donc le volume réactionnel total à 3 ml cette méthode de dosage permet de doser 2 nM de diacétyle.

REACTION DE L'ACETOINE AVEC LES REACTIFS PRECONISES

L'acétoïne ou acétylméthylcarbinol est considérée comme le précurseur biologique du diacétyle.

$CH_3 - CO - CO - CH_3$ — **Diacétyle**

$CH_3 - CHOH - CO - CH_3$ — **Acétoïne**

Pour cette raison, l'acétoïne est souvent présente dans les mêmes milieux que le diacétyle. Dans certains cas, il est intéressant de doser ces deux produits simultanément (3) ou séparément après passage sur résine échangeuse d'ions (14). Nous avons donc testé la réactivité de l'acétoïne dans les conditions précédemment décrites.

A concentration égale, l'acétoïne donne des colorations dix fois moins intenses que le diacétyle avec le réactif arsénicosulfurique.

En présence d'un faible pourcentage d'acétoïne, nous pourrons donc négliger son interférence.

En milieu phosphoferrique, l'acétoïne est oxydée complètement en diacétyle, cette méthode permet donc de doser la somme diacétyle-acétoïne.

Compte tenu de ces résultats, nous avons évité l'oxydation de l'acétoïne par la suppression du chlorure ferrique. En milieu phosphorique et en présence de phénazone comme seule substance intensifiante, l'acétoïne n'interfère plus dans le dosage du diacétyle. Cette dernière méthode, bien que légèrement moins sensible que celles préconisées ci-dessus, nous paraît donc appréciable par sa spécificité, selon les premiers essais que nous avons effectués. Nous poursuivons actuellement nos recherches dans ce sens.

En résumé, les méthodes décrites offrent donc diverses possibilités :

— Dosage de la somme diacétyle-acétoïne.

— Dosage du diacétyle et de l'acétoïne après séparation.

— Dosage spécifique du diacétyle en présence d'acétoïne.

Laboratoire
de la Maîtrise de Conférence
de Chimie Biologique.

1875

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ANALYTICAL BIOCHEMISTRY 22, 154–160 (1968)

Separation of Diacetyl, Acetoin, and 2,3-Butylene Glycol by Salting-Out Chromatography

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Received February 10, 1967

Lack of a method for quantitative separation of mixtures of compounds in microgram quantities has hampered studies of the mechanisms by which diacetyl (2,3-butanedione), acetoin (3-hydroxy-2-butanone), and 2,3-butylene glycol (2,3-butanediol) are formed from pyruvate, and the metabolic interrelationships among these compounds. Diacetyl and acetoin have commonly been determined as the sum of both, by the colorimetric procedure of Westerfeld (1) or by the steam-distillation procedure of van Niel *et al.* (2) as modified by Michaelian and Hammer (3) or Prill and Hammer (4). The latter procedure can be used to determine diacetyl and acetoin differentially, but large quantities of the compounds are required and the method has inherent disadvantages, including possibilities of thermal decomposition, chemical rearrangement, and oxidation of the compounds being separated. Gas chromatography can be used for determining diacetyl and acetoin, but this method requires clarification of biological test systems and is not readily adaptable to quantitative studies.

Acetoin, diacetyl, and 2,3-butylene glycol are neutral, water-soluble, structurally related, organic nonelectrolytes, representing three levels of oxidation of the same four-carbon skeleton. This paper presents a procedure for separating them quantitatively by salting-out chromatography, which is the separation of water-soluble nonelectrolytes by elution with an aqueous salt solution through a column of ion-exchange resin. Preliminary results with this method substantiate its usefulness (5–7). The procedure is based on the report of Sargent and Rieman (8), who found that separation of organic nonelectrolytes was greatly improved when a salt solution was used instead of water as eluant on columns of ion-exchange resin. They reported separating several series of closely related compounds with the new procedure. Additionally, the present paper includes modifications of the method reported by Happold and Spencer for determining 2,3-butylene glycol (9).

MATERIALS AND METHODS

Columns. Analytical-grade Dowex 1-X8 anion-exchange resin in the chloride form, 200–400 mesh, washed alternately with 1 *N* NaOH and 1 *N* HCl, was used for experiments involving columns of two sizes with different physical and chemical parameters. Column I had a diameter of 1.9 cm and was packed to a bed height of 27 cm with resin slurried in 3.0 *M* $(NH_4)_2SO_4$. The resin in this column was converted to the sulfate form by washing with 3.0 *M* $(NH_4)_2SO_4$ until tests for chloride ions in the effluent were negative. Column II had a diameter of 3.5 cm and was packed to a bed height of 54 cm with resin slurried in saturated Na_2SO_4. This column was washed with saturated Na_2SO_4 until tests for chloride ions in the effluent were negative.

Separation, Elution, and Collection. All separations were made at room temperature; 1 ml samples of solutions containing 25 to 100 μg of each of the compounds to be separated were placed on top of the resin beds. Diacetyl, acetoin, and 2,3-butylene glycol were separated and eluted by washing columns I and II, respectively, with 0.5 *M* $(NH_4)_2SO_4$ at a flow rate of 0.5 ml/min and 0.5 *M* Na_2SO_4 at a flow rate of 1.5 ml/min. Effluents were collected and distributed into test tubes by a fraction collector. Fraction sizes were determined by a drop counter, synchronized with the collector.

Conversion of 2,3-Butylene Glycol to Acetoin. Column II, with 0.5 *M* Na_2SO_4 as eluant, proved advantageous in the separation and determination of 2,3-butylene glycol. After separation, the glycol was converted to acetoin by the following procedure, based on the oxidation of 2,3-butylene glycol with bromine water, first reported by Happold and Spencer (9): (*1*) To 5 ml of the glycol fraction plus 4 ml of water in a red, light-resistant, 15 mm × 15-cm test tube capped with a 12 mm rubber septum, 4 ml of bromine water was added with a syringe. (*2*) The mixture was heated for 3 min in the dark in a bath of boiling water. (*3*) Next, the mixture was transferred to a water bath at room temperature and held for 15 min in the dark. (*4*) Finally, a small amount of Na_2SO_3 (on a spatula point) was added to neutralize excess bromine water.

Determination of Diacetyl or Acetoin. With diacetyl, acetoin, and 2,3-butylene glycol separated, and the glycol oxidized to acetoin, the Westerfeld method (1) was used for their determination.

Chemicals. Diacetyl and acetoin were obtained from Eastman Organic Chemicals; 2,3-butylene glycol and esterified α-acetolactate were obtained from K & K Laboratories, Jamaica, New York. An infrared spectrum determined for the compound received from K & K Laboratories indicated that it was the acetoxy ethyl ester of α-acetolactate.

1914

α-Acetolactate was prepared from the ester by saponifying with two equivalents of cold 0.025 *N* NaOH, added slowly to the system held at 5°C and continuously purged with a stream of nitrogen. The creatine and recrystallized α-naphthol, used as reagents for the Westerfeld test, were respectively obtained from Calbiochem and Fisher Chemical Co.

RESULTS AND DISCUSSION

Nature of the Eluant. The following eluant salts were compared on columns with the dimensions of column I except that the resin was left in the chloride form for studying the chlorides: $(NH_4)_2SO_4$, NH_4Cl,

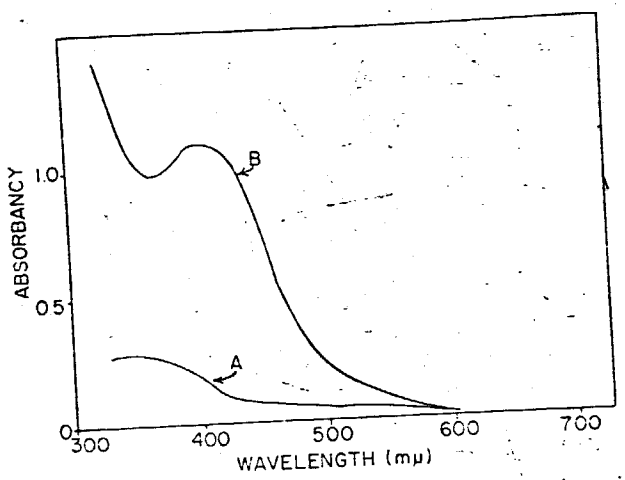

FIG. 1. Absorption spectra of eluant salt solutions after oxidation with bromine water and addition of Westerfeld reagents; 5 ml of a 0.5 *M* eluant-salt solution was oxidized with bromine water (see text). Westerfeld reagents were added, and the absorption spectrum was determined with a Beckman model DB spectrophotometer: (A) Na_2SO_4; (B) $(NH_4)_2SO_4$.

NaCl, and Na_2SO_4. For each test, 1 ml of an aqueous solution containing 100 μg each of diacetyl, acetoin, and 2,3-butylene glycol was placed on the column.

Each of the four salts, at a concentration of 0.5 *M* and a flow rate of 0.5 ml/min, with a fraction size of 1.6 ml (32 drops), separated diacetyl and acetoin, but only $(NH_4)_2SO_4$ completely separated acetoin and 2,3-butylene glycol. The compounds emerged from the column in decreasing order of polarity, irrespective of the salt used: 2,3-butylene glycol, acetoin, and diacetyl. Recoveries were quantitative with each of the salts, showing that adsorption of the compounds to the column was not a

problem. Recoveries of the three compounds in over 100 determinations ranged from 95 to 104%.

The compounds appeared earlier in the effluent volume with the ammonium salts, and in more compact bands. Resolution was somewhat better with the sodium salts and with the sulfates. An increase in concentration of each of the eluant salts resulted in an increase in elution volume for each component of the mixture, but resolution of the compounds was not improved. No further attempts were made to get better resolution of acetoin and 2,3-butylene glycol on column I with the three salts not giving excellent resolution.

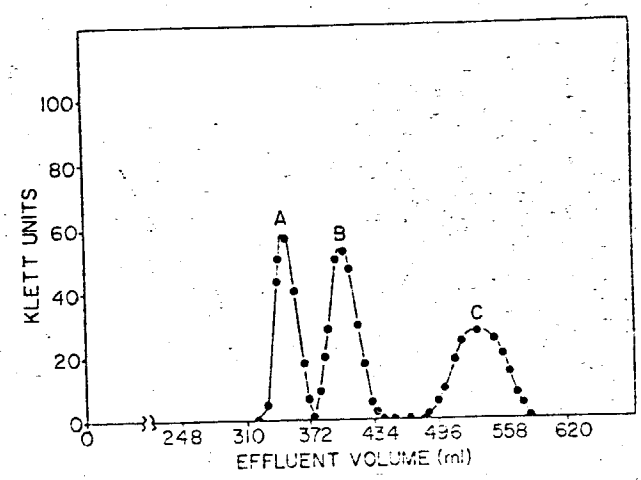

FIG. 2. Elution pattern of a mixture of 2,3-butylene glycol, acetoin, and diacetyl chromatographed on Dowex 1-X8 anion-exchange resin in the sulfate form. A 1 ml aqueous solution containing 100 μg each of 2,3-butylene glycol (A), acetoin (B), and diacetyl (C) was added to a 3.5 × 54 cm column. Elution was with 0.5 *M* Na_2SO_4 at a flow rate of 1.5 ml/min. Fractions of 6 ml were collected, and those containing glycol were oxidized to acetoin (see text). Subsequently, all fractions were analyzed by the Westerfeld procedure (1).

Analysis of Fractions Containing Diacetyl or Acetoin. The Westerfeld method (1) was used. With 0.5 *M* Na_2SO_4 as eluant, no difficulties were encountered. With 0.5 *M* $(NH_4)_2SO_4$ as eluant, no difficulties were encountered if the aliquots used in the Westerfeld test were no larger than 3 ml. Larger aliquots resulted in a white precipitate.

Analysis of Fractions Containing 2,3-Butylene Glycol. Difficulties were encountered in the oxidation and determination of 2,3-butylene glycol separated on column I by washing with $(NH_4)_2SO_4$. Upon addition of the Westerfeld reagents, following oxidation of the glycol to acetoin

with bromine water (9), a masking emerald-green color was encountered. The green color appeared upon addition of the reagents and likely resulted from their reacting with products from a reaction of $(NH_4)_2SO_4$ with the bromine water. Absorption spectra in Figure 1 indicate such a reaction.

One method found for avoiding the green color was to ensure complete oxidation of the $(NH_4)_2SO_4$ by increasing the heating period required for oxidizing the glycol. We found a 45 min heating period satisfactory. Another method was to use 0.5 *M* Na_2SO_4 as eluant in place of the $(NH_4)_2SO_4$. With this substitution, a 3 min heating period was found to be satisfactory for oxidizing the glycol, but column I did not give

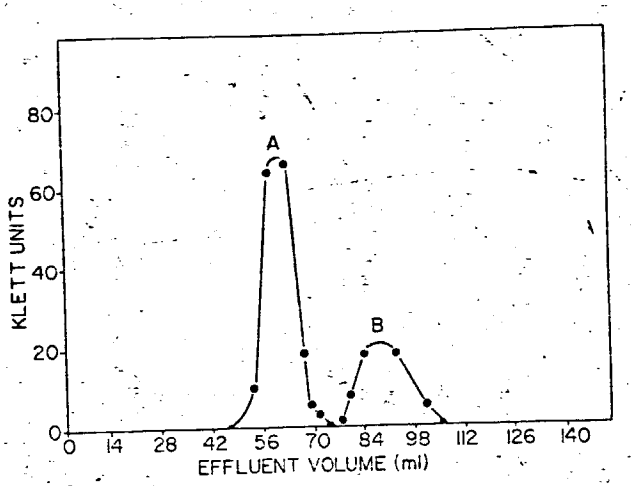

FIG. 3. Elution pattern of a mixture of acetoin and diacetyl chromatographed on Dowex 1-X8 anion-exchange resin in the sulfate form. A 1 ml aqueous solution containing 100 μg each of acetoin (A) and diacetyl (B) was added to a 1.9 × 27 cm column. Fractions of 1.5 ml were collected during elution with 0.5 *M* $(NH_4)_2SO_4$ at a flow rate of 0.5 ml/min. Fractions were analyzed by the Westerfeld procedure (1).

clear-cut separation of acetoin and 2,3-butylene glycol with 0.5 *M* Na_2SO_4 as eluant.

The possibility was investigated of using a larger column with 0.5 *M* Na_2SO_4 as eluant. Column II, described in the section on methods, was prepared and tested. This larger column accomplished excellent separation of all three compounds, with the flow rate increased to 1.5 ml/min and the fraction size to 6.2 ml (128 drops). Figure 2 shows the separation of diacetyl, acetoin, and 2,3-butylene glycol. Figure 3 shows the separation of diacetyl and acetoin with column I. Where 2,3-butylene glycol is

not involved or its determination is not required, column I with 0.5 *M* $(NH_4)_2SO_4$ as eluant is satisfactory.

Interfering Substances. Application of a column chromatographic procedure to complex biological test systems requires investigation of the possibility that other substances might interfere with the results. Pyruvate, citrate, and sugars are ruled out by the fact that they do not react with the reagents of the Westerfeld test to give the typical red color. Acetaldehyde gives an atypical color, but 24 hr is required (10), and we found acetaldehyde to appear in an effluent volume region different from that of diacetyl, acetoin, or 2,3-butylene glycol. Thiamine is not normally present in the large amounts reported to produce a red-colored complex with reagents of the Westerfeld test (10).

A compound of considerable concern was α-acetolactate. This acid is stable above neutrality (11). Thus, for testing α-acetolactate, converted to the free-acid form as indicated in the section on methods, the eluant salt solutions were adjusted to pH 7.0, to avoid the possibility of on-column decarboxylation. α-Acetolactate did not move in the same effluent volume regions as diacetyl, acetoin, or 2,3-butylene glycol.

Columns I and II are being applied in studies dealing with diacetyl biosynthesis and the metabolic interrelationships among diacetyl, acetoin, and 2,3-butylene glycol (6, 7). Considerable data accumulated to date show that the addition directly to the columns of complex biological materials, e.g., cell-free extracts, does not interfere with their efficiency in quantitatively separating diacetyl, acetoin, and 2,3-butylene glycol.

SUMMARY

Salting-out chromatography was found suitable for separating microgram quantities of diacetyl, acetoin, and 2,3-butylene glycol at room temperature. One milliliter of a solution containing 25 to 100 μg of each of the compounds to be separated is applied to the top of a column of Dowex 1-X8 anion-exchange resin in the sulfate form. The compounds are separated and eluted from a 1.9 × 27 cm column with 0.5 *M* $(NH_4)_2SO_4$ at a flow rate of 0.5 ml/min, or from a 3.5 × 54 cm column with 0.5 *M* Na_2SO_4 at a flow rate of 1.5 ml/min. The compounds emerge from the columns quantitatively in decreasing order of polarity: 2,3-butylene glycol, acetoin, and diacetyl. The separated 2,3-butylene glycol is oxidized to acetoin with bromine water. With the compounds separated and the glycol converted to acetoin, the Westerfeld method can be used to complete the quantitative determinations.

ACKNOWLEDGMENT

This investigation was supported in part by research grant EF-138 from the National Institutes of Health, U. S. Public Health Service.

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Acta virol. 7 : 472—474, 1963

Inactivation of Influenza Viruses by α - Dicarbonyl Compounds

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Received February 5, 1963

It was found that derivatives of glyoxal, α-oxyaldehydes and related compounds inhibit virus multiplication in embryonated eggs (Tiffany *et al.*, 1957; Moffet *et al.*, 1957; De Bock *et al.*, 1957; McLimans *et al.*, 1957). For example, injection of 2.5 mg. kethoxal into the allantoic cavity shortly before or some hours after infection with 50 EID_{50} of Newcastle disease virus protected a high proportion of chick embryos against death. The substance showed a similar protective effect against infection with A type influenza virus. It was also demonstrated that the drug exhibits a virucidal effect and thus its activity was explained by direct action on the virus (McLimans *et al.*, 1957). Spectrophotometric investigation of the interaction of glyoxal derivatives and nucleotides suggested that these compounds react in a specific way with the guanine base (Staehelin, 1959). The glyoxal molecules probably penetrate first through the protein coat of the virus and then react with the ribonucleic acid of the intact virus. Comparative experiments have shown that infectious ribonucleic acid isolated from tobacco mosaic virus was inactivated at a markedly higher rate by glyoxals than by formaldehyde, but intact virus was inactivated more rapidly by the smaller formaldehyde molecules.

In the present work we studied the kinetics of virus inactivation by various compounds of the group mentioned with the aim of elucidating the relationship between the structure of the compounds and their inactivating effect. We were especially interested in comparing the effects of diacetyl (1,2-diketon) and glycerinaldehyde (α-oxyaldehyde) with those of glyoxals.

Materials and Methods

Virus. FM1 influenza virus strain was used in its 160th egg passage. The virus was passaged by intraallantoic inoculation of 11 days old chick embryos. The harvested infective allantoic fluid used in the inactivation experiments was clarified by centrifugation at 3,000 rev/min. for 5 minutes and contained $10^{7.2}$ EID_{50} of virus per ml.

Inactivating compounds. The following substances were tested for their ability to inactivate influenza virus: phenylglyoxalhydrate, glyoxalbisulphite, 4-biphenylglyoxalbisulphite, polymeric glyoxal, glycerinaldehyde, diacetyl, acetylacetone and ninhydrin. The first 3 compounds were synthesized in our Institute, the others were commercial preparations.

Virus inactivation. 0.1 ml. portions of infective allantoic fluid were mixed with 0.9 ml. of a solution containing 1 mg. of the inactivating substance per ml. The mixtures were kept at room temperature for 7.5, 15, 30, 60 and 120 minutes, respectively, and then diluted to 10^{-7}—10^{-9}. Chick embryos were then inoculated with 0.25 ml. amounts of the diluted mixtures, using 6 eggs for each dilution. Control experiments in doembryonated eggs carried out as described previously (Sprössig and Schabinski-Stepan, 1956) showed that, in such dilutions, 7 out of the 8 compounds tested did not exhibit any inactivating action on the virus or a toxic effect on the host cells. Only diluted ninhydrin solutions showed a toxic effect on chorioallantoic membrane cells.

The inoculated chick embryos were incubated at 35° C for 48 hours and then chilled at 4° C for 24 hours. Thereafter the allantoic fluids were harvested and 0.1 ml. portions mixed with 0.4 ml. of phosphate buffered saline and 0.5 ml. of a 0.5% chicken erythrocyte suspension. Haemagglutination was read after one hour at room temperature. EID_{50}/ml. titres were calculated by the method of Reed and Muench (1938).

Results and Discussion

The inactivation rate of virus varied considerably depending on the compound used (Fig. 1). Under constant conditions of testing, phenylglyoxalhydrate showed the most marked inactivating effect on influenza virus. Gradually less effective were 4-biphenylglyoxalbisulphite and glyoxalbisulphite. This decrease in the inactivating effect was expected as the blocked aldehyde groups could not react with the guanine bases of ribonucleic acid. The reaction can proceed only after splitting off the bisulphite, i.e. after the aldehyde groups become free again. 4-biphenylglyoxalbisulphite was successfully used by Magrassi *et al.* (1960) in supressing influenza infection in mice.

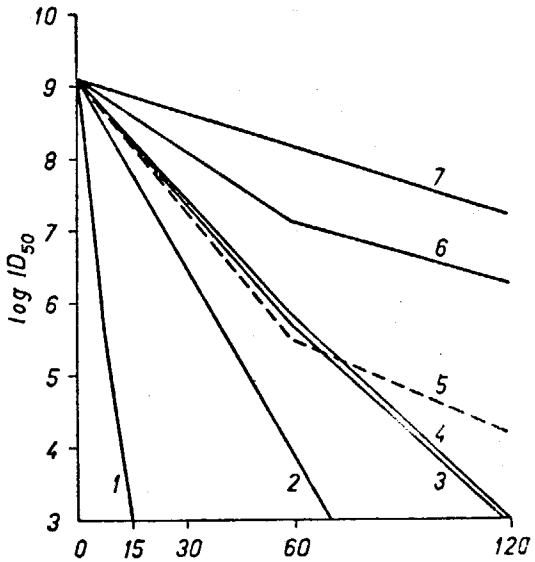

Fig. 1.

Course of inactivation of influenza virus strain FM_1 by: 1 — phenylglyoxal hydrate; 2 — 4 biphenylglyoxal bisulphite; 3 — glycerinaldehyde; 4 — diacetyl; 5 — glyoxalbisulphite; 6 — acetylacetone; 7 — polymeric glyoxal.

Abscissa: reaction time in minutes; ordinate: titre of virus in log ID_{50}/ml. values

The relatively high inactivating effect of diacetyl, a 1,2-diketon, without any signs of a toxic effect, was of special interest. In contrast to this compound, acetylacetone, a 1,3-diketon, in which the 2 carbonyl groups are separated by a methylene bridge, was much less effective. This fact indicated that the neighbourhood of both carbonyl groups is essential for the inactivating effect.

As distinct from these 2 aliphatic diketons, the cyclic 1,2,3-triketon, ninhydrin, showed in addition to a virus inactivating effect also a marked toxic effect and therefore this substance could be tested in embryonated eggs only after additional dilution. The results of the experiments with ninhydrin are therefore not included in Fig. 1. Glycerinaldehyde, an α-oxyaldehyde, showed an inactivating effect similar to that of diacetyl. The poor effect of polymeric glyoxal is explained by blocking of the active aldehyde groups.

The results obtained clearly showed that influenza viruses can be inactivated, in addition to glyoxals and α-oxyaldehydes, also by α-diketons. It must be emphasized that α-diketons, in contrast to glyoxals, are resistant a-

polymerization and oxidation. These properties of α-diketons can be of great importance in their application as inactivating agents.

Further experiments will be directed to the elucidation of the action of diacetyl, other α-diketons and related compounds on other viruses.

Summary

Influenza virus can be inactivated in addition to glyoxal compounds and α-oxyaldehydes also by the 1,2-diketon diacetyl in concentrations that were not toxic for cells of the chorioallantoic membrane of embryonated eggs. The separation of the two neighbouring carbonyl groups by a methylene bridge leads to nearly complete loss of antiviral activity.

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BACTERIAL BUTYLENE GLYCOL DEHYDROGENASE AND DIACETYL REDUCTASE*

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(Received for publication, June 14, 1954)

The early literature indicates much confusion as to the origin of acetylmethylcarbinol and butylene glycol in microbiological fermentations. Hoppold and Spencer (1) have reviewed much of this early work and have studied in detail the conditions for the formation of acetylmethylcarbinol and butylene glycol from glucose and pyruvate by *Aerobacter aerogenes*. Juni (2) has demonstrated that acetoin arises from pyruvate via acetolactic acid as intermediate with *A. aerogenes* and other bacterial cells. Evidence that the butylene glycol-acetoin system is diphosphopyridine nucleotide-linked has been obtained by DeMoss *et al.* (3), who observed the reduction of DPN[+] by butylene glycol and the reoxidation of DPNH by acetoin in extracts of *Leuconostoc mesenteroides*. The work reported here deals with the isolation and partial purification of two enzyme systems from *A. aerogenes* and *Staphylococcus aureus*, one catalyzing the reversible oxidation by DPN[+] of butylene glycol to acetoin and the other catalyzing an essentially irreversible reduction by DPNH of diacetyl to acetoin. These enzymes are referred to as butylene glycol dehydrogenase and diacetyl reductase respectively.

Experiments with Bacterial Extracts

In preliminary experiments with crude extracts of *A. aerogenes* it was observed that the addition of 2,3-butylene glycol caused a rapid reduction of DPN[+], and that the reduction was reversed by the addition of either acetoin or diacetyl. Extracts of *S. aureus* also catalyzed a rapid oxidation

* Aided by grants from the National Institutes of Health, United States Public Health Service, the American Cancer Society (recommended by the Committee on Growth of the National Research Council), and by a contract (N6onr279, T. O. 6) between the Office of Naval Research and New York University College of Medicine.

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[1] The following abbreviations are used: DPN[+] and DPNH oxidized and reduced diphosphopyridine nucleotide; Tris, trishydroxymethylaminomethane. The organism *Micrococcus pyogenes* (var. *aureus*) is referred to as *Staphylococcus aureus*.

of DPNH by diacetyl; the reduction of DPN by butylene glycol was, however, quite slow. Neither extract could bring about a reduction of DPN by acetoin. With butylene glycol as the substrate, analysis of the reaction mixture for acetoin and diacetyl indicated only acetoin to be present in amounts approximately equivalent to the DPN reduced.[2]

TABLE I

Reduction of Diacetyl and Acetoin

The reaction mixtures contained 0.3 μmole of DPN, 200 μmoles of glucose, 50 units of glucose dehydrogenase, 1.0 μmole of iodoacetate, 0.1 ml. of *S. aureus* extract with 3.0 mg. of protein and 50 μmoles of potassium phosphate buffer, pH 7.0, in a total volume of 1.0 ml. In addition, 18.6 μmoles of diacetyl were present in Experiment 1, or 13.3 μmoles of acetoin in Experiment 2. Incubation in air at 30°.

Experiment No.	Time	Diacetyl	Acetoin	Butylene glycol
	min.	*μmoles*	*μmoles*	*μmoles*
1	0	18.6	0	0
	30	3.5	9.5	4.7
	60	0	7.5	10.1
	90 (No glucose)	18.5	0	0
2	0	0	13.3	0
	90	0	8.0	5.3
	90 (No glucose)	0	13.3	0

Further experiments were carried out with diacetyl and acetoin as substrates and the glucose dehydrogenase system to reduce DPN according to the following reactions:

(1) $\text{Glucose} + \text{DPN}^+ + \text{H}_2\text{O} \rightleftharpoons \text{gluconate}^- + \text{DPNH} + 2\text{H}^+$

(2) $\text{Acetoin (or diacetyl)} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{2,3-butylene glycol (or acetoin)} + \text{DPN}^+$

Sum (1) + (2): $\text{2 glucose} + \text{H}_2\text{O} + \text{acetoin (or diacetyl)} \xrightleftharpoons{\text{DPN}} \text{gluconate}^- + \text{H}^+ + \text{2,3-butylene glycol (or acetoin)}$

Typical experiments are shown in Table I. In these experiments the reaction mixture described was incubated for various periods and then deproteinized by addition of 1.0 ml. of 12 per cent trichloroacetic acid. Suitable aliquots were removed for the determinations. Iodoacetic acid was added to prevent fermentation of glucose. With diacetyl as substrate, complete reduction to acetoin and butylene glycol took place in 30

[2] No diacetyl was formed from acetoin, in the presence of DPN[+], even when the bacterial extracts were supplemented with pyruvate and lactic dehydrogenase, or with α-ketoglutarate, NH_3, and glutamic dehydrogenase. This should have shifted an unfavorable equilibrium through reoxidation of any DPNH that might have been formed.

1955

to 60 minutes. With acetoin as substrate, butylene glycol was produced in amounts equivalent to the acetoin removed. There was no significant disappearance of either diacetyl or acetoin in the absence of enzyme.

The above results suggested the existence of two enzyme systems in the bacterial extracts, one catalyzing the reversible oxidation of butylene glycol to acetoin and the other the irreversible reduction of diacetyl to acetoin. However, due to the similarity of structure of acetoin and diacetyl, and the fact that with each compound a carbonyl group is reduced to a hydroxyl group, it was possible that a single enzyme might act on both compounds. In order to decide this question the *A. aerogenes* extract was purified utilizing the rate of reduction of DPN by butylene glycol as an assay, and the *S. aureus* extract was purified utilizing the rate of oxidation of DPNH by diacetyl as the assay. The former activity will be referred to as butylene glycol dehydrogenase and the latter as diacetyl reductase. Both activities were purified about 80-fold. At each stage of purification of both extracts, the rate of oxidation of DPNH by acetoin was also measured. With the *A. aerogenes* extract it was found that the ratio of diacetyl reduction to acetoin reduction decreased from 1.5 to 0.5. With the *S. aureus* extract this ratio increased from 4.5 to 22.5. It appears fairly conclusive that the two enzymatic activations are not the same.

Butylene Glycol Dehydrogenase

Assay—For assay of this activity, the samples in quartz cells, $d = 1.0$ cm.) contained 150 μmoles of potassium phosphate buffer, pH 7.0, 40 μmoles of butylene glycol, 0.3 μmole of DPN, enzyme, and water to a final volume of 3.0 ml. The reaction was started by the addition of butylene glycol. The increase in optical density at 340 mμ between 15 and 30 seconds after making the last addition (measured in the Beckman spectrophotometer against a blank cell containing all components except DPN) was used to calculate the enzyme activity. One enzyme unit is defined as the amount of enzyme causing an increase in optical density of 0.01 per minute. Within the limits of enzyme concentration used the activity is proportional to protein concentration. For measurement of diacetyl and acetoin reduction the assay was the same, except that the rate of oxidation of DPNH (0.3 μmole) by either substrate (40 μmoles) was used. The protein content of the enzyme solutions was determined spectrophotometrically by measuring the absorption of light at wave-lengths 280 and 260 mμ. The protein concentration was calculated from the absorption at 280 mμ with a correction for the nucleic acid content from the data given by Warburg and Christian (4).

Purification—The preparation of the *A. aerogenes* extract has been described in a previous paper (5). All steps were carried out in the cold.

A solution of 0.2 per cent protamine sulfate in deionized water was added

to the *A. aerogenes* extract in the proportion of 0.16 ml. per ml. of extract. After being stirred for 10 to 15 minutes, the mixture was centrifuged and the precipitate discarded. Solid ammonium sulfate was added to the supernatant fluid to 55 per cent of saturation. The suspension was stirred for 15 minutes, centrifuged, and the supernatant fluid discarded. The precipitate was dissolved in a volume of 0.01 M potassium phosphate buffer, pH 7.0, approximately one-tenth of the volume before addition of ammonium sulfate. The solution was then dialyzed 4 hours against 50 to 100 volumes of the same buffer with one complete change of buffer after 2 hours. The enzyme solution was chilled to 0° and ethanol slowly added to a concentration of 40 per cent by volume while the solution was being cooled further to −10°. 0.01 M zinc acetate was then added to a concentration of 0.002 M. The suspension was centrifuged at −5° to −10° at 15,000 to 20,000 r.p.m. for 30 minutes in the high speed head of the International centrifuge and the precipitate was discarded. 0.01 M zinc acetate was further added to the supernatant fluid to a concentration of 0.008 M and the suspension centrifuged as before. The precipitate was triturated with 0.01 M potassium phosphate buffer, pH 7.0, and centrifuged. The clear colorless supernatant fluid contained about 12 per cent of the original units with an 80-fold purification. These solutions have been kept at −14° to −18° for over 2 years with partial retention of activity. A summary of the purification steps is shown in Table II.

Specificity—In the forward direction butylene glycol dehydrogenase was tested on the following substances, all of which were inactive: ethanol, glycerol, ethylene glycol, isobutanol, malic acid, isoamyl alcohol, glucose, isopropanol, and tartaric acid.

Diacetyl Reductase

The assay and enzyme unit are the same as for the butylene glycol dehydrogenase, except that DPNH and diacetyl are the reactants.

Purification—The preparation of the *S. aureus* extract has been previously described (5). All steps were carried out in the cold.

A solution of 0.2 per cent protamine sulfate in deionized water was added to the *S. aureus* extract in the ratio of 0.16:1.0 by volume. After stirring 10 to 15 minutes, the suspension was centrifuged and the precipitate discarded. The pH of the supernatant fluid was adjusted to 5.7 by the addition of 10 per cent acetic acid. Calcium phosphate gel (23.5 mg. of $Ca_3(PO_4)_2$ per ml.) was added to the solution in the proportion of 3 ml. of gel for 10 ml. of enzyme solution. After being stirred for 15 minutes, the solution was centrifuged and the supernatant fluid discarded. The gel was then stirred with 10 ml. of 10 per cent ammonium sulfate, adjusted to pH 8.0 with aqueous ammonia, and centrifuged. This process was

1955

repeated. The supernatant fluids were combined, solid ammonium sulfate was added to 30 per cent saturation, and the mixture was centrifuged. The precipitate was discarded and solid ammonium sulfate was added to the supernatant solution to 60 per cent saturation. The suspension was centrifuged and the supernatant fluid discarded. The precipitate was

TABLE II

Purification of Butylene Glycol Dehydrogenase of A. aerogenes

115 gm. of cells (wet weight).

Steps	Volume	Units	Protein	Specific activity	Yield	$\frac{D}{A}$ ratio*
	ml.		*mg.*	*units/mg. protein*	*%*	
Extract	375	1,290,000	10,250	115	100	1.5
Protamine supernatant solution	365	1,160,000	2,020	306	90	1.5
$(NH_4)_2SO_4$ ppt. (0–0.55 saturation)	41	760,000	670	1120	59	0.8
Zinc acetate-ethanol fraction	15	170,000	18	8850	12	0.5

For description of the unit, see the text.

* Rate of diacetyl reduction to rate of acetoin reduction in the presence of DPNH.

TABLE III

Purification of Diacetyl Reductase of S. marcescens

20 gm. of cells (wet weight).

Steps	Volume	Units	Protein	Specific activity	Yield	$\frac{D}{A}$ ratio*
	ml.		*mg.*	*units/mg. protein*	*%*	
Extract	60	96,000	600	160	100	4.5
Protamine supernatant solution	60	94,000	174	540	98	7.5
$Ca_3(PO_4)_2$ eluate	20	68,000	178	380	71	13.0
$(NH_4)_2SO_4$ ppt. (0.3–0.5 saturation)	10	48,000	43	1120	50	23.0
" " (0.25–0.5 ")	5	30,000	23	1327	42	22.5

* Rate of diacetyl reduction to rate of acetoin reduction in the presence of DPNH.

dissolved in one-half the previous volume of 0.1 M potassium phosphate, pH 7.0; solid ammonium sulfate was added to 25 per cent saturation, the suspension was centrifuged, and the precipitate discarded. Solid ammonium sulfate was further added to the supernatant fluid to 50 per cent saturation, and the suspension centrifuged. The precipitate was dissolved in 0.1 M K_2HPO_4, resulting in a clear colorless solution which has been stored at −14° to −18° over 2 years with partial retention of activity. The purification is summarized in Table III.

Equilibrium Constant of Butylene Glycol-Acetoin System

The equilibrium constant of Reaction 3 (K = [acetoin] [DPNH] [H^+]/[2,3-butylene glycol] [DPN^+]) was determined at pH 7.4 and 8.4 with different concentrations of reactants. Butylene glycol and acetoin were

(3) 2,3-Butylene glycol + DPN^+ ⇌ acetoin + DPNH + H^+

incubated with DPN (0.0306 μmole per ml.) enzyme and either potassium phosphate buffer for the series at pH 7.4 or Tris buffer for the series at pH

TABLE IV

Equilibrium Constant of Reaction 2,3-Butylene Glycol + DPN^+ ⇌ Acetoin + DPNH + H^+

The initial concentration of DPN^+ was 0.0306 × 10^{-5} mole per liter throughout. No DPNH initially.

Experiment No.	pH	Initial concentration of reactants: moles per liter × 10^{-3}		Concentration of reactants at equilibrium: moles per liter × 10^{-3}			K
		Butylene glycol	Acetoin	DPNH	DPN^+	Acetoin	
							mole × liter⁻¹
1	7.4	13.3	0.166	0.0109	0.0197	0.177	2.93 × 10^{-10}
2	7.4	13.3	0.083	0.0153	0.0153	0.098	2.94 × 10^{-10}
3	7.4	26.6	0.083	0.0195	0.0110	0.195	2.73 × 10^{-10}
4	8.4	13.3	0.083	0.0258	0.0038	0.110	2.72 × 10^{-10}
5	8.4	6.7	0.166	0.0217	0.0089	0.188	2.72 × 10^{-10}
6	8.4	6.7	0.333	0.0172	0.0134	0.359	2.67 × 10^{-10}
7	8.4	6.7	0.666	0.0127	0.0179	0.679	2.86 × 10^{-10}
8	8.4	6.7	0.832	0.0108	0.0198	0.843	2.73 × 10^{-10}
Average							2.74 × 10^{-10}

8.4 (each 50 μmoles per ml.) in a final volume of 3.0 ml. The blank cell contained all of the components except DPN; d = 1.0 cm.; temperature, 27°. After taking a zero time reading of the optical density at 340 mμ prior to the addition of enzyme, 500 units of butylene glycol dehydrogenase (specific activity 8850) were added and the optical density recorded until constant values were obtained, indicating attainment of equilibrium: this occurred within 30 minutes. The concentrations of DPN and DPNH at equilibrium were determined from the optical density at 340 mμ and the change in the concentration of acetoin calculated from the DPNH formed. The concentration of butylene glycol at equilibrium was assumed to be equal to the initial concentration because the change was negligible by comparison with the amount of butylene glycol initially present. The molecular extinction coefficient of DPNH was taken as

1955

6.22×10^6 (cm.^{2} × mole^{-1}) (6). From a number of determinations, shown in Table IV, the average value for K was calculated to be 2.71×10^{-16} (moles × liter^{-1}).

DISCUSSION

It seems probable that diacetyl reductase is a component of the "diacetyl mutase" of Green *et al.* (7). The failure to obtain oxidation of acetoin in the presence of diacetyl reductase, DPN$^+$ glutamic dehydrogenase, α-ketoglutarate, and NH_4^+ ions[2] indicates that the diacetyl-acetoin system is considerably more electropositive than the glutamic-α-ketoglutarate + NH_3 system for which the E_0' has been calculated to be about −0.196 volt (8, 9). However, Doisy and Westerfeld (10) have demonstrated that acetoin and butylene glycol significantly increased acetylation of *p*-aminobenzoic acids by rabbits, and Dolin *et al.* (11) have reported that *Streptococcus faecalis* will cleave diacetyl to acetate and acetyl CoA. Sebek and Randles (12) have observed that *Pseudomonas fluorescens* can oxidize butylene glycol and acetoin to diacetyl. All of these observations indicate that diacetyl can be formed from acetoin in biological systems. If this is so, it seems unlikely that this reaction would be catalyzed by a pyridine nucleotide-linked enzyme system. The oxidation of acetoin and reduction of diacetyl might be analogous to the corresponding reactions of glycolic and glyoxylic acids (13), in which the former reaction (glycolic acid oxidation) is catalyzed by a non-pyridine nucleotide enzyme and the latter (glyoxylic acid reduction) by a DPN-linked one.

Methods

2,3-Butylene glycol, acetoin, and diacetyl were commercial products. Glutamic and glucose dehydrogenases were prepared as previously described (8, 14). DPN$^+$ was assayed spectrophotometrically with the glucose dehydrogenase system. DPNH was prepared enzymatically by reduction of DPN$^+$ with alcohol in the presence of yeast alcohol dehydrogenase (15) and was precipitated and kept as the barium salt (16). It was assayed spectrophotometrically through oxidation by α-ketoglutarate and NH_4^+ ions in the presence of glutamic dehydrogenase. Other preparations and analytical methods were as previously described (5).

SUMMARY

Enzymes catalyzing the reversible oxidation of butylene glycol to acetoin (butylene glycol dehydrogenase) and the reduction of diacetyl to acetoin (diacetyl reductase) have been isolated and partially purified from extracts of *Aerobacter aerogenes* and *Staphylococcus aureus*. Both enzymes

are pyridine nucleotide-linked. The equilibrium constant of the reaction catalyzed by butylene glycol dehydrogenase has been determined.

The authors wish to thank Dr. Severo Ochoa for his interest and advice.

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1955

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Nature 157: (3985) 336-337, (1946)

Fermentative Formation of Diacetyl

According to Martius' recent hypothesis[1], dehydrogenation would always constitute the primary phase of pyruvic acid breakdown in the animal organism. Dehydrogenation is followed by decarboxylation, with the formation of the ketene-like radical $\underset{|}{CH_3}-\underset{|}{C}=O$, which may act as hydrogen acceptor, being reduced to acetaldehyde. This radical could also react with the acetaldehyde present, to form diacetyl, which again could act as hydrogen acceptor, and be reduced to acetylmethylcarbinol:

$$\underset{|}{CH_3}-\underset{|}{C}=O + CH_3.CHO \rightarrow CH_3.CO.CO.CH_3 \xrightarrow{+H_2} CH_3.CH(OH).CO.CH_3.$$

Lipmann's work[2] has already showed that diphosphothiamine acts both as cocarboxylase and also as an active component in the dehydrogenation of pyruvic acid. However, detailed information has been lacking of the different phases of pyruvic acid dehydrogenation.

Recent investigations by Myrbäck and co-workers[3] now seem to have cleared up the main features of this problem. They have shown that diphosphothiamine in yeast exists also in the oxidized form, as disulphide (diphosphothiamine-SS). This indicates that thiamin really forms a redox-system of well-known type, like cysteine-cystine or GSH - GSSG, as suggested already by Zima, Ritsert and Moll[4].

Myrbäck's work also gives a starting-point for the experimental investigation of Martius' hypothesis. In baker's yeast all the thiamine exists in the disulphide form which, in anaerobic fermentations, is easily and quantitatively reduced, chiefly into cocarboxylase. On the other hand, the thiamine in brewer's yeast is mostly in the form of cocarboxylase, which remains quantitatively unchanged even during aerobic, aerated fermentations. Likewise, the disulphide form in it remains unreduced in anaerobic fermentations.

If, then, the disulphide form of diphosphothiamine acts as an active component in the dehydrogenation of pyruvic acid, and if the dehydrogenation of pyruvic acid is an essential condition for the formation of aroma substances—diacetyl, acetylmethylcarbinol and 2,3-butylene glycol—this fact ought to manifest itself in parallel fermentations with baker's and brewer's yeasts under anaerobic and aerobic conditions. In anaerobic fermentations, brewer's yeast would be expected to produce more aroma substances than baker's yeast, while the reverse ought to be true in aerobic, aerated fermentations. The accompanying table shows that this is indeed the case.

Yeast	Fermentation	Hours	Saccharose fermented %	Aroma substances (mgm. per 100 gm. fermented sugar)		
				Acetylmethylcarbinol	2,3-Butylene glycol	Total
Baker's	anaerobic	26	99·0	0·0	25·5	25·5
Brewer's	"	26	96·0	0·0	64·2	64·2
Baker's	aerobic	21·5	99·6	68·6	186·5	255·1
Brewer's	"	27·5	98·2	12·2	87·2	99·4

The fact that diacetyl has not been found in sugar fermentations by yeast can be ascribed to an immediate reduction of the primarily produced diacetyl. In order to lessen the possibility of such reduction, pyruvic acid was used as substrate. With dry brewer's yeast, formation of diacetyl under strictly anaerobic conditions could in fact be demonstrated by the analytical method of Langenbeck, Wrede and Schleckermann[5].

These experimental results support Martius' hypothesis that dehydrogenation of pyruvic acid is an essential condition for the formation of the aroma substances, and that in fermentations this process through the stages diacetyl → acetylmethylcarbinol → 2,3-butylene glycol. They likewise support Myrbäck's view that the disulphide form of diphosphothiamine takes an active part in the dehydrogenation of pyruvic acid.

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Nature 220 (5169): 792-3,

Mechanism of Diacetyl Formation in Yeast Fermentation

DIACETYL is an important component in the flavour of food and it is known to be the chief component of the aroma of butter. We have shown that it is also found in distilled alcoholic beverages, such as whisky and cognac. The formation of diacetyl in yeast fermentation has long been known[1] and extensive studies have been made of its appearance and influence on the aroma of beer. There is still much confusion about the mechanism of its formation[2-4], however, partly because of analytical difficulties. Maule *et al.*[5] have proved that the colorimetric determination of diacetyl requiring fractionation by distillation may give values which are erroneous and too high in comparison with those obtained by gas chromatography with an electron capture detector.

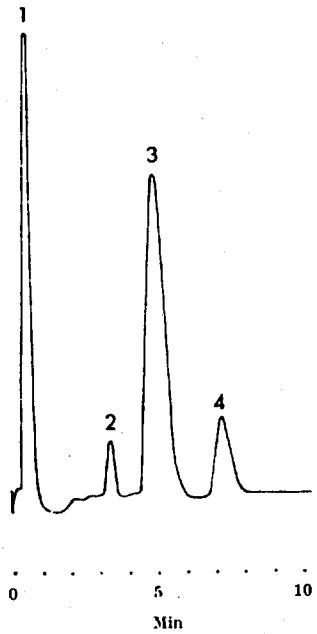

Fig. 1. Head-space vapour chromatogram of glucose (8 per cent) solution fermented for 3 h by baker's yeast (8 per cent) at 30° C. The vapour sampling has been carried out after removing the yeast and equilibrating the solution for 15 min at 40° C. Conditions: electron capture detector; column (length 2 m) contains 40 cm of diglycerol (20 per cent) on 'Celite 545' and 155 cm of TCEP (20 per cent) on 'Celite 545'. 1, Gas; 2, ethanol; 3, diacetyl; 4, 2,3-pentanedione.

The oxidation of acetoin to diacetyl is repeatedly presented as the way of its formation, but Portno[6] has clearly proved that diacetyl is not formed by the oxidation of acetoin in yeast fermentation. We have studied the appearance of diacetyl in yeast fermentation in completely anaerobic conditions, by application of the head-space technique and an electron capture detector, recommended by Harrison *et al.*[7] for the determination of vicinal diketones. A gas chromatogram, typical of the head-space vapour of anaerobic glucose fermentation by baker's yeast, is reproduced in Fig. 1. It can be estimated that the medium contained 4 mg/l. of diacetyl and 0·3 mg/l. of 2,3-pentanedione.

Lewis and Weinhouse[8] showed that baker's yeast can synthesize α-acetolactate from pyruvate, and we found[9] that appreciable amounts of all the keto acids synthesized by baker's yeast are transferred from the yeast cells into the fermenting medium. For this reason, we have studied the anaerobic decomposition of synthetic α-acetolactic acid[10] and its ethyl ester in a solution corresponding to the fermentation conditions. We used the head-space technique and an electron capture detector and observed the spontaneous formation of diacetyl. The amounts of diacetyl formed from acetolactic acid and its ethyl ester in a nitrogen atmosphere in a phosphate-buffered solution are presented in Table 1. Both compounds gave diacetyl although the decomposition rate of the free acid was markedly higher. Even a small reduction of *p*H, from 6 to 5·5, increased the rate of formation of diacetyl and the same effect was achieved if the temperature was increased. The molar amount of diacetyl appearing during the breakdown of acetolactic acid was about 4 per cent of the acetolactic acid added. Previous investigations by Yoshizawa[11] also seem to indicate spontaneous formation of diacetyl in fermentation conditions.

Table 1. SPONTANEOUS FORMATION OF DIACETYL FROM ACETOLACTIC ACID AND ETHYL ACETOLACTATE

h	Diacetyl (mg/l.) From acetolactic acid (1·7 mM) *p*H 5·5	*p*H 6·0	From ethyl acetolactate (5 mM) *p*H 5·5
0·5	0·8	0·3	0·01
1	3·9	2·9	0·02
2	4·8	4·2	0·05
3	5·1	4·8	0·08
8	5·5	5·5	0·13

Phosphate buffer of Sorensen; anaerobic conditions at 40° C; head-space technique applied.

With 1-^{14}C and 2-^{14}C-pyruvate, Holzer *et al.*[12,13] have shown that pyruvate decarboxylase (*EC* 4.1.1.1) from brewer's yeast, together with pyruvate, delivers "active pyruvate"(α-lactyl-2-thiaminepyrophosphate) and that "active acetaldehyde" (α-hydroxyethyl-2-thiaminepyrophosphate) is formed by the decarboxylation of "active pyruvate". The "active acetaldehyde" gives acetoin with acetaldehyde and pyruvate decarboxylase of yeast; with pyruvate and pyruvate oxidase system from mitochondria of baker's yeast it gives α-acetolactate and acetyl-CoA, and with α-ketobutyrate, α-aceto-α-hydroxybutyrate is formed. Carlson and Brown[14] have further shown that hydroxyethylthiaminepyrophosphate occurs in several micro-organisms, including baker's yeast, and that this evidently serves as an acetaldehyde-donor. We have studied the formation of diacetyl by means of pyruvate decarboxylase, isolated from brewer's yeast by the method of Ullrich *et al.*[15]. When the purified enzyme was used, the formation of diacetyl could not be observed in a weak pyruvate concentration (0·05 M), but when the concentration was increased to 0·5 M, diacetyl was rapidly formed in completely anaerobic conditions (Table 2). When acetyl-CoA (Sigma) was used in addition to pyruvate decarboxylase, diacetyl was formed, even in a pyruvate concentration of 0·05 M (Table 3).

Table 2. FORMATION OF DIACETYL FROM 0·5 M PYRUVATE BY PYRUVATE DECARBOXYLASE

h	Diacetyl (mg/l.)
0·5	0·05
1	0·2
2	0·9
3	1·8
5	2·2

Medium containing pyruvate decarboxylase (1,000 EU/100 ml.; specific activity 22 EU/mg protein), $MgSO_4 \cdot 7H_2O$ (0·02 M) and TPP ($1{\cdot}5 \times 10^{-5}$ M), buffered to *p*H 6·0 with Sorensen's phosphate buffer; anaerobic conditions at 40° C; head-space technique applied.

Table 3. FORMATION OF DIACETYL FROM 0·05 M PYRUVATE BY PYRUVATE DECARBOXYLASE TOGETHER WITH ACETYL-CoA

h	Diacetyl (mg/l.)
0·5	0·1
1·5	0·3
2·5	0·5
4·5	0·7
10·5	1·5

Medium containing acetyl-CoA (2 mg/100 ml.), pyruvate decarboxylase (1,000 EU/100 ml.; specific activity 23 EU/mg protein), $MgSO_4 \cdot 7H_2O$ (0·02 M) and TPP ($1{\cdot}5 \times 10^{-5}$ M), buffered to *p*H 6·0 with Sorensen's phosphate buffer; anaerobic conditions at 40° C; head-space technique applied.

1963

The compound which appears in the medium and produces diacetyl behaves like α-acetolactic acid because, as in the experiments with synthetic acetolactic acid, both a lowering of the *p*H and a rise in the temperature increased the rate of diacetyl formation. Collins *et al.*[16] have found that addition of acetyl-CoA is a presupposition for the formation of diacetyl in cell-free extracts of *Streptococcus diacetilactis* and *Leuconostoc citrovorum*, and quite recently[4] of some other specimens of bacteria and of a strain of *Saccharomyces cerevisiae*, but they reject the concept that α-acetolactic acid acts as a precursor of diacetyl.

When the yeast was present in glucose fermentation by baker's yeast, diacetyl and 2,3-pentanedione appeared in minor amounts, and only in rather acid solutions (*p*H about 3-5); moreover, the amounts did not increase until the yeast had been removed (Fig. 1). The more neutral the fermentation medium, the larger are the amounts produced. Here again, with synthetic acetolactic acid and in the enzymatic experiments, the rise in temperature or the reduction of *p*H increases the formation rate of the diketones. If baker's yeast is added to a solution in which appreciable amounts of diacetyl and pentanedione had appeared after removal of the yeast, the amounts of these diketones again diminish markedly. The effect of baker's yeast in consuming the diacetyl and the results obtained by the head-space technique thus confirm our earlier findings[1].

We consider that the experimental results show that diacetyl is formed in yeast fermentation by decomposition of the α-acetolactic acid synthesized by yeast, which was transferred from the cells into the fermenting medium. 2,3-Pentanedione is apparently formed in an analogous way from α-aceto-α-hydroxybutyric acid. The suggestion that diacetyl in yeast fermentation is formed by decomposition of acetolactate also explains the observation of Owades *et al.*[17], later confirmed[1,18], that the addition of valine inhibits the formation of diacetyl by yeast, while valine causes a feed-back inhibition on acetolactate formation, by the inhibition of α-acetohydroxyacid synthetase[19].

We thank Mrs Saara Brummer for assistance in the experimental work and Mr Lauri Jalkanen for the preparation of pyruvate decarboxylase.

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Received August 19, 1968.

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1973

1973

FRUIT JUICE CONSTITUENTS

Composition of Commercial, Segment, and Peel Juices of Florida Oranges

J. Agric. and Food Chem. 5(1):49-52, (1957)

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Comparative composition studies were conducted throughout a season. Peel juices were always highest in pH, Brix-acid ratio, soluble pectic substances, ascorbic acid, flavonoids, diacetyl, and color, and lowest in acidity and fluorescence. Peel juices were usually highest in soluble solids, specific gravity, and viscosity. During the early part of the season, sucrose was lowest and reducing sugars were highest in peel juices. Peel juices added at a level of 3% in reconstituted concentrate were detected with significance by a taste panel.

THE YIELD of single strength orange juice per 90-pound box of oranges for the season of 1940-41 was 27.46 No. 2 (18-ounce) cans. By the 1953-54 season, after a progressive increase, this yield had risen to 39.83 cans per box (4). This is an increase from 3.86 to 5.60 gallons per box or a gain of 45%. Without a doubt, much of this increase was due to more efficient extraction and finishing equipment and some might have been due to inherently juicier fruit resulting from improved grove and handling practices. Nevertheless, it seemed possible that this trend, if due to increasing extraction and finishing pressures, might eventually result in the incorporation of substantial amounts of peel and rag extractives in the segment juice. With the exceptions given below, [illegible] is known about the over-all effects of incorporated peel juice on the quality of commercially extracted segment juice. It was to supply some of these missing data that the present study was undertaken.

Olsen, Huggart, and Asbell (9) found [illegible] increased pressure during juice [illegible] and the use of pulp cutback [illegible] increased the tendency for frozen [illegible] orange juice to gel in the [illegible] and clarify upon reconstitution.

[illegible] and Rouse (2) extracted orange [illegible] with four makes of juice extractors [illegible] observed small variations in Brix-[illegible] total solids, and acid con[illegible]. Wide variations were found in [illegible] yield, pulp by volume, water-[illegible] solids, pectinesterase, flavo[illegible] and pectin.

[illegible] (10) has mentioned the possibility of obtaining bitter flavors from the peel, seeds, or pulpy portion of the orange by overextraction.

Hall (5) found that the juice from the outermost parts of the edible portions of the fruit was higher in soluble solids and lower in acid than was that from the innermost portions.

The general plan of the work was to make certain physical and compositional comparisons of commercially extracted orange juice, peel juice, and hand-pressed segment juice. Only soluble constituents were considered and the juices were clarified by filtration before analysis. It was thought that if these three juices were collected simultaneously at a single plant at weekly intervals for an entire season, the analyses should furnish some basis for estimating the effect of current extraction and finishing procedures and the probable effects of increased extraction and finishing pressures, if they were contemplated in the future.

Experimental Procedure

Samples were collected at weekly intervals throughout the 1954-55 operating season from a commercial concentrate plant in central Florida. This period extended from December 14 to June 16, with the exception of the month of March, when no oranges were handled at this plant because of insufficient quantity of fruit of suitable maturity. Samples of fresh commercially extracted juice, fruit for hand juice extraction, and peel juice from peel oil recovery operations were obtained each time. The fresh commercial juice was taken directly from the finishers. The fresh fruit was taken to the laboratory, halved, and lightly extracted on a glass reamer, care being taken to include a minimum of rag and pulp. This was termed segment juice. The peel juice came from an auxiliary operation in which cold-pressed orange oil was recovered. For this purpose fresh peel from the extractors passed directly to fluted Pipkin rolls, which expressed an emulsion of peel oil in peel juice. The emulsion passed through finishers which removed pulp, seeds, and pieces of peel and then to high-speed centrifugals where cold-pressed peel oil was recovered and the juice discarded. There was no dilution with water. Samples of this juice were collected for study.

All juices were placed in 46-ounce cans and frozen as soon as possible after the samples were obtained. All samples were held at −18° C. until the end of the season, when they were thawed, filtered clear with Hyflo Supercel, refrozen in 4-ounce cans, and held until analyzed.

Early and midseason varieties were being processed during December, January, and February. During the first part of this period the fruit was principally of early (usually Hamlin) varieties and during the latter part principally midseason (usually Pineapple) varieties. Generally both kinds were used and blended in making the final product. It was impossible to determine at a particular moment which variety [illegible] being processed and this should be borne in mind in evaluating the [illegible]

During the April-June period (late season) nearly all the fruit entering processing plants was of the Valencia variety.

Analytical Methods

All colorimetric measurements in this study were made with a Lumetron Model 401 colorimeter. Refractive index measurements were made with a Bausch & Lomb Precision refractometer; and per cent soluble solids by weight was estimated using sucrose refractive index tables.

Acidity was determined by titrating with standard alkali to the phenolphthalein end point and calculating as per cent citric acid by weight. Density measurements permitted expression of the acid content on a weight percentage basis. The pH was determined with a Model N Beckman battery-operated, glass-electrode meter.

Reducing sugars were determined by the Lane Eynon (*1*) procedure and expressed as invert sugar. Total sugars were determined by the same method after acidifying and allowing the solution to stand overnight to invert the nonreducing sugars. Nonreducing sugars were calculated by multiplying the differences by 0.95 and were reported as per cent sucrose.

Pectic substances were determined by the method of McCready and McComb (*8*), in which the precipitated and deesterified pectin was treated with concentrated sulfuric acid and carbazole, the color development was measured using a 530-mμ filter, and comparisons were made with a standard curve.

Ascorbic acid was determined by titrating 5 ml. of juice with standardized dichlorophenolindophenol dye solution after the addition of 5 ml. of 0.5% oxalic acid solution.

Flavonoids were estimated by the Davis (*5*) procedure, using a reference curve obtained with solutions of known hesperidin content.

Diacetyl and acetoin were estimated together as diacetyl by a modification of the method used by Hill, Wenzel, and Baretto (*6*), in which 100 ml. of juice was distilled until 25 ml. of distillate was collected. After mixing, 10 ml. of distillate was treated with 5 ml. of 1-naphthol solution and 2 ml. of alkali-creatine reagent in a colorimeter tube. The mixture was shaken for 15 seconds, allowed to stand for 10 minutes, and again shaken for 15 seconds. The tubes were read using a 550-mμ filter. No calibration curve was prepared and the results are expressed as absorbances, which are approximately proportional to the sum of the diacetyl and acetoin contents.

Ash was determined by evaporating 100 ml. of juice in a platinum crucible to a thick sirup, which was gradually charred and fire fly ignited at [illegible] in a muffle furnace. After cooling and moistening, it was dried and again ignited at 525° C. overnight.

Specific gravity values were obtained by weighing portions in a calibrated Reischauer flask at 20° C. and comparing with the weight of water at the same temperature.

Relative viscosities were estimated on a Stormer viscometer at 20° C. by comparing the values thus obtained with those for distilled water at the same temperature.

Color was evaluated by diluting the samples with an equal volume of water and comparing with a water blank using a 420-mμ filter. The results are presented as absorbance.

Fluorescence was determined with a Coleman Model 12 electronic photofluorometer, using 2 ml. of juice mixed with 20 ml. 95% ethyl alcohol. The mixtures were allowed to stand for a short time and filtered. The filtrate was used in the cuvette of the instrument. The B-1 filter was used in the No. 1 position and the PG-1 filter in the No. 2 position. The instrument had been set to give a reference reading of 90 with a standard quinine sulfate solution (3 mg. per liter in 0.1 *N* sulfuric acid). Meter readings are reported.

Taste tests were made to estimate the relative flavor potencies of the peel juice at different times during the season. The season was divided into four equal periods and the weekly samples from each period were pooled in equal amounts. Each of these composites was added in varying amounts to a reconstituted orange concentrate of good quality and presented to a taste panel in comparison with controls containing no peel juice. The triangular method of presentation was used and the order, codes, and selection of odd samples were randomized. All samples were presented at the same temperatures in ruby-colored glasses. A total of 20 judgments was obtained for each comparison. The table of Kramer (*7*) was used in estimating the significance of the data.

Results and Discussion

With the exception of ash and taste data, all results of analytical procedures are given in Figure 1. Overlapping of varieties during the early season no doubt accounts for some of the wide compositional variations found. Later season fruit is almost exclusively Valencia, and consequently, less variability would be expected. Usually, this is borne out in the data.

As would be expected, the soluble solids contents of both early and late season juices increased as the season progressed. During the early part of the season the solids content of the peel juice was [illegible] than that of the other juices, but [illegible] the late season [illegible].

The acidity [illegible] peel juice [illegible] Later, [illegible] not [illegible] the expected [illegible] juice acidity [illegible] 0.1 to 0.3% and showed a slight increase [illegible] season progressed, which is the reverse of the usual finding for canned juice. For peel juices, pH values remained very constant, but for the segment and commercial juices they rose noticeably during the latter part of the season. Brix-acid ratio for peel juices ranged from 22.1 to 29.3%, and segment juices from 10.5 to 16.4%.

As might be expected, there was an upward trend in reducing sugars with time in both the early and late parts of the season. Here, during most, the content of early season peel juice at all times exceeded that of the other juices, but with late season juices this was not the case.

Sucrose contents increased with time, but the content of the early season peel juices was uniformly lower than that of the others.

Total sugars, consequently, all showed an upward trend with time. The total sugar contents of late season juices were very similar and offered no clues as to the identity of the substance or substances responsible for the high soluble solids findings in peel juice.

The hand-prepared segment and commercially extracted juices showed no particular change in soluble pectic substances throughout the season; but the peel juices, always higher in these constituents than the others, showed a marked upturn late in the spring. The commercial juices contained more pectic substances than did segment juices during the late season.

As expected, segment and commercial juices were much higher in ascorbic acid during the early part of the season than later. Peel juices at all times exceeded the corresponding segment and commercial juices in ascorbic acid but decreased in this constituent throughout the entire season. Thus the first sample of peel juice taken in June contained only about 5 mg. more of ascorbic acid per 100 ml. than did the other juices. The difference had been nearly 50 mg. per 100 ml. in December.

In flavonoid content, the segment and commercial juices were remarkably constant at 300 to 500 mg. per liter throughout the season; but peel juices ranged from 1030 to 2930 mg. per liter, with the lower limit coming at the end of the season. The test employed [illegible] certain flavonoids, regardless of whether they influence flavor or not. Hesperidin, which gives the Davis reaction and is the principal flavonoid of orange juice, is not very bitter, probably be-

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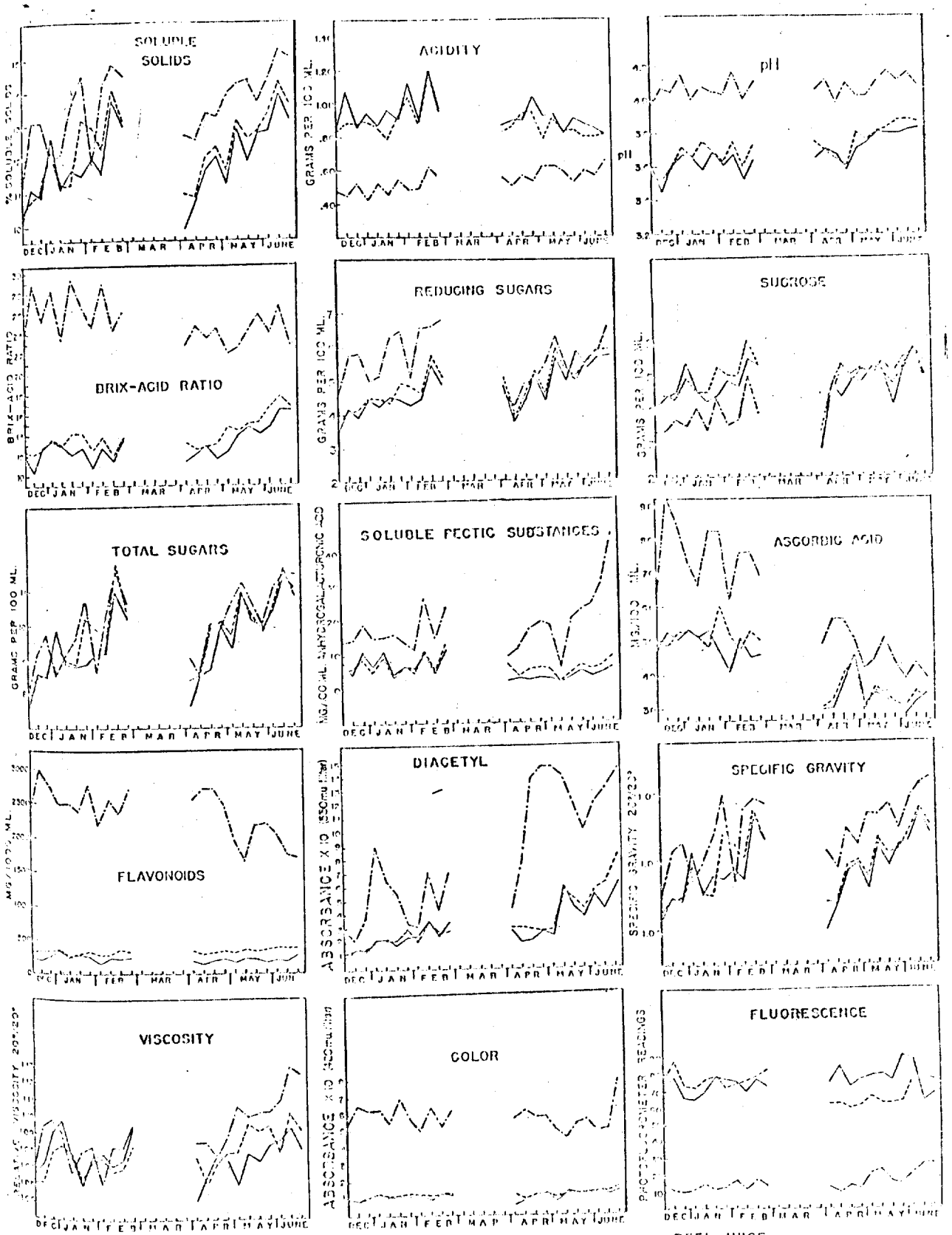

SEGMENT (HANDPRESSED) JUICE———, COMMERCIAL JUICE-----, PEEL JUICE—·—·

Figure 1. Comparisons of composition of commercial, segment, and peel juices of Florida oranges

cause of its low solubility in water. On the other hand, the Davis test does not indicate flavonoids having no free phenolic groups.

The method for determining diacetyl plus acetoin has been employed to serve as an index of bacterial activity in fresh juice. These compounds, however, may be normal constituents of juices, as the test was given by all types of juices and all were handled rapidly without much opportunity for fermentation. It is difficult to explain the diacetyl-acetoin values given by the peel juices, unless these actually contained more of these substances as normal constituents, or, if the fermentation theory is to be credited, the cleanup operations on the oil line may have been less frequent and rigorous than on the juice line and greater build up of fermented material occurred. The values show an upward trend in all juices during both early and late parts of the season, but the rise was particularly marked during the latter part, especially with peel juice, although it was at all times the highest with respect to diacetyl.

Although the total sugar contents of the three juices were about the same for the latter part of the season, the soluble solids of the peel juices were from 1 to 2% higher than those of the other juices. To determine if the nonsugar constituents responsible for this excess were mineral salts, ash determinations were made on both segment and peel juices collected on May 4 and May 11 (Table I). Although the data show somewhat greater mineral content for the peel juices, the excess was not enough to account for the high solids, unless the salts involved acids of very high equivalent weights.

Total nitrogen determinations on the same quarterly composites of peel juices used for tasting and also on corresponding composites of segment juices gave, respectively, the following results expressed as crude protein (N × 6.25): first quarter, 0.428 and 0.364%; second quarter, 0.564 and 0.440%; third quarter, 0.645 and 0.440%; fourth quarter, 0.733 and 0.554%. This comparison does not indicate that the unidentified nonsugar part of the peel juice solids is of a nitrogenous nature, as the small differences observed do not go very far toward accounting for the much greater differences in solids.

In general, specific gravity variations closely followed the data on soluble solids. Similar high values were obtained for the late season peel juice, showing conclusively that the high solids values were due not merely to some minor nonsugar constituent having an abnormal influence on refractive index, but to considerable amounts of some other substance or substances that influenced solution densities as well.

To characterize the juices further,

Table I. Ash in Segment and Peel Juices

Type of Juice	Date of Collection	Ash, %
Segment	3-4-55	0.155
Segment	5-11-55	0.164
Peel	5-4-55	0.502
Peel	5-11-55	0.508

relative viscosity was determined. While these values were nearly always greater for the peel juices than for the other types during the latter part of the season, the differences were small and were probably the result of higher concentrations of known constituents rather than of any appreciable amount of substances like dextrins. The viscosity curves show some resemblance to those for soluble pectic substances, but the correlation is not impressive.

The color curves resemble those of the flavonoids closely enough to suggest that the flavonoids contribute to the total color in filtered orange juices.

Peel juices were far below the segment and commercial juices in fluorescence. With the exception of a slight upturn for peel juice at the end of the season, there was little in the way of trends. The most striking feature lies in the comparison of the peel juice fluorescence curve with the color curve for these juices. These curves show strong negative correlation: when the color is high, the fluorescence is low. If the color is related to flavonoid content, perhaps the flavonoids present have a quenching effect on fluorescence. Whether this is due to their absorption of the incident ultraviolet light or to absorption of the emergent visible light is not known. If a connection between the flavonoids and the quenching effect could be established, it would suggest a possible basis for estimating flavonoids. Grapefruit segment, commercial, and peel juices all fluoresced so brilliantly that, even at one fourth the concentration of the orange juices, the meter needle was thrown off scale.

The results of the taste tests are given in Table II. In all cases, 20 judgments were made by the laboratory panel.

In all instances the taste panel was able to detect the presence of 3% of peel juice in reconstituted orange juice with significance. Except juice [illegible] for the first two quarters of the [illegible] were identified with the [illegible] while the composite of the [illegible] were identified with the [illegible] ances. When tested full strength, [illegible] clarified peel juice had a distinct [illegible] flavor characterized as being bitter, astringent, or harsh.

Although flavonoids are probably involved in the bitter flavor of peel juice, it has not been possible to show to what extent this is true. Two other classes of compounds that might account for the bitter flavor are alkaloids and tannins. In previous work at the laboratory on peel juices not included in the present series, it was not possible to detect alkaloids. Tannins were found to be present in concentrations of less than 0.05%, which is lower than that in many nonbitter fruit juices.

Acknowledgment

The authors wish to acknowledge the generous counsel and assistance of W. C. Scott, W. H. Miller, and J. C. Lastinger of this Laboratory.

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Received for review April 23, 1956. Accepted August 1, 1956. Division of Agricultural and Food Chemistry, 129th Meeting ACS, Dallas, Texas, April 1956. The use of trade names in this article is for identification only and implies no endorsement of manufacturer or product. U. S. Citrus Products Station is one of the laboratories of the Southern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

Table II. Taste Perception of Composite Peel Juices in Reconstituted Orange Juice Concentrate

Composite Sample	% Composite Added to Juice	Judgments: Correct	Judgments: Incorrect	Significance, % Level
Dec. 14–Jan. 11	3.0	12	8	5.0
Jan. 25–Feb. 23	3.0	12	8	5.0
April 6–May 4	3.0	14	6	0.1
May 18–June 15	3.0	15	[illegible]	1.0

APPLIED MICROBIOLOGY, June 1970, p. 883-889
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Vol. 19, No. 6
Printed in U.S.A.

Enzymatic Removal of Diacetyl from Beer[1]

III. Enzyme Protection and Regeneration of Cofactor

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Received for publication 27 February 1970

Use of diacetyl reductase, a reduced nicotinamide adenine dinucleotide (NADH)-requiring enzyme, to eliminate diacetyl off-flavor in beer was studied. The crude enzyme was extracted from *Aerobacter aerogenes* and partially purified by ammonium sulfate precipitation or Sephadex chromatography. In the semipure state, the enzyme was inactivated by lyophilization; in a crude state, the lyophilized extract remained stable for at least 4 months at −20 C. A 50% reduction in specific activity within 5 min was observed when crude diacetyl reductase was suspended (5 mg of protein/ml) in phosphate buffer at *p*H 5.5 or below; a similar inactivation rate was observed when the crude enzyme was dissolved in a 5% aqueous ethyl alcohol solution. Effective crude enzyme activity in beer at a natural *p*H of 4.1 required protection of the enzyme in 10% gelatin. Incorporation of yeast cells with the gel-protected enzyme provided regeneration of NADH. Combinations of yeast, enzyme, and gelatin were tested to obtain data analyzed by regression analysis to determine the optimal concentration of each component of the system required to reduce the level of diacetyl in spiked (0.5 ppm) beer to less than 0.12 ppm within 48 hr at 5 C. The protected enzyme system was also effective in removing diacetyl from orange juice (*p*H 3.8) and some distilled liquors.

Diacetyl has long been considered a serious off-flavor component in beer (18). It has been the subject of several recent reports (3, 5, 6) and also has been reported as a growing problem in frozen orange juice (2, 8, 9, 10). Marketing demands for beer have shifted in favor of a mild-flavored product, making the diacetyl problem even more active. Without the masking effect of stronger flavor components, diacetyl becomes increasingly noticeable and objectionable (13). Changes in the composition of wort, acceleration of production, and introduction of continuous fermentation have created conditions favorable to formation of diacetyl off-flavor.

Brewers have been only partially successful in controlling diacetyl levels in beer, usually at the expense of production efficiency. Extended lagering of the beer assures more complete diacetyl removal, but the longer holding periods and storage requirements make the process burdensome. The addition of fresh whole yeast cells to fermented beer (Kräusening) is another means of reducing the diacetyl content, but yeast autolysis may produce additional off-flavors in the finished product. Addition of valine to discourage diacetyl production by feedback inhibition also has been advocated (6, 13). Research carried out in the Department of Microbiology at Oregon State University has revealed the ability of several bacterial species to destroy diacetyl in milk cultures (4, 15, 16). By using the organism *Aerobacter aerogenes* 8724, Seitz et al. (16) demonstrated a diacetyl destroying enzyme identified as diacetyl reductase. In a preliminary report, Bavisotto et al. (1) presented findings suggesting the possible commercial use of diacetyl reductase to control diacetyl off-flavor in beer. Subsequently, Tolls et al. (17) studied methods of using the enzyme in eliminating diacetyl from beer and pointed out limitations of the enzyme in flavor control. The present report further substantiates possible industrial use of the enzyme by providing a means to stabilize the enzyme in beer and to eliminate the use of substrate quantities of the expensive cofactor reduced nicotinamide adenine dinucleotide (NADH).

MATERIALS AND METHODS

Enzyme preparation. The organisms used as sources of enzyme were *A. aerogenes* 8724 and *Streptococcus diacetilactis* 18-16. Both are maintained in the culture

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collection of the Department of Microbiology, Oregon State University; the *A. aerogenes* strain was originally obtained from the American Type Culture Collection, where the *S. diacetilactis* strain also has been deposited (ATCC no. 15346). Both organisms were maintained and grown by using citrate broth described by Sandine et al. (14).

Cells were grown in 8-liter quantities by using aerated carboys (nonaerated in the case of *S. diacetilactis*) or an aerated and *p*H-regulated New Brunswick microfermentor (capacity of 12 liters). Cells were harvested after 36 to 48 hr of growth with a Sorvall RC-2 refrigerated centrifuge operated at 5,000 × *g* for 15 min. The cells were then washed three times and resuspended in 0.1 M potassium phosphate buffer at *p*H 7.0. Cell suspensions (50-ml quantities with the consistency of light cream) were sonically treated for 20 min in a Raytheon 10-kc sonic oscillator, and the cell debris was removed by centrifugation at 15,500 × *g* for 1.5 hr. The resulting supernatant fluid was dialyzed through three 2-liter distilled water changes over a 30-min period and then lyophilized or stored frozen as the crude, unpurified extract. Protein determinations for each extract were done by the method of Lowry et al. (7).

Crude extracts of diacetyl reductase were partially purified by using a column (2.5 by 45 cm) packed with G-200 Sephadex gel in 0.1 M potassium phosphate buffer at *p*H 7.0. Samples (4.0 ml) were added to the column, and 50-drop portions were collected every 5 min; 25 samples were assayed for enzyme activity. In other attempts at enzyme purification, protein precipitation by ammonium sulfate was carried out. The purified samples were further analyzed for activity maintenance after lyophilization and extended storage at room temperature, 5 C, and −20 C. Activity was also analyzed as a function of decreasing *p*H (7.0 to 3.0) and in a 5% aqueous ethyl alcohol.

Enzyme assays. The Gilford model 2000 continuous-recording spectrophotometer was used to measure enzyme activity recorded as the decrease in absorbancy of NADH at 340 nm. The total system (standard assay conditions) contained 2.7 ml of potassium phosphate buffer at *p*H 7.2, 0.1 ml of diacetyl reductase (5 mg/ml), 0.2 ml of NADH at a concentration of 2 mg/ml, and 0.1 ml of diacetyl at a concentration of 860 μg/ml. The resulting absorbancy decrease plots were converted to units of enzyme activity by two methods. The first used a definition of one unit of enzyme as the amount of enzyme which under standard assay conditions caused a 50% reduction of the absorbancy when $1/T$, the reciprocal of time in seconds, equalled 10^{-6} (1). The other means of determining enzyme activity was to record the change in absorbancy with time (chart speed, 2 inches/min), measured on the linear portion of the curve. Specific activity in each case was determined by calculating the units of enzyme present per milligram of enzyme extract tested.

Diacetyl determinations. Diacetyl was assayed by using the colorimetric method of Owades and Jakovac (11) as modified by Pack et al. (12).

Use of diacetyl reductase in beer. The enzyme system used for studies on diacetyl removal from beer consisted of gelatin (Swifts' Superclear G10) to protect the enzyme, yeast cells (Fleischmann's dry yeast, Standard Brands, Inc., New York) to regenerate NADH, and the crude lyophilized enzyme. The mixture was prepared by warming 10 g of gelatin in 50 ml of distilled water to 40 C, at which temperature it was held until the gelatin was dissolved. After cooling to just above room temperature, the *p*H of the gel was adjusted to 7.0 ± 0.1 with 1 M NaOH before addition of the yeast and enzyme. One gram of yeast suspension and 0.1 g of dissolved, lyophilized enzyme preparation were then added, mixed, and spread as a thin film on sheets of polyethylene. After 24 hr at room temperature (25 C), the gel dried to a transparent, flexible film that could then be peeled from the polyethylene, cut into pieces approximately 1 cm square, and stored at 5 C.

Beer samples were spiked to 0.5 ppm of diacetyl by the addition of diluted, pure diacetyl (K & K Laboratories, Jamaica, N.Y.). The gel-yeast-enzyme was added to the beer at the rate of 360 mg per 120 ml of beer; each 120 ml of beer sample contained 324 mg of gelatin, 32.4 mg of yeast, and 3.2 mg of enzyme. The beer samples then were incubated for periods of time ranging from 12 to 120 hr at 25 C and at 5 to 7 C. Final diacetyl concentrations after incubation were determined by using the method of Owades and Jakovac (11). Each component of the gel-yeast-enzyme system was prepared separately and in combination with one of the other components, and analyzed in beer to determine the role of each in the diacetyl removal system. The standards set for the satisfactory performance of the enzyme system in beer required that diacetyl be reduced from 0.5 to 0.1 ppm in a minimal amount of time or that it be reduced to 0.2 ppm or less within 48 hr. All tested combinations were incubated in beer at 5 to 7 C for 48, 72, and 96 hr and analyzed in duplicate during separate trial periods (four to six tubes per trial).

Procedures used to measure leakage of yeast and enzyme from the diacetyl removal system into beer included plate counts on potato dextrose agar and reuse experiments to show that elements of the gel-yeast-enzyme system remained intact and active through repeated uses in fresh-beer samples.

Use of diacetyl reductase in orange juice and distillers' products. The same conditions used to measure the ability of the enzyme system to remove diacetyl from beer were employed to test the effectiveness of diacetyl reductase in removing the compound from orange juice samples or distilled liquors. The orange juice was obtained commercially as the frozen concentrate; the liquor samples were supplied by Hiram Walker and Sons, Peoria, Ill.

RESULTS

Enzyme assays. Table 1 shows specific activity values of crude and partially purified enzyme fractions prepared from *A. aerogenes*. The apparent protective influence of protein and the inactivating effect of lyophilization may be noted. Also, less than two- and fourfold purification was obtained by ammonium sulfate and Sephadex chromatog-

raphy, respectively; freezing and thawing had little affect on enzyme activity.

The influence of storage temperature and time on the activity of diacetyl reductase fractions appear in Table 2. These results indicate that crude, lyophilized, and ammonium sulfate-precipitated enzyme fractions need to be stored at −20 C to maintain activity for extended periods.

The activity of diacetyl reductase as a function of decreasing *p*H and in 5% aqueous ethanol is shown in Fig. 1. The lability of the enzyme to increasing concentrations of hydrogen ion is apparent; 5% ethanol also was harmful to the enzyme. At *p*H 7.0 and 6.0, only about 5 min was required for the complete reduction of the diacetyl (29 ppm). The protective effect of gelatin against this adverse influence of *p*H is illustrated in Table 3; even at *p*H 4.1, complete removal of diacetyl (29 ppm) was affected by enzyme mixed in 1.5% gelatin in less than 1 hr. It also may be seen that 2.5% gelatin was too high a concentration to allow the reaction to proceed. The gelatin also protected against inactivation by 5% alcohol.

TABLE 1. *Effect of freezing and lyophilization on specific activity of crude and partially purified fractions of diacetyl reductase*[a]

Treatment	Fraction		
	Crude extract	50% AS[b] precipitate	Sephadex eluate
Unfrozen	86.5		
Frozen	85.6	117.6	285.2
Lyophilized	81.6	25.2	4.0

[a] Results are expressed as units (change in absorbancy per minute) per milligram of enzyme protein. Assays were run on freshly prepared enzyme immediately after freezing or lyophilization.

[b] Ammonium sulfate.

Use of diacetyl reductase in beer. Table 4 shows the effect of the individual components (gelatin, enzyme, or yeast) on the level of diacetyl remaining in beer (*p*H 4.1) after incubation at 5 C for 2, 4, and 9 days. Gelatin or enzyme alone were inactive, but yeast alone was active. Yeast and enzyme were no more active than yeast alone, suggesting the enzyme was not functional under these conditions, again because of the *p*H effect. Gelatin and enzyme also were inactive because there was no NADH for the enzyme. The complete system was the most active, reducing all the diacetyl in 4 days even at this low temperature.

The effect of varying the gelatin, yeast, and enzyme concentrations on the rate of diacetyl reduction in beer by using the protected enzyme system may be seen in Fig. 2–4, respectively. It is clear that the yeast cells are the most important variable, though the gelatin and enzyme concentrations also are important.

When these data were submitted to computer regression analysis, the equation and correlation coefficient shown in Table 5 resulted. From the equation, the optimal concentration of the three components was determined.

Use of diacetyl reductase in orange juice and distillers' products. Diacetyl analyses on distillers' samples along with proof data are given in Table 6. Successful reduction of diacetyl reductase was only measureable with the low-proof samples, since no color reaction occurred when diacetyl analyses were made on the three high-proof samples.

TABLE 2. *Effect of storage temperature and time on specific activity of crude and partially purified fractions of diacetyl reductase*[a]

Time (weeks)	Fraction and temp								
	Frozen crude extract[b]			Lyophilized crude extract[c]			50% AS[d] precipitate		
	22 C	5 C	−20 C	22 C	5 C	−20 C	22 C	5 C	−20 C
0	85.6	—	—	81.6	—	—	117.6	—	—
1	10.1	75.5	—	—	—	—	37.4	85.5	—
2	0	14.0	—	—	—	—	0	16.0	—
4	0	15.0	—	—	—	—	0	16.0	—
8	0	7.3	—	24.2	—	—	0	9.3	—
12	0	3.4	79.0	—	—	76.6	0	4.0	109.6

[a] Results are expressed as in Table 1; —, indicates not determined.

[b] Frozen extracts were placed at the temperature indicated and held for the period shown before assay.

[c] Lyophilized enzyme was stored at 22, 5, and −20 C and assayed after the periods indicated.

[d] Ammonium sulfate.

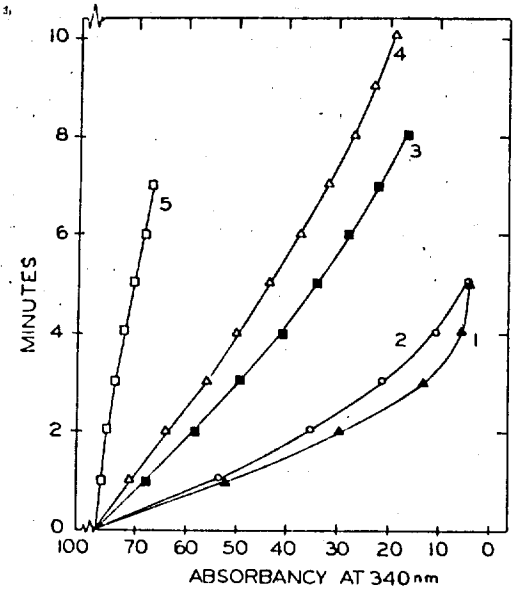

FIG. 1. *Activity of diacetyl reductase at pH levels between 7.0 and 4.2 and in the presence of 5% ethanol. Curves represent decrease in absorbancy as the result of NADH oxidation in the presence of diacetyl. Curves 1 to 5 are pH 7.0, pH 6.0, 5% ethanol, pH 5.1, and pH 4.3, respectively.*

TABLE 3. *Effect of gelatin concentration on the specific activity and rate of diacetyl reduction by diacetyl reductase system in phosphate buffer at different pH values*[a]

Trial[b]	Gelatin	pH	Specific activity	Removal time
	g/liter			*min*
1	15	7.0	3,480	19
2	20	7.0	3,280	20
3	15	4.1	1,528	44
4	20	4.1	2,186	31
5	25	4.1	—[c]	NR[d]
6	15	7.0	22,440	3
7	15	6.0	20,240	3
8	15	5.1	3,820	11
2	15	4.3	1,114	>60
10	15	7.0[e]	5,600	8

[a] The complete system contained 5 mg of enzyme per ml prepared in the concentrations of gelatin indicated. Cuvettes contained 0.4 mg of NADH, and the reaction was started by adding 86 μg of diacetyl.

[b] Trials 6 through 10 employed a more active enzyme preparation than trials 1 through 5.

[c] Not determined.

[d] No reaction.

[e] 5% ethanol present.

TABLE 4. *Effect of the various components of the diacetyl reductase system individually and in different combinations on the rate of diacetyl reduction in beer at 5 C*[a]

System	Diacetyl remaining		
	48 hr	96 hr	216 hr
	ppm	*ppm*	*ppm*
Gel alone	0.50	0.50	0.50
Enzyme alone	0.50	0.50	0.[illegible]0
Yeast alone	0.33	0.20	0.0
Gel and enzyme	0.50	0.50	0.30
Gel and yeast	0.15	0.08	[illegible]
Gel, yeast and enzyme	0.13	0.0	[illegible]
Yeast and enzyme	0.32	0.19	[illegible]

[a] The initial concentration of diacetyl was 0.5 ppm, with the amount present at different times determined by the Owades and Jakovac method.

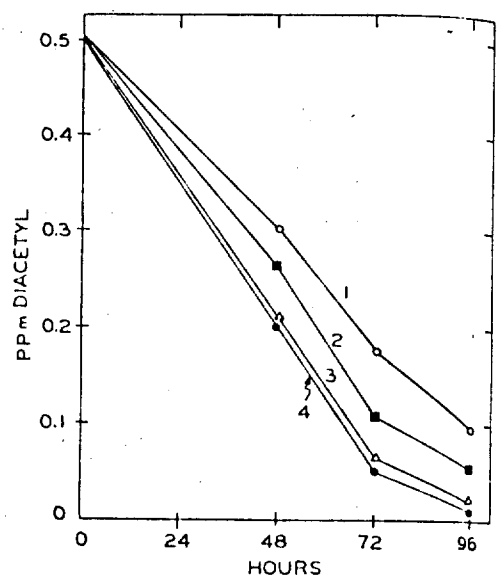

FIG. 2. *Effect of gelatin concentration on rate of diacetyl reduction by diacetyl reductase system in beer at 5 C. Curves 1 to 4 are 2.5, 5.0, 10.0, and 15.0% gelatin, respectively.*

In orange juice, the protected enzyme system functioned, but complete removal of diacetyl was not achieved (Fig. 5).

DISCUSSION

The results presented in Tables 1 and 2 on the stability of crude diacetyl reductase preparations from *A. aerogenes* emphasize that partially puri-

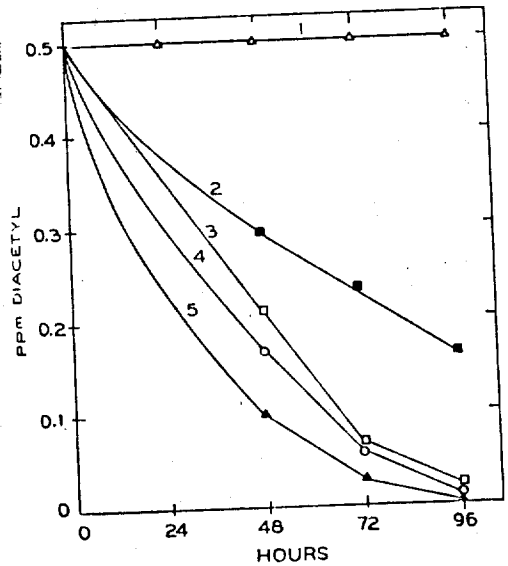

FIG. 3. *Effect of yeast concentration on rate of diacetyl reduction by diacetyl reductase system in beer at 5 C. Curves numbered 1 to 5 are 0.0, 0.5, 1.0, 1.5, and 2.0% yeast, respectively.*

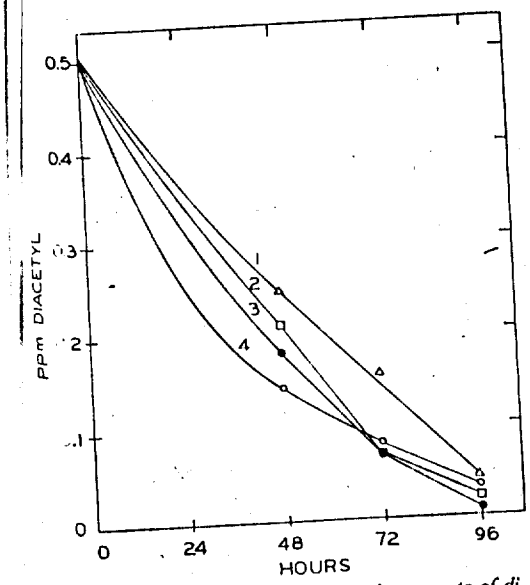

FIG. 4. *Effect of enzyme concentration on rate of diacetyl reduction by diacetyl reductase system in beer at 5 C. Curves numbered 1 to 4 are 0.0, 0.1, 0.2, and 0.3% enzyme, respectively.*

fied enzyme becomes much more labile to freezing and lyophilization. The crude extract, however, is quite stable to these treatments but, unfortunately maintains activity for extended periods only when stored at −20 C. This imposes a storage inconveinence regarding commercial use of the enzyme. Whether this limitation will apply to the gelatin protected enzyme system will require further study. However, preliminary results have revealed that gel-yeast-enzyme mixtures stored at 25, 5, or −20 C lose no activity up to 4 months of storage.

Although initial experiments were complicated by flakes of gelatin-containing enzyme which made absorbancy readings somewhat inaccurate, the data (Table 3) suggested that gelatin would be a suitable protective agent for the enzyme in adverse *p*H and alcohol environments. Although this was true (Fig. 2), some method of regenerating cofactor for the enzyme was needed to avoid the necessity of adding substrate quantities of NADH. When it was noted that yeast cells alone and, more especially, when incorporated into gelatin were active in reducing diacetyl in beer (Table 4), it became apparent that yeasts were providing a constant source of reduced cofactor to allow the enzyme to function. This may occur through the coupling of the enzyme to alcohol or lactic dehydrogenase (1). Figure 3 emphasizes dramatically the dependency of diacetyl reductase on the presence of yeast cells when exogenous NADH is not provided. In fact, even in the absence of enzyme (Fig. 4), the yeast cells function quite well in reducing diacetyl since they contain diacetyl reductase (17) in addition to the NADH-regenerating system.

These findings suggest the possibility of using yeast cells immobilized in gelatin in the absence of added diacetyl reductase to control diacetyl off-flavors in fermented berverages. This could be done by using the strains of yeasts being used in the actual fermentation to minimize the possibility of contaminating beer with a foreign yeast. In this regard, the gelatin flasks prepared in this study, which contained enzyme and yeast cells, were tested for leakage of cells into beer. In three experiments in which 120 ml of beer was incubated at 5 C for 96 hr with 360 mg of the enzyme system, an average of 3 cells per ml were recovered; when an equivalent amount of yeast was similarly plated, 35,000 cells per ml were recovered. Thus, 99.991% of the yeasts are retained in the gelatin, even though the flakes were prepared by cutting the film. Preparation of the mixture in droplet form would probably prevent any leakage, and further work on this is in progress. Where it might be desirable to use yeast cells exclusively for diacetyl control, studies need to be made on conditions necessary in preparation of the cells to minimize cost and maximize the activity of the cells for diacetyl reductase and NADH regeneration.

TABLE 5. *Multiple regression equation for determining the optimal concentration of enzyme system components to reduce diacetyl in 120 ml of beer to less than 0.12 ppm within 48 hr at 5C*

Multiple regression equation	Multiple correlation coefficient (R^2)	Optimal component concn (mg/120 ml of beer)		
		Yeast (x_1)	Enzyme (x_2)	Gelatin (x_3)
$y = 0.6748 - 0.009787x_1 + 0.0000924x_1^2 - 0.032564x_2 + 0.001865x_2^2 - 0.000941x_3 + 0.000001858x_3^2 + 0.0005312x_1x_2 - 0.00000861x_1x_3 - 0.00002293x_2x_3$	0.727	67.74	1.67	420.41

TABLE 6. *Diacetyl content and proof of distillers' products*

Sample	Proof	Diacetyl (ppm)[a]	Diacetyl remaining after 120 hr
F12-1	3.6	2.7	0
F12-2	115	1.1	--[b]
F15-1	5.5	2.7	0.6
F15-2	116	1.1	--[b]
Rye distillate	138	5.1	--[b]

[a] Diacetyl values were furnished by supplier.
[b] No color reaction occurred.

In the brewing industry, a variety of chill-proofing (proteolytic) enzymes are currently used to enhance the clarity of the finished product. Usually, less than a pound of these agents is used per barrel of beer, and it was of interest to compare this customary rate of enzyme addition with that found necessary in the present study for the diacetyl reductase system. From the regression analysis (Table 5), the optimal concentration of the enzyme component was about 68 mg per 120 ml of beer; this is equivalent to 0.15 lb per barrel. Where the total weight of the enzyme system is concerned (490 mg/120 ml), this is equivalent to about 1.1 lb per barrel of beer. Although this may seem like a sizable quantity, the gelatin-yeast-enzyme system is recoverable from the beer after the diacetyl is reduced and does not actually represent an addition to the beer that might affect quality after processing. Furthermore, it was determined in this study that the gelatin-protected system was reusable after recovery from treated beer. In a typical experiment in which the initial use reduced the diacetyl in beer from 0.5 ppm to none in 96 hr, the first reuse left 0.23 ppm with complete removal by 192 hr. Even a third reuse of the recovered flakes lowered the diacetyl to 0.07 ppm after 192 hr.

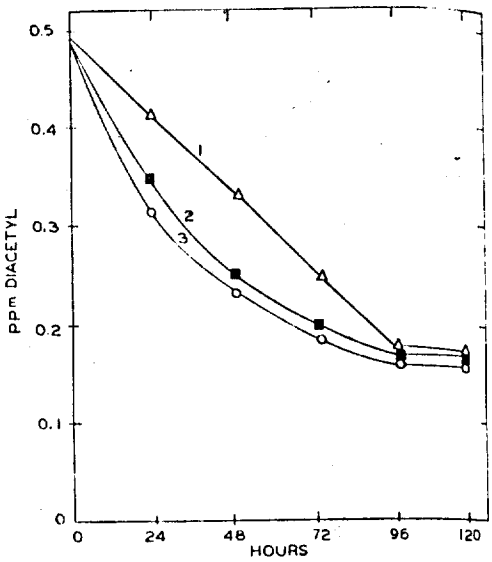

FIG. 5. *Effect of gelatin concentration on rate of diacetyl reduction by diacetyl reductase system in orange juice at 5 C. Curves numbered 1 to 3 are 10, 15, and 20% gelatin, respectively.*

Attempts to contain the gelatin flakes in gauze bags suspended in the beer to facilitate easy removal resulted in some inhibition of diacetyl removal; reduction of diacetyl from 0.5 to 0.23 ppm occurred in 96 hr, whereas control beer with the flasks evenly distributed throughout contained no diacetyl after this period. Thus, it would appear that the protected enzyme system should be free to mix with the beer for maximal diacetyl removal efficiency.

It is clear from the results of this study (Table 6 and Fig. 5) that further research is necessary to establish the applicability of diacetyl reductase to the removal of diacetyl from orange juice and

distillers' products. The preliminary data would appear to justify further studies in this regard. The suitability of the protected enzyme system, however, to control this flavor defect in beer seems well established. Results on the application of the system on a commercial scale will be reported in the near future.

ACKNOWLEDGMENTS

The regression analysis was performed by K. E. Rowe, Department of Statistics, Oregon State University.

This research was supported by a grant from Charles Pfizer and Co., Inc.

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APPLIED MICROBIOLOGY, Apr. 1970, p. 649-657
Vol. 19, No. 4
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Enzymatic Removal of Diacetyl from Beer

II. Further Studies on the Use of Diacetyl Reductase[1]

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Received for publication 26 January 1970

Diacetyl removal from beer was studied with whole cells and crude enzyme extracts of yeasts and bacteria. Cells of *Streptococcus diacetilactis* 18-16 destroyed diacetyl in solutions at a rate almost equal to that achieved by the addition of whole yeast cells. Yeast cells impregnated in a diatomaceous earth filter bed removed all diacetyl from solutions percolated through the bed. Undialyzed crude enzyme extracts from yeast cells removed diacetyl very slowly from beer at its normal *p*H (4.1); at a *p*H of 5.0 or higher, rapid diacetyl removal was achieved. Dialyzed crude enzyme extracts from yeast cells were found to destroy diacetyl in a manner quite similar to that of diacetyl reductase from *Aerobacter aerogenes*, and both the bacterial and the yeast extracts were stimulated significantly by the addition of reduced nicotinamide adenine dinucleotide (NADH). Diacetyl reductase activity of four strains of *A. aerogenes* was compared; three of the strains produced enzyme with approximately twice the specific activity of the other strain (8724). Gel electrophoresis results indicated that at least three different NADH-oxidizing enzymes were present in crude extracts of diacetyl reductase. Sephadex-gel chromotography separated NADH oxidase from diacetyl reductase. It was also noted that ethyl alcohol concentrations approximately equivalent to those found in beer were quite inhibitory to diacetyl reductase.

Diacetyl in beer causes an off-flavor which has been described as "lactic-diacetyl, buttery, or sarcina-like" (28). In addition, diacetyl is considered to be an off-flavor in wines (10, 20) and in citrus juices (2, 16). Different causes for this flavor defect have been suggested. In 1903, Claussen (5) described the causative agent of diacetyl production in beer to be a bacterium belonging to the genus *Pediococcus*. Shimwell and Kirkpatrick (26) studied diacetyl formation in beer and concluded that the causative agent was not a member of the genus *Pediococcus* but was in the genus *Streptococcus*. More recently, Burger et al. (3) reported that yeast cells produced diacetyl as a by-product during fermentation of wort used in beer manufacture. They also claimed that *Lactobacillus pastorianus*, a common bacterial contaminant in beer during the lagering stage, produced diacetyl. Kato and Nishikawa (14) also claimed that "beer sarcina" (a term used synonymously with pediococci), brewers' yeast, and *L. pastorianus* all produced diacetyl in beer. Several lactobacilli capable of producing diacetyl in wine were described by Fornachon and Lloyd (10). Other species of bacteria capable of producing diacetyl in wine were described by Pilone et al. (20). It also has been reported (3) that diacetyl appears in beer exposed to air for prolonged periods of time at certain stages of processing. This presumably is due to oxidation of α-acetolactic acid to diacetyl as described by Inoue et al. (12) and Suomalainen and Ronkainen (27).

The trend towards manufacture of light, mild-flavored beer in the United States has intensified the diacetyl off-flavor problem for the brewing industry. In this regard, Drews et al. (8) recommended that light beer should not contain more than 0.07 ppm of diacetyl and that 0.2 ppm was the flavor threshold level of such beer. Russian beer, however, was shown in a survey published by Denshchikov et al. (6) to range from 0.40 to 0.96 ppm of diacetyl.

During studies on diacetyl biosynthesis by a lactic streptococcus (23), Seitz et al. (25) noted diacetyl-destroying activity in cell-free extracts. It subsequently was found (24; W. E. Seitz et al., Bacteriol. Proc., p. 23, 1962) that *Aerobacter aerogenes* also was very active in destroying diacetyl, and a preliminary study on the use of

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diacetyl reductase from this bacterium to remove diacetyl from beer has been made (1). The present research is an extension of this latter work and concerns the limitations of the enzyme to control this flavor defect in the brewing industry.

MATERIALS AND METHODS

Diacetyl determinations. The colorimetric assay for diacetyl described by Owades and Jakovac (17) and modified by Pack et al. (19) was used.

Cultures. Yeasts of the genus *Saccharomyces* and bacteria of the *Pediococcus*, *Acetobacter*, *Aerobacter*, and *Streptococcus* genera used in this study (Table 1) were obtained from the stock culture collection of the Department of Microbiology, Oregon State University; from the American Type Culture Collection (ATCC), Washington, D.C.; and from Charles Pfizer & Co., Inc. All cultures were maintained in yeast-complete-medium (YCM), citrate broth (CB), or on wort agar (WA). YCM (*p*H 7.0) contained the following ingredients in g/liter: glucose, 20.0; tryptone, 20.0; and yeast extract, 10.0. Wort broth (*p*H 4.8) contained the following ingredients in g/liter: malt extract (Difco), 15.0; peptone (Difco), 0.78; maltose, 12.75; dextrin, 2.75; glycerol, 2.35; dipotassium phosphate, 1.0; and ammonium chloride, 1.0. CB was prepared as described by Sandine et al. (22). WA was available from Difco. YCM agar was prepared by adding 15 g of agar per liter of medium.

TABLE 1. *Yeast and bacterial cultures used*

Organism	Medium[a]	Organism	Medium
Saccharomyces cerevisiae var *ellipsoides*	CB	Bakers' yeast granules[b]	
S. cerevisiae		*Pediococcus cerevisiae*	WA
2091	YCM	10791	
2000–3	YCM	*Acetobacter pasteurianus*	WA
I	YCM	6033	
2094-N	CB	*A. melanogenus* 9937	WA
1538	CB	*Streptococcus diacetilactis*	CB
2094-P	YCM	18-16	
PH3	CB	*Aerobacter aerogenes*	
2000	YCM	8724	CB
T	YCM	8308	CB
S. carlsbergensis	CW	12658	CB
Bakers' yeast cake[c]		OSU	CB
		Streptococcus faecalis	
		OSU	CB
		10C1	CB

[a] Abbreviations: CB, citrate broth; YCM, yeast-complete-medium; CW, commercial wort, Blitz Weinhard Co.; WA, wort agar.
[b] Fleischmann's brand.
[c] Red Star brand.

Diacetyl production and destruction. Two procedures were used to follow the appearance and loss of diacetyl. The first involved the use of 2 gal of wort prepared by adding one 3-lb can of Blue Ribbon malt extract (Premier Malt Products, Inc., Milwaukee, Wis.) and 3 lb of sucrose to 5 gal of water. The wort was then inoculated with 0.25 oz of Fleischmann's dry yeast. Frequent agitation was used to hasten the start of the fermentation which was allowed to proceed for 7 days at 14 to 16 C. The *p*H and amount of diacetyl in the fermenting wort was determined at specific times using 20 ml portions at each sampling.

For the second method, two 250-ml flasks of wort broth were inoculated with a 5% inoculum of actively growing *Saccharomyces cerevisiae* 2091, a brewers' yeast strain. The temperature was maintained at 10 C and the flasks were shaken occasionally to hasten the start of the fermentation. The diacetyl concentration in the fermenting medium was determined as described above.

Diacetyl production by yeast strains. To examine variability between yeasts in their ability to produce diacetyl, eight brewers' strains of *S. cerevisiae* were inoculated in duplicate into 20 ml of sterile wort broth in culture tubes (25 by 250 mm, Corning no. 9820). After incubation for 63 hr at 10 C, one sample was removed for counting the yeast cells; diacetyl was determined on the duplicate 20-ml portion.

Diacetyl removal by heat-inactivated and live cells. Whole cells of bacteria or yeasts were heat inactivated by rapidly bringing a cell suspension to 98 C and then rapidly cooling in ice water to 25 C. Suspensions of live and heat-inactivated cells (0.25 g) were incubated in triplicate for a given length of time at 25 C in the presence of diacetyl (20 μg/ml) under the conditions shown in Table 2; reduced nicotinamide adenine dinucleotide (NADH) was omitted in experiments with whole cells.

Diacetyl removal using whole yeast cells in dialysis tubing. A heavy Fleischmann's yeast cell suspension was washed several times with 0.1 M phosphate

TABLE 2. *Experimental design for the assay of cells and enzyme extracts for diacetyl reductase activity by the modified Owades and Jakovac apparatus*

Component	Tube no.			
	1-3	4-6	7-9	10-12
	ml	*ml*	*ml*	*ml*
Buffer (0.1 M KH_2PO_4)[a]	19	18	17.5	17
Enzyme (5 mg/ml)[b]		1	0.5	1
Reduced nicotinamide adenine dinucleotide (4 mg/ ml)			1	1
Diacetyl (20 ppm)	1	1	1	1

[a] Beer was substituted for buffer in experiments so indicated in the Results section.
[b] Whole cells (0.25 g) were used in place of enzyme in experiments so indicated in the Results section.

buffer (*p*H 7.2) and then with beer (*p*H 4.3). Quantities (50 ml) of the suspension were placed in cellophane dialysis tubing which was then immersed in beer (1 liter) held at 3.6 C, previously spiked to contain 0.5 ppm of diacetyl Samples (20 ml) were taken daily up to 6 days and tested for diacetyl. In some cases, the samples were also tested organoleptically by members of a taste panel.

Diatomaceous earth—yeast cell filtrations. A glass column (5 by 60 cm) with a coarse-porosity fritted glass filter disc was used in experiments designed to study filtration of beer as a means of diacetyl removal. The column was packed with a suspension of Johns-Manville Hyflosuper-cel (a commercial grade of diatomaceous earth used in beer filtrations). When sufficient diatomaceous earth suspension had settled out to the desired filter bed height (18 cm), the remainder was poured off the top of the column. A refrigerated Gilson fraction collector was set to collect 5 ml of effluent liquid per tube. A solution of diacetyl (0.5 ppm) was then passed through the column to determine the void volume; the diacetyl content of eluate fractions was determined by the modified method of Owades and Jakovac (17).

A suspension of both diatomaceous earth and yeast cells was then prepared. The two yeasts used in these experiments were Fleischmann's yeast and *S. carlsbergensis*. With the Fleischmann's yeast, 20 g of dry yeast granules was mixed with 200 g of diatomaceous earth in 2,000 ml of distilled water (or beer). Since, as determined by plate count, 1 g of dry yeast was equivalent (on a per-cell basis) to 2.5 g of wet-packed yeast, 50 g of the wet-packed brewers' yeast was mixed with 200 g of diatomaceous earth to obtain equal ratios of the constituents. The filter bed was prepared as described above. A diacetyl solution (0.5 ppm) was then passed through the filter to determine the extent of diacetyl removal by the live yeast cells impregnated in the column.

Bacterial cell-free crude extract preparation. Bacteria were grown from a 1% inoculum in 2 to 40 liters of sterile medium for 24 hr at 30 C. CB was the medium most frequently used, but glucose broth (omitting sodium citrate) was also used.

After growth, the cells were harvested with the use of a continuous-flow attachment for the Sorvall RC-2 refrigerated centrifuge at 12,100 × *g* with a flow rate of [illegible] ml per min. The packed cells were recovered from the collection tubes by resuspension in 0.1 M potassium phosphate buffer at *p*H 7.2. The cells were washed three times in buffer and then resuspended in buffer to a volume of 50 ml.

Crude enzyme extracts were prepared by disrupting the cells in a Raytheon 10 kc sonic oscillator for 20 min. Cell debris was removed by centrifugation at 27,750 × *g* for 1.5 hr in the refrigerated centrifuge. The supernatant fluid was dialyzed against three, 4 liter changes of distilled water, with each dialysis lasting 8 hr. The crude enzyme was then lyophilized and stored at −20 C until used. Protein determinations on the extract were done by the method of Lowry et al. (15).

Yeast cell-free crude extract preparation. Yeasts were grown from a 1% inoculum in 2-liter amounts of sterile CB or YCM broth for 24 hr at 30 C. In some cases, such as with Fleischmann's yeast, the yeast was used as supplied commercially and not grown in CB or YCM.

After growth, the cells were harvested with the use of the large-capacity centrifuge head (GSA) of the Sorvall RC-2 refrigerated centrifuge at 4,080 × *g* for 10 min. The packed cells were recovered by resuspension in 0.1 M potassium phosphate buffer at *p*H 7.2. The cells were washed three times in buffer and then resuspended with sufficient buffer to make the suspension heavy but still pipettable.

A 10-ml amount of the heavy yeast cell suspension was added to the cylinder well of an Eaton cell press (9) which had been prechilled to dry-ice temperature. The cylinder remained in contact with the dry ice for 15 min to freeze the suspension. The piston was placed in the cylinder and a pressure of 10,000 lb/inch² was applied by means of a hydraulic press. The frozen cells, extruded through a small orifice in the bottom of the cylinder, were collected in a metal centrifuge tube. This material was thawed and then centrifuged at 27,750 × g for 1.5 hr. The supernatant fluid, when not used immediately, was dialyzed, lyophilized, and stored at −20 C. Protein determinations on the extract were done by the method of Lowry et al. (15).

Assay of crude cell-free extracts for diacetyl reductase. Enzyme assays were carried out by two methods. The first method involved the use of either a Cary (model 11) or a Gilford (model 2000) continuous recording spectrophotometer to measure the activity of the crude enzyme extracts by following changes in the absorbancy at 340 nm caused by the oxidation of NADH during diacetyl reduction. The reactions were initiated by the addition of diacetyl to solutions containing enzyme, NADH, and buffer. After the blank was adjusted to zero, the absorbancy following the addition of NADH was recorded. The diacetyl solution was then added to the cuvette, and the reaction was allowed to proceed at 25 C. The time in seconds (T) required for 50% reduction of the initial absorbancy was used for the calculation of the enzyme units present (1).

The second method involved the use of the Owades and Jakovac (17) apparatus to measure colorimetrically the amount of diacetyl present. Table 2 shows the experimental design for the assay of crude enzyme extracts by this method. Tubes 1–3 were used to determine the initial diacetyl concentration. Tubes 4–6 were used to detect enzyme activity in the absence of the cofactor, NADH. Tubes 7–9 and 10–12 were used to measure the enzyme activity for two different enzyme concentrations in the presence of cofactor.

Effect of alcohol on diacetyl reductase activity. Diacetyl reductase in crude extracts from *A. aerogenes* was assayed according to the procedure described above. Various alcohol concentrations were obtained by direct addition of absolute ethyl alcohol to the reaction mixture in the cuvettes.

Sephadex chromatography. Thermal denaturation, ammonium sulfate fractionation, disc electrophoresis, and Sephadex chromatography were used to separate

diacetyl reductase from endogenous NADH oxidase activity found in cell-free crude extracts of *A. aerogenes* 8724. Only the latter was successful. A Sephadex column (2.5 by 45 cm) was packed with type G-200 gel according to the technical information supplied by Pharmacia Fine Chemicals, Inc. A 0.1 M potassium phosphate buffer system was employed. Blue dextran 2000 was used to determine the void volume; elution data were expressed as fraction numbers after the void volume was eluted. A 4-ml amount of a solution (40 mg per ml) of the extract was added to the top of the column. The concentration of protein remaining in each of the 50-drop (1.35 ml) fractions eluted from the column was followed by absorbancy readings at 280 nm with a Gilford model 2000 spectrophotometer. These fractions were then assayed for NADH oxidase and diacetyl reductase activity.

RESULTS

Diacetyl production and destruction. Figure 1 shows a diacetyl production and destruction curve

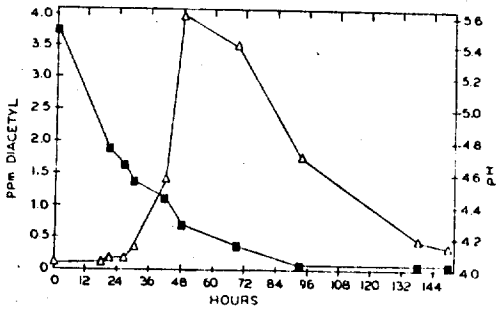

FIG. 1. *Diacetyl produced (△) and pH attained (■) at 14 to 16 C by Fleischmann's yeast after different times of incubation in 2 gal of wort.*

TABLE 3. *Cell population and diacetyl produced by various strains of Saccharomyces cerevisiae after incubation at 10 C in wort broth for 63 hr*

Strain	Standard plate count/ml	Diacetyl produced		
		Amt	Amt per cell × 10^9	Relative to strain 2000
		ppm	ppm	
None[a]		0.08		
2000-3[b]		0.10		
2000	1.70×10^7	0.54	32	1.0
1538	1.18×10^7	0.59	50	1.6
PH3	1.73×10^7	0.62	36	1.1
2091	8.00×10^6	0.63	79	2.5
I	1.25×10^7	0.70	56	1.8
T	1.17×10^7	0.79	67	2.1
2094-N	1.83×10^7	1.00	55	1.7

[a] Uninoculated wort broth.
[b] Strain 2000-3 grew poorly in this medium.

typical of the yeast fermentations at 14 to 16 C; the *p*H of the wort indicates the extent of the fermentation. Diacetyl production was maximum at 48 hr and then decreased with time. In another experiment conducted at 10 C, the diacetyl peak occurred at 96 hr. One batch of beer prepared from inoculated wort was bottled after the 6th day of the fermentation. After 2 more weeks of storage at 20 C, the yeasts settled to the bottom of the bottles and the diacetyl was found to have disappeared completely.

Table 3 shows the amount of diacetyl produced by the eight strains of *S. cerevisiae*. It may be seen that under these conditions, a 2.5-fold difference in diacetyl production occurred between the lowest (strain 2000) and highest (strain 2091) diacetyl producers.

Studies on diacetyl removal. The effect of using live, whole cells and heat-inactivated cells of yeast and *S. diacetilactis* to remove diacetyl from an aqueous buffered solution is shown in Table 4. The use of Fleischmann's yeast resulted in the greatest diacetyl removal (90%). The mixture of brewers' yeast strains resulted in the removal of 75% of the diacetyl, whereas *S. diacetilactis* 18-16 destroyed 70% but in much less time. Heat-inactivated cells of each of the above suspensions were not capable of reducing any of the added diacetyl.

Diacetyl was removed from beer by yeast cells contained in a dialysis tubing (Fig. 2). Flavor-panel results of one such experiment indicated that extensive yeast autolysis had occurred during a 3-day reaction time. Nevertheless, the diacetyl level of the beer was decreased by this technique.

The diatomaceous earth filter beds also eliminated all the diacetyl in the solutions tested, and yeast autolysis was not a problem. Figure 3 com-

TABLE 4. *Ability of live whole cells and heat-killed cells to remove diacetyl (30 ppm) from a 20-ml aqueous solution buffered at pH 7.2 at a temperature of 25 C*

Cell type	Cell concn[a] (g/tube)	Reaction time (hr)	Per cent diacetyl removed	
			Heat-killed cells	Live cells
Brewers' yeast[b]	0.24	275	0	75
Bakers' yeast[c]	0.24	275	0	90
S. diacetilactis 18-16	0.27	3	0	70

[a] Cell weight was based on wet-packed cells.
[b] *Saccharomyces cerevisiae* 2000, I, and T were mixed in equal amounts.
[c] Fleischmann's yeast.

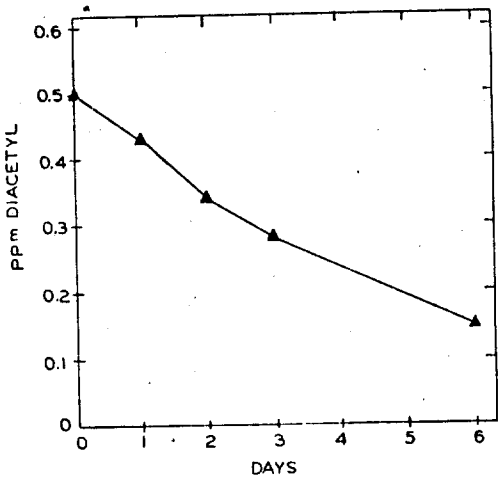

FIG. 2. *Ability of 50 ml of a heavy suspension of live whole yeast cells contained in dialysis tubing to remove diacetyl from beer (pH 4.3) at a temperature of 3.6 C.*

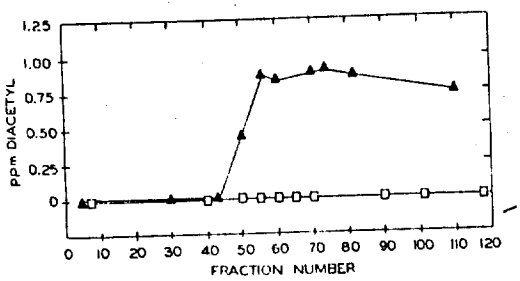

FIG. 3. *Comparison of the ability of diatomaceous earth (▲) and diatomaceous earth impregnated with yeast cells (□) to prevent the penetration of diacetyl through filter beds 18 cm deep and 5 cm in diameter.*

ures the ability of two different filter beds to move diacetyl. Using a diatomaceous earth filter d (5 by 18 cm), it was found that the first traces diacetyl percolating through the bed occurred fraction 43. The column with the yeast cells pregnated in the diatomaceous earth allowed diacetyl to penetrate. The flow rate of the lumn was approximately 12 drops per min. aster flow rates were obtained with a larger olumn 12.5 cm in diameter with a filter bed only 2 cm deep. As a result of the increased surface area and the shallower bed, the flow rate was too rapid for the fraction collector counter. To correct this, the flow rate was adjusted to 5 drops per sec; here again, all of the diacetyl was destroyed.

Diacetyl reductase activity of crude cell-free extracts. Initial attempts to demonstrate that crude enzyme extracts of yeasts were capable of removing diacetyl from beer were not encouraging. Table 5 shows results with extracts of Fleischmann's yeast; at *p*H 4.3 and at 5 C, 42 mg of extract was able to destroy only 9% of the diacetyl from a 1.1-ppm initial concentration in 64 hr. The low *p*H of the beer caused much of the crude enzyme extract to precipitate. At this *p*H with a reaction time of 2 hr at 30 C, no measureable diacetyl destruction was apparent. When (Fig. 4) the *p*H of the beer was raised with sodium hydroxide to 5.25, essentially the same concentration of extract removed more than 80% of the diacetyl at 30 C in 1 hr. It was also shown that as the concentration of the undialyzed crude

TABLE 5. *Ability of undialyzed crude enzyme extract of Fleischmann's yeast to remove diacetyl from beer (pH 4.3) incubated at the times and temperatures indicated*[a]

Beer sample	Crude enzyme extract (mg/ml)	Reaction time (hr)[b]	Temp	Initial diacetyl concn (ppm)	Per cent diacetyl removed[c]
A	21	2	30 C	1.31	0
B	42	2	30 C	1.31	0
C	42	64	5 C	1.10	9

[a] Diacetyl was added to increase the concentration to the levels indicated.
[b] In each case, after a short time the low *p*H caused the enzyme extract to precipitate.
[c] Triplicate analyses were made.

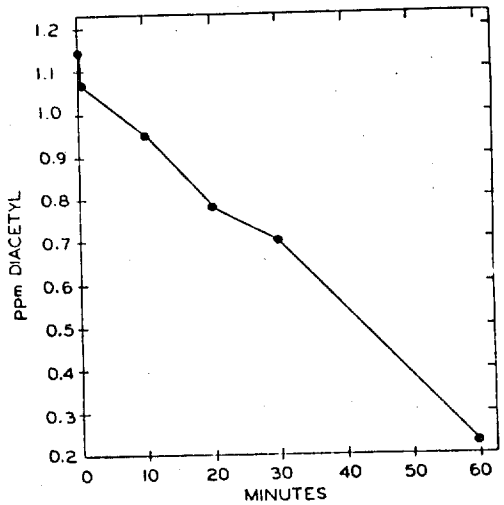

FIG. 4. *Ability of undialyzed crude enzyme extract (42.5 mg) from Fleischmann's yeast to remove diacetyl from beer at a pH of 5.25 and a temperature of 30 C, reacting for the times indicated.*

enzyme extract from Fleischmann's yeast was increased, the amount of diacetyl removed in 45 min at *p*H 7.2 and at 30 C increased (Fig. 5). The addition of a heavy suspension of heat-inactivated Fleischmann's yeast cells to the crude enzyme extract had no effect on the ability of the extract to destroy diacetyl.

A comparison of the ability of crude enzyme extract of *A. aerogenes* 8724 and Fleischmann's yeast to remove diacetyl from an aqueous solution at *p*H 7.2 at 30 C is shown in Table 6. Without NADH, yeast crude enzyme extract removed about 5% of the diacetyl, whereas the bacterial extract removed almost 20%. In the presence of NADH, better than 50% of the diacetyl was destroyed by the extract of the yeast, whereas the bacterial extract reduced 100% of the diacetyl.

Diacetyl reductase was not limited to strain 8724 of *A. aerogenes*; three other strains tested were also active (Table 7).

Figure 6 shows the effect of ethyl alcohol concentration on the ability of diacetyl reductase to remove diacetyl from an aqueous solution when assayed with the continuous recording spectrophotometer. A concentration of 3.3% alcohol inhibited the enzyme 42%; 10% alcohol inhibited 69%, and 16.7% alcohol inhibited 80% of the enzyme activity.

In all of the cell-free crude enzyme extracts tested, an endogenous level of NADH oxidation was apparent, even in the absence of the substrate diacetyl. Juni and Heym (13) referred to the

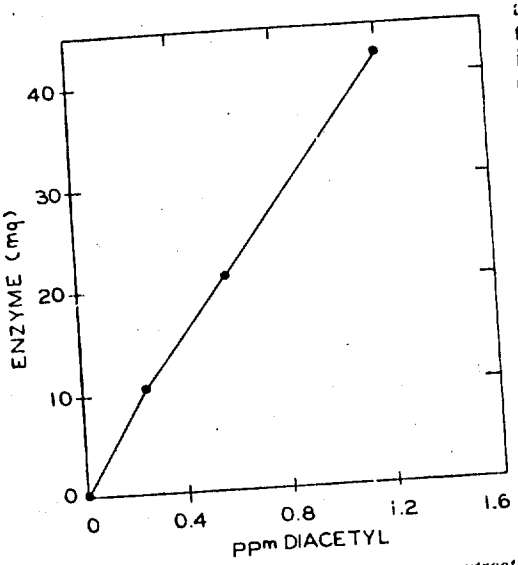

FIG. 5. *Ability of undialyzed crude enzyme extract from Fleischmann's yeast to remove diacetyl from an aqueous solution at pH 7.2 in 45 min at a temperature of 30 C.*

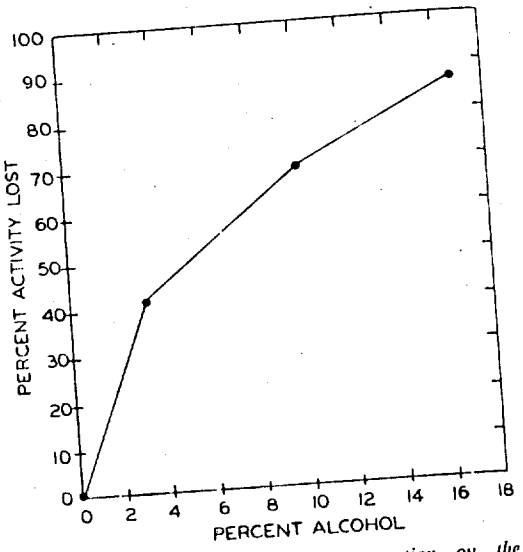

FIG. 6. *Effect of alcohol concentration on the activity of diacetyl reductase from A. aerogenes.*

TABLE 6. *Effect of enzyme extracts from Aerobacter aerogenes 8724 or Fleischmann's yeast on diacetyl present in pH 7.2 phosphate buffer after incubating for 80 min at 30 C*

System[a]	Concn of diacetyl (ppm)		
	No enzyme present	Yeast crude extract[b]	Bacterial crude extract[b]
Buffer + diacetyl	2.05	1.95	1.65
Buffer + diacetyl + reduced nicotinamide adenine dinucleotide	2.06	1.00	0

[a] Sufficient 0.1 M potassium phosphate buffer was added in each case to bring the final volume to 20 ml.

[b] Dialyzed, crude extract (50 mg) was used.

TABLE 7. *Comparison of Aerobacter aerogenes strains for diacetyl reductase activity*

Strain	Protein concn (mg/ml)	Total activity (units/ml)	Specific activity (units/mg of protein)
8308	6.50	61,500	9,900
8724	4.70	27,800	5,900
12658	6.70	62,500	9,300
OSU	6.30	66,700	10,600

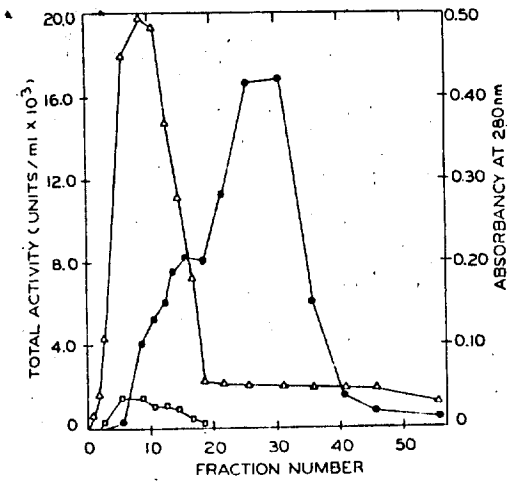

FIG. 7. *Sephadex elution pattern showing activity of NADH oxidase (□), diacetyl reductase (●), and relative absorbancy (△) of each 1.35-ml fraction eluted from a column (2.5 by 36.0 cm) of Sephadex G-200.*

endogenous activity as NADH oxidase. Even though their dehydrogenase enzyme preparation was partially purified, they still observed significant NADH oxidase activity. In the present study, attempts were made to separate the NADH oxidase from diacetyl reductase. Thermal denaturation and ammonium sulfate fractionation experiments were found to inactivate diacetyl reductase more readily than NADH oxidase. Disc electrophoresis experiments showed at least three sites of NADH oxidation on the polyacrylamide gels; however, no new bands of NADH oxidation were observed in the presence of diacetyl.

Sephadex chromatography was useful in separating NADH oxidase and diacetyl reductase activities. Figure 7 shows that the first 20 fractions contained most of the NADH oxidase activity, whereas fractions 20–40 were almost entirely free of the oxidase but were enriched for diacetyl reductase.

DISCUSSION

Diacetyl production and destruction (Fig. 1) are typical of all brewers' yeast fermentations. Strain, propagation methods, fermentation conditions, and composition of the wort all are important concerning diacetyl production. However, it has been said that other factors affecting the production of diacetyl are of secondary importance to the choice of yeast strain selected for the fermentation (21).

Other workers have reported that yeast strains may produce different amounts of diacetyl (14, 18), but none of these reports related diacetyl production to the amount of growth. To make the results more meaningful, the relative diacetyl production per cell was used as the basis of comparison (Table 3); one strain produced nearly 2.5 times as much diacetyl as the strain producing the least diacetyl. Differences as great as this may be significant, especially when mild-flavored beer is being produced. Owades et al. (18) suggested one possible reason (feedback inhibition) for strain variation in diacetyl production when they noted that yeasts differed significantly in their ability to absorb valine from wort.

Burger et al. (4) reported that both bakers' yeast and brewers' yeast were capable of removing diacetyl from beer. They also noted that heat-treated yeast cells were not capable of removing diacetyl. These results were confirmed in the present study (Table 4). It also was shown that yeast cells are not alone in their ability to remove diacetyl, but that some bacterial cells also have this capacity; *Streptococcus diacetilactis* 18-16 removed diacetyl more rapidly than yeast cells.

The diacetyl-destroying ability of whole yeast cells was utilized in experiments designed to remove diacetyl off-flavor from beer. Even though yeast cells held in dialysis tubing were capable of removing diacetyl, yeast autolysis off-flavors resulted from the lengthy exposure time. However, one application of the use of live yeast cells for diacetyl removal was successful. Yeast cells, when impregnated in a diatomaceous-earth filter bed, removed all the diacetyl from solutions percolated through the bed.

The possibility of treating beer by filtration to remove diacetyl is suggested by these results. Beer is filtered through diatomaceous earth twice after fermentation in most brewery operations, as it leaves the aging tank and when it is pumped from the finishing tank to the holding tank. Yeast cells could be incorporated in the diatomaceous earth at the first filtration step if so desired. Yeast cells recovered from the fermenter could be used in the filtration process. Also, the prolonged exposure of yeast cells to the beer, which is prevalent when breweries practice kräusening, would be avoided so that yeast autolysis problems would not occur. Due to the difference in settling rates (diatomaceous earth settling more quickly than yeast cells), if two filtration beds were used alternately, while one bed was filtering beer the other bed could be fluidized, the old yeast could be washed away, and fresh yeast could be impregnated into the bed. This practice would aid in preventing yeast autolysis off-flavor from occurring.

Dialyzed crude enzyme from yeast was found

to destroy diacetyl in a manner quite similar to that of diacetyl reductase obtained from *A. aerogenes*. The enzymes from both organisms were able to destroy some diacetyl without NADH addition. Also, with each enzyme, diacetyl reduction was greatly stimulated by the addition of NADH. The endogenous diacetyl-destroying activity observed in each extract was probably the result of residual NADH which had not been removed by dialysis.

Juni and Heym (13) described an active 2,3-butanediol dehydrogenase which they found in yeasts and bacteria. This enzyme was also capable of oxidizing NADH in the presence of diacetyl. Seitz et al. (25) also noted these activities in *S. diacetilactis* and pointed out that whereas the 2,3-butanediol dehydrogenase was reversible, the diacetyl reductase was irreversible; this also was true for the enzymes of *A. aerogenes* (24). Thus, it seems that these two activities are catalyzed by different enzymes; otherwise one might expect the diacetyl reductase to be reversible also. Juni and Heym (13) suggested these activities (diacetyl and acetoin reduction) were catalyzed by the same enzyme. Proof of either alternative is lacking, however, but is being studied in these laboratories.

It seems that diacetyl mutase described by Green et al. (11) and pyruvic acid oxidase studied by Dolin (7) are not the same as diacetyl reductase. These enzymes required the addition of thiamine pyrophosphate and magnesium ion, were not stimulated by NADH addition, and were inactivated by dialysis and lyophilization.

The 42% inhibition of diacetyl reductase activity resulting from a 3.3% ethyl alcohol solution provides one more reason why diacetyl reductase is not suitable for use in beer in which the alcohol content is generally about 3.6%. The low *p*H of beer presents a problem with respect to the commercial use of diacetyl reductase. Clearly, some method of protecting the enzyme from hydrogen ions will be necessary, and such studies are in progress. A means of regenerating NADH also will be desirable.

Gel electrophoresis results indicated that at least three different NADH-oxidizing enzymes were associated with the crude diacetyl reductase from *A. aerogenes*. These could be removed from diacetyl reductase by Sephadex chromatography, but even their presence in the crude extracts did not prevent the use of the enzyme to remove diacetyl from beer under the conditions of this study. However, use of the enzyme to remove diacetyl from beer under commercial conditions will require either the use of whole cells or some other system to protect the enzyme.

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Letter from Chief of Food & Drug Division USDA

2089

Simulated flavor

Prof. H. A. Ruelee,
Head of Dept. of Dairy Husbandry,
State College of Agriculture,
Urbana, Illinois.

March 3, 1936.

Dear Professor Ruelee:

I am sorry that I have not had an darlier opportunity to answer Your letter of February 15. You inquire whether the addition to butter in the churn of a steam distillate from butter starter has the approval of this Administration. The context indicates that you are postulating no sort of label declaration of the added constituent.

With the thought that it may not have come to your attention, I am enclosing a trade notice on the subject of the addition of flavors to butter, which was issued November 22, 1933. With this as the starting point it is, perhaps, in order to develop briefly the views of this Administration as to just what constitutes "artificial flavor" within the meaning of this notice.

The prohibition of the Food and Drugs Act against label statements as to the identity or general character of a food, which are false or misleading in any particular, has been construed with great strictness by the United States Supreme Court. If an article of food "is not the identical thing that the brand indicates it to be, it is misbranded." Consumer understanding is naturally the prime criterion of identity in articles of food, and especially so in products like butter which are still made to a considerable extent in the hone. It is doubtless this concept which Congress had in mind when they used the expression "usually known as butter" in the statutory standard which is quoted in the enclosed notice.

The long time use of starters, to control the development of acidity and flavor, in the ripening of cream prior to churning will, I think, establish this practice as normal in manufacturing the product "usually known as butter."

The addition of certain volatile constituents of starters toward the end of the butter making process, however, seems to me quite clearly the addition of artificial flavor within the meaning of our notice. I use the term "artificial" in the sense of not so normal to the product as the consumer understands it. We have

BUTTER - added flavor

always held, in considering the increasingly numerous proposals to incorporate this or that substance in food products in order to enhance their commercial value, that it was entirely immaterial whether or not these substances were super-imposed on or derived from similar materials present in the normal product. To hold otherwise would be to abandon the very sheet anchor in the maintenance of the integrity of food names.

The argument is invariably advanced that the addition makes the food product a superior one. Our reply is that, if this is so, the argument for informative labeling is allthe stronger.

Whether the addition to butter of the products you suggest, and others of somewhat similar character, will enhance the flavor of an inferior butter is, perhaps, still an open question. Should the work which our chemists are doing on the subject indicate that such was the case in any instance, the adulteration would theb become one of such a character that no informative labeling could correct it,

You have already inferred, no doubt, that the position we have taken on the addition of certain starter constituents near the end of the butter manufacturing process, would be the same whether starter constituents or starter itself were used in the churn for purposes of flavor enhancement. While, as you say, such a practice mau not involve adulteration as the layman understands this term, we must leave to the courts the final decision as to whether a product so made is entitled to the unqualified designation "butter" in the light of consumer understanding.

Very truly yours,

W. C. Campbell,

Chief.

Enclosure
TN 11-22-33 Butter Mfrs.

WBW:MCG
mm

USDA

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DAIRY AND EGG PRODUCTS SECTION

Prof. H. A. Ruelee,
Head of Dept. of Dairy Husbandry,
State College of Agriculture,
University of Illinois,
Urbana, Illinois.

APR 2 - 1936

Dear Professor Ruelee:

I have your letter of March 18 raising certain additional questions on the matter of the addition of flavor to butter by processes other than the normal and recognized use of starters which develop acidity and perhaps flavor by bacterial growth.

Perhaps I did not make it quite clear in my earlier letter that any other use of starter or distillate from starter was regarded by us as a flavoring rather than a "starting" of the cream. In other words, its prime purpose is to impart a flavor to the finished product rather than to develop acidity and flavor by bacterial multiplication.

I have given extended consideration to your question as to what would be proper labeling of the flavored article. The statutory definition and standard quoted in the notice sent you on March 3, is unique among our food standards in that it has the full authority of Congress behind it. You will note that it is very explicit in requiring butter to be made exclusively from milk or cream or both. The only other substances permitted are common salt and coloring matter. With such a mandate before us, we are constrained to take the position that the addition of flavoring materials of whatever character produces an article which is no longer butter within the meaning of the statute.

As soon, therefore, as our chemists have worked out methods for detecting added flavoring materials it is our intention to immediately inaugurate appropriate regulatory action against the flavored article wherever we find it in interstate commerce, and without reference to whether the product is sold as butter or as butter containing artificial flavoring.

Very truly yours,

W. G. Campbell
Chief.

WBS-HCG

BUTTER - added flavor

CHANGES IN THE BIACETYL CONTENT OF CREAMED COTTAGE CHEESE CAUSED BY ORGANISMS ASSOCIATED WITH SPOILAGE

Food Res. 22 (2): 170-175, (1957)

CHARLES S. WALES[a] AND L. G. HARMON

Michigan State University Agricultural Experiment Station, East Lansing[b]

(Manuscript received October 11, 1956)

Biacetyl produced during the fermentation of citric acid by *Leuconostoc citrovorum* and/or *Leuconostoc dextranicum* is primarily responsible for some of the desirable flavor and aroma in dairy products, including cottage cheese. Acetylmethylcarbinol, produced by the same fermentation scheme under more anaerobic conditions, is readily oxidized to biacetyl. Krishnaswamy and Babel (*5*) found 1.06 to 2.55 p.p.m. of biacetyl in dry cottage cheese curd made by the long-set method. Elliker (*3*) observed that *Streptococcus lactis* had no effect on biacetyl in butter, but that several species of *Pseudomonas* destroyed biacetyl. Parker and Elliker (*6*) reported rapid decreases in biacetyl and losses of aroma in cottage cheese inoculated with cultures of *Pseudomonas viscosa* and *Pseudomonas fragi*; whereas, *Alcaligenes metacaligenes* was less active in biacetyl destruction. They reported the biacetyl was converted to acetylmethylcarbinol and possibly 2, 3-butylene glycol.

Twenty-four different organisms capable of causing spoilage in cottage cheese were isolated and identified by Bonner (*1*). These organisms represented 7 genera of bacteria, 3 genera of molds, and 2 genera of yeast. Varying degrees of flat flavor were encountered in cheese inoculated with these organisms and stored at 40° and 50° F. An investigation of methods of increasing biacetyl in cottage cheese suggested the desirability of determining the amounts of biacetyl usually occurring in creamed cottage cheese, and the effect of several different organisms on the biacetyl and acetylmethylcarbinol content of cottage cheese stored at 40° and 50° F.

EXPERIMENTAL PROCEDURE

Thirty samples of commercial fresh creamed cottage cheese secured from processing plants or retail markets were examined organoleptically and analyzed for biacetyl and acetylmethylcarbinol content.

Twelve organisms representing bacteria, yeast, and molds capable of causing spoilage were inoculated into cottage cheese. The organisms were *Geotrichum candidum*, *Mucor plumbeus*, *Rhodotorula flava*, *Pseudomonas fluorescens*, *Pseudomonas tralucida*, *Ps. fragi*, *Achromobacter butyri*, *Achromobacter eurydice*, *Alcaligenes metalcaligenes*, *Bacillus firmus*, *Micrococcus flavus*, and *Escherichia coli*.

One lot of cottage cheese curd was divided into 13 equal portions. An appropriate amount of creaming mixture containing 14% butterfat and 4% salt was also divided into 13 equal parts. One lot of cheese was creamed and used as a control. Each of the remaining lots of cream was inoculated with 0.1 ml. of a 24-hour trypticase soy broth culture of one of the above microorganisms. The inoculated lots of cream were added to the separate portions of curd and the creamed cheese was packaged into 12-oz. cartons.

[a] Dairy Industry Supply Association fellowship winner, assigned to Michigan State University, 1955-56.

[b] Mich. State Univ. Agr. Expt. Sta., Journal Article No. 1961.

It is impossible to sterilize cottage cheese without resorting to procedures which alter the physical characteristics of the curd or interfere with the normal activity of subsequent inoculants. An attempt was made to introduce sufficient inoculation of the known contaminant to dominate the fermentation, and in each instance the spoilage was characteristic of the organism inoculated. The number of organisms added ranged from 300 molds per ml. in the cream inoculated with *Mucor plumbeus*, to 450,000 coliforms per ml. in the cream inoculated with *E. coli*. One-half of the packages from each lot was stored for 12 days at 40° F. and the other half stored for 6 days at 50° F. Spoilage rendered longer storage times impractical.

Biacetyl, acetylmethylcarbinol, pH, and organoleptic determinations were performed initially and at 3-day intervals on each sample of creamed cottage cheese. Biacetyl and acetylmethylcarbinol were determined by the Prill and Hammer method (*7*), using a 25-g. sample of cheese. Color intensity was measured on a Cenco-Sheard-Sanford photometer. Duplicate determinations were frequently performed and agreed within 1 p.p.m. The pH was determined on thoroughly macerated cheese, with a Beckman Model G pH meter.

RESULTS AND DISCUSSION

Amount of biacetyl and acetylmethylcarbinol in fresh creamed cottage cheese. Biacetyl contents of 30 samples of commercial fresh creamed cottage cheese ranged from 0.7 to 10.4 p.p.m., with most samples containing from 3 to 8 p.p.m. A flat flavor was usually apparent in samples containing less than 3 p.p.m. The amount of acetylmethylcarbinol varied from 15 to 50 p.p.m. with most samples containing from 32 to 38 p.p.m. Hammer (*4*) reported that butter usually contained about ten times more acetylmethylcarbinol than biacetyl.

Effect of various organisms on the biacetyl content of creamed cottage cheese. Data for samples stored 12 days at 40° F. and 6 days at 50° F. are shown in Figures 1 to 4 and 5 to 8, respectively. Most of the organisms used in this work caused a reduction in the biacetyl content of cottage cheese stored at 50° F. In the samples held at 40° F., most of the organisms caused very slight reductions in biacetyl during the first 6 days and only moderate decreases thereafter.

The biacetyl content of all the cheese at 0 day was 6.4 p.p.m. In the control sample stored at 40° F. there was a gradual increase in biacetyl to a maximum of 9.4 p.p.m. on the 9th day, after which there was a moderate decrease to 8.5 p.p.m. on the 12th day. A slight decrease between the 3rd and 6th day was within the range of experimental error. In the control sample stored at 50° F. the biacetyl value increased to 10.2 p.p.m. on the 3rd day and decreased to 4.6 p.p.m. on the 6th day. These results agree in general with those of Krishnaswamy and Babel (*5*), who observed an increase in the biacetyl content after 4 days and a decrease after 7 days, when good quality dry curd was stored at 40° F.

Ach. butyri is the only organism which caused an increase in biacetyl at both storage temperatures (Figures 3 and 7). At 40° F. the biacetyl content of the cheese inoculated with *Ach. butyri* increased continuously throughout the holding period, exceeding the control on the 6th day and thereafter. The cheese inoculated with *Ach. butyri* was the only sample in which the biacetyl content increased on the 3rd day, although the value was below the corresponding control.

The *Pseudomonas* organisms caused greater decreases in biacetyl at 40° F. than any other organisms studied (Figure 2). However, at

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Effect of microorganisms on the biacetyl content of creamed cottage cheese stored 12 days at 40° F.

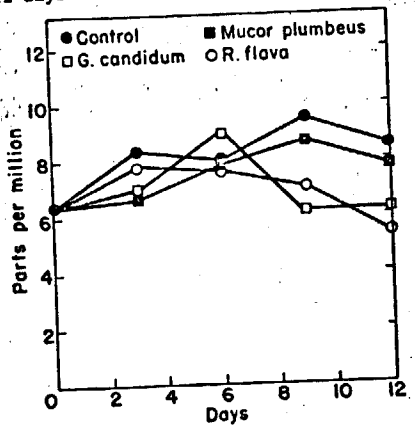

Figure 1. Variations in the biacetyl content of creamed cottage cheese inoculated with G. candidum, Mucor plumbeus, or R. flava.

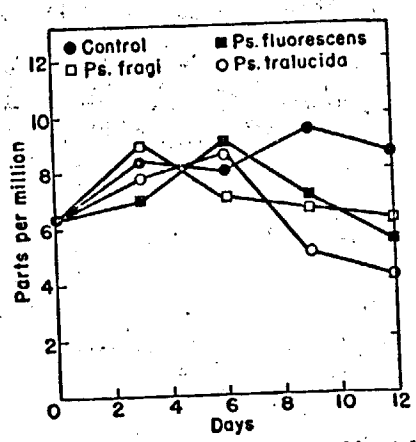

Figure 2. Variations in the biacetyl content of creamed cottage cheese inoculated with Ps. fragi, Ps. fluorescens, or Ps. tralucida.

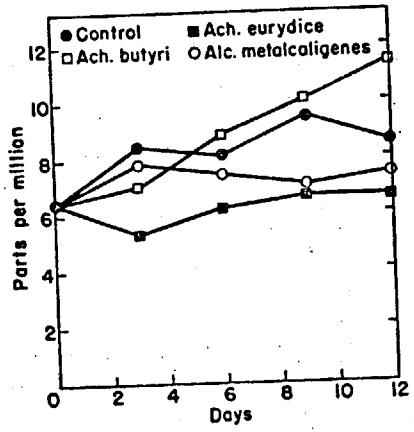

Figure 3. Variations in the biacetyl content of creamed cottage cheese inoculated with Ach. butyri, Ach. eurydice, or Alc. metalcaligenes.

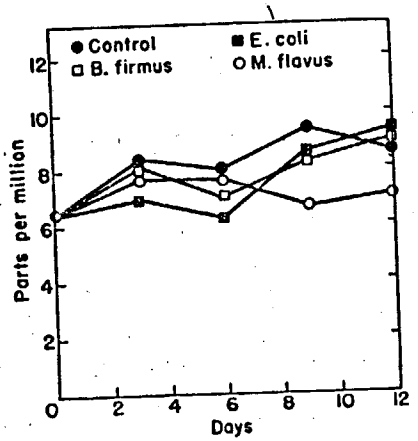

Figure 4. Variations in the biacetyl content of creamed cottage cheese inoculated with B. firmus, E. coli, or M. flavus.

(Figure 6) these decreases were equalled or excelled by the yeast and molds. *Alc. metalcaligenes* caused only a slight decrease in biacetyl at both 40° and 50° F. (Figures 3 and 7). These results agree with those of Parker and Elliker (*6*), except these workers observed greater destruction of biacetyl at 50° F. by several strains of *Pseudomonas* than encountered in the work reported herein.

At both 40° and 50° F. the biacetyl content of the cheese inoculated with *M. flavus* and *B. firmus* (Figures 4 and 8) remained relatively constant, with the terminal values being slightly greater than the control.

Effect of mircoorganisms on the biacetyl content of creamed cottage cheese stored 6 days at 50° F.

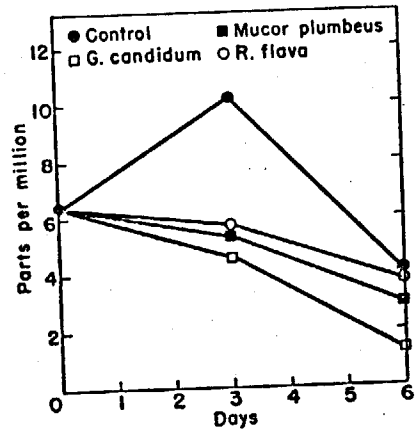

Figure 5. Variations in the biacetyl content of creamed cottage cheese inoculated with G. candidum, Mucor plumbeus, or R. flava.

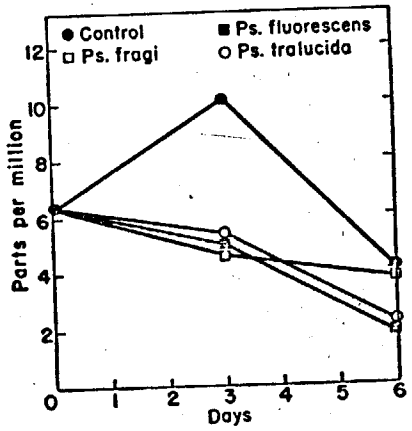

Figure 6. Variations in the biacetyl content of creamed cottage cheese inoculated with Ps. fragi, Ps. fluorescens, or Ps. tralucida.

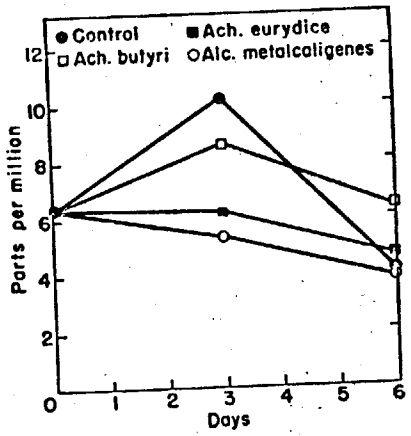

Figure 7. Variations in the biacetyl content of creamed cottage cheese inoculated with Ach. butyri, Ach. eurydice, or R. flava.

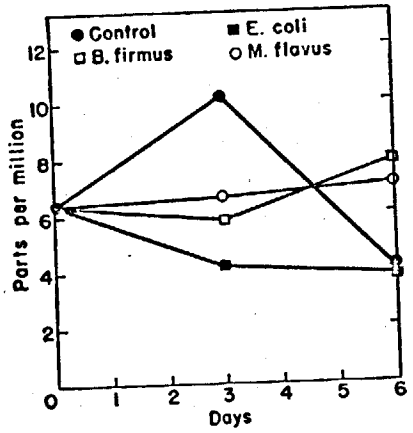

Figure 8. Variations in the biacetyl content of creamed cottage cheese inoculated with B. firmus, E. coli, or M. flavus.

Biacetyl was not completely destroyed in any sample. Cheese containing definite slime formation contained biacetyl in amounts ranging from 0.4 to 8.0 p.p.m.

Effect of various organisms on the acetylmethylcarbinol content of creamed cottage cheese. The acetylmethylcarbinol content of the control sample of creamed cottage cheese stored at 40° F. was 45, 50, and 40 p.p.m. at 0, 6, and 12 days, respectively. None of the organisms caused significant change in the acetylmethylcarbinol content of the cheese stored at 40°

2146

In the control cheese stored at 50° F. there was a decrease in acetylmethylcarbinol during the first 3 days and a substantial increase between the 3rd and 6th day. Values at 0, 3, and 6 days were 45, 25, and 49 p.p.m., respectively.

There was an enormous increase in acetylmethylcarbinol in the cheese inoculated with *E. coli* and stored at 50° F. The strain of *E. coli* used in this work conformed to the characteristics described by Breed *et al.* (2), except gelatin was liquefied and no acid was produced from xylose. Laboratory tests showed the organism was Voges-Proskauer—negative.

Smaller increases in acetylmethylcarbinol occurred in samples inoculated with *Ach. butyri* and *Ps. tralucida*, and held at 50° F. The latter organism caused a corresponding decrease in biacetyl. Similar results were observed with *Pseudomonas desmolyticum*.

Cheese inoculated with *Rh. flava, Ach. eurydice, Ps. fragi, Ps. fluorescens, G. candidum*, and *Mucor plumbeus*, and stored at 50° F., showed continuous uniform decreases with virtual disappearance of acetylmethylcarbinol at the 6th day. The acetylmethylcarbinol content of samples inoculated with *B. firmus, M. flavus*, and *Alc. metalcaligenes* conformed closely to the control throughout the 6-day storage interval.

Effect of pH on changes in biacetyl and acetylmethylcarbinol. The pH value of all samples of creamed cottage cheese was 4.85 at 0 days and varied from 5.4 to 4.5 and from 5.2 to 4.5 among the samples stored at 40° and 50° F., respectively. Decreases in pH were more frequent in the samples at 50° F. Increases in pH during storage were greatest in cheese inoculated with *Ach. butyri, Alc. metalcaligenes*, and *Ps. tralucida*. Decreases in pH were greatest in cheese inoculated with *R. flava* and *G. candidum*. Variations in biacetyl and acetylmethylcarbinol were not consistently related to pH of the inoculated samples studied.

Effect of biacetyl on flavor. Most deterioration in organoleptic quality occurred as a result of spoilage induced by the organisms inoculated into the cheese. Flat flavors which occurred as a result of decreases in biacetyl were less prominent than other off-flavors, due to action of microorganisms. Although *Ach. butyri* produced an increase in biacetyl, cheese inoculated with this organism possessed an objectionable aromatic odor at the end of the storage periods. Organoleptic quality could not be correlated to the biacetyl content of the inoculated samples of cheese.

SUMMARY

Biacetyl content of fresh creamed cottage cheese ranged from 0.7 to 10.4 p.p.m. The acetylmethylcarbinol content of the same samples varied from 15 to 50 p.p.m.

Several organisms commonly identified with spoilage in cottage cheese were individually inoculated into creamed cottage cheese which was stored at 50° and 40° F. for 6 and 12 days, respectively. The biacetyl content of the cheese inoculated with *Achromobacter butyri* increased during storage. The *Pseudomonas* organisms caused greater decreases in biacetyl than any other organisms studied.

No appreciable changes occurred in the acetylmethylcarbinol content of inoculated cheese stored at 40° F. Cheese inoculated with *Rhodotorula flava, Achromobacter eurydice, Pseudomonas fragi, Pseudomonas fluorescens, Geotrichum candidum*, and *Mucor plumbeus*, and stored at 50° F., showed continuous decreases in acetylmethylcarbinol.

Variations in biacetyl and acetylmethylcarbinol were not consistently related to pH of the inoculated samples studied. Neither could organoleptic quality be correlated to the biacetyl content of the inoculated samples of cheese.

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2146

2154

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J. Food Sci. 33(3) 294-7, (1968)

Volatile Compounds from Heated Glucose

SUMMARY—Volatiles from anhydrous glucose heated in air at 250°C for 30 min were collected in a trap maintained at the temperature of solid carbon dioxide. A concentrated ether extract of the distillate was shown by gas chromatography to contain at least 100 compounds. Among those identified and heretofore unreported from heated glucose were the gamma lactone of 4-hydroxy-2-pentenoic acid, 1-(2'-furyl)-propane-1,2-dione (acetylfuroyl), 3-methylcyclopentane-1,2-dione, phenol and methylfuroic acid.

INTRODUCTION

A NUMBER OF PUBLICATIONS have recently appeared on the application of gas chromatography to the analysis of volatile products from the thermal decomposition of carbohydrates and closely related substances: Bailey *et al.* (1962), Bryce *et al.* (1963), Doerr *et al.* (1966), Fiddler *et al.* (1966), Gianturco *et al.* (1963, 1964), Greenwood *et al.* (1961), Graham (1966), Heyns *et al.* (1966a), Sugisawa (1966), and Tai *et al.* (1964). These studies indicate the presence of a wide variety of compounds, but generally only the low boiling classes have received major attention. Compounds of intermediate volatility have not been as extensively studied despite some indication that they may be more important contributors to flavor.

Since glucose was present in substantial amounts in the materials previously studied, it was felt that heating glucose itself in an isolated system would give valuable information on the origin of the volatile flavor compounds.

Among the variables that may affect the composition of volatiles from glucose are heating time, temperature, and the presence of oxygen. On the basis of achieving an adequate collection of volatiles in a reasonable time, a temperature roughly intermediate between 150°C (Sugisawa, 1966) and 300°C (Heyns *et al.*, 1966a) and a heating period of 30 min were chosen. The heating was carried out in atmospheres of both air and nitrogen.

EXPERIMENTAL

Heating and distillation

Two g of anhydrous glucose (Baker analyzed reagent) were placed in a 50 ml (₩ 45/50) thre-neck distillation flask in which a coarse capillary and a thermometer were fixed by means of Teflon adapters (Fig. 1). A side-arm of the flask led into a trap which was surrounded by dry crushed ice. The system was connected to a water aspirator or to a cylinder of "pre-purified grade" N, to obtain either the air or N atmosphere over the heated glucose. Heat was provided by an electric heating mantle which was thermostatted by a Jelrus automatic controller (manufactured by the Jelrus Co., Inc., New York, N. Y.) to maintain an internal flask temperature of approximately 250°C. The total distillation time was 30 min.

* Present address: International Flavor and Fragrances, Union Beach, N. J.

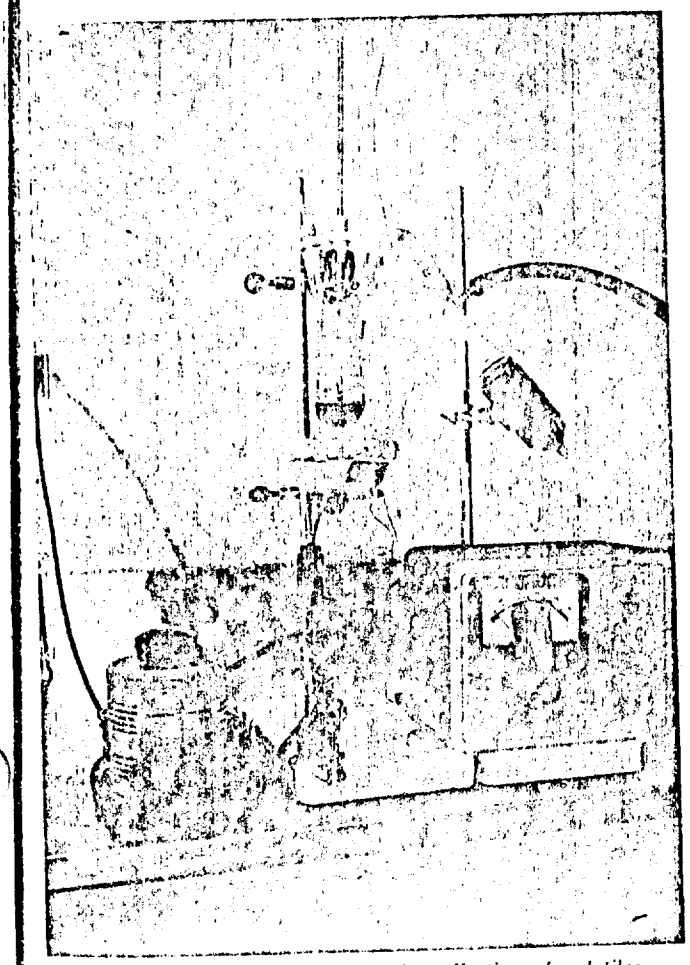

Fig. 1. Distillation system for the collection of volatiles.

Extraction and concentration

The frozen distillate, usually less than 1 ml, was diluted to about 3 ml with distilled water, transferred to a 150 × 16 mm test tube, enough anhydrous Na_2SO_4 added to saturate the solution, and extracted with an equal volume of redistilled, anhydrous ether. The ether phase was then pipetted into a 5 ml beaker, and the ether slowly evaporated under a stream of N to about one-third the original volume, after which, it was transferred with a pipet to a 3 ml conical bottom, glass-stoppered centrifuge tube for further evaporation under N. The final concentration was arbitrary, but evaporation was terminated when there was a noticeable increase in viscosity of the ether extract. The final volume was about 0.5 ml. This concentrate had a refrigerated storage life of 7–10 days at 3°C, after which, it tended to form a precipitate. Stability could be increased somewhat by redilution with anhydrous ether prior to storage.

When it was desired to eliminate the masking effect of the ether solvent on compounds of very short retention times during subsequent gas chromatography, the undiluted distillate was transferred directly to the centrifuge tube and diluted with 2.0–2.5 ml of a saturated Na_2SO_4 solution, then centrifuged at 4000 rpm until an oily, brown ring separated at the surface. From this layer, 0.5 μl samples were withdrawn for direct injection into the gas chromatograph-mass spectrometer unit.

Analysis

One to five micro-liter samples of the concentrated ether extract were taken for gas chromatography. Mass spectra were obtained, using a coupled gas chromatograph (Perkin-Elmer Model 226)—mass spectrometer (Hitachi-Perkin-Elmer Model RMU-6A) unit with a capillary column, 200 ft long, 0.02 in. ID, and coated with a mixture of diethyleneglycolsuccinate and H_3PO_4 (2%). The column effluent was split with equal portions being directed to the flame ionization detector, and to the mass spectrometer, the latter via a Watson-iBemann, helium separator (Watson *et al.*, 1965). The operating parameters were as follows:

Gas chromatograph:	
flow rate	Helium carrier gas at 15 ml/min.
program	30°C for 5 min, then 2°C/min to 160°C
injection block temperature	275°C
Mass spectrometer:	
inlet temperature	200°C
ion source pressure	4×10^{-6} mm
ion source temperature	250°C
acceleration voltage	2.5 Kv
target current	60uA
electron multiplier voltage	2.5 Kv
scan speed	12 sec. (m/e 12-400)
exit slit	0.15 mm

Trapping for infrared analysis was attempted by the method of Edwards *et al.* (1965), from a Perkin-Elmer 810 gas chromatograph with a 6 ft long, ⅛-in. OD column packed with 10% polypropyleneglycol on 60/80-mesh Chromosorb W. The outlet temperature was kept at 250°C, and the injection block temperature at 275°C. The column was held at 65°C for 5 min, and then ballistically programmed to 200°C where it remained until completion of the analysis. Twenty % of the carrier gas, N was diverted to the flame ionization detector, and 80% to the outlet.

The presence of formaldehyde and CO_2 was established in separate experiments by bubbling the sweep gas from the heated glucose into a solution of 1% chromotropic acid in concentrated H_2SO_4 (Frisell *et al.*, 1958) and 0.05% $Ba(OH)_2$ solution respectively. The development of a purple color in the former and a precipitate in the latter was taken as evidence of the presence of these compounds.

RESULTS AND DISCUSSION

On the basis of comparative gas chromatography, there was no evidence that the heating atmosphere significantly influenced the composition of the collected volatiles.

Since primary emphasis in this study was placed on compounds of intermediate volatility, no special attempt

Table 1. Compounds identified in the volatile fraction of heated glucose.

G.C. No.	Compounds	Method of identification I.R.	G.C.	M.S.	Remarks
	CO_2				See experimental
	Formaldehyde				See experimental
1	Furan		++	++	
2	2-Methylfuran		++	++	
2	Butadione (Diacetyl)		++	++	
4	Acetic acid		++	++	
6	2-n-Propylfuran			+	Heyns *et al.* (1966b)
8	Furfural (2-furaldehyde)	++	++	++	
9	2-Acetylfuran		++	++	
12	3-Furaldehyde			+	Heyns *et al.* (1966b)
15	5-Methyl-2-furaldehyde		++	++	
16	5-Methyl-2-acetylfuran			+	
24	4-Hydroxy-2-pentenoic acid, gamma lactone		++	++	
27	1-(2′-furyl)-propane-1,2-dione (acetylfuroyl)		++	++	
29	3-Methylcyclopentane-1,2-dione		++	++	
31	Phenol		++	++	
42	Methylfuroic acid (one of various positional isomers.)				By mass spectrum interpretation only

++ Agreement with authentic compounds.
+ Mass spectral data only.

was made to conserve the more volatile components during distillation. Many of these have recently been reported by Qua *et al.* (1964), Heyns *et al.* (1966a), and Sugisawa (1966).

At least 100 compounds were recovered by the method of distillation and concentration employed, as shown by a typical gas chromatogram (Fig. 2). Table 1 lists the compounds identified and their method of identification.

Identifications were made by first obtaining mass spectra of the eluting peaks to ascertain probable structures which were subsequently confirmed or rejected on the basis of comparison with mass spectra of authentic samples. Confirmation of a specific structure was then determined by comparison of gas chromatographic retention times with authentic samples.

Except for furfural, attempts to obtain useful infrared spectra from the packed column were unsuccessful, possibly because of the low concentrations involved, and the low efficiency of recovery from the injected sample.

Of the compounds identified, the gamma lactone of 4-hydroxy-2-pentenoic acid 1-(2′-furyl)-propane-1,2-dione ,acetylfuroyl), 3-methylcyclopentane-1,2-dione, phenol and methylfuroic acid are not heretofore known to have been reported as arising from heated glucose. The mass spectra of these compounds are presented in Fig. 3.

The existence of phenol as a volatile compound from heated glucose is observed with special interest, due to its known toxic properties. The presence of the gamma lactone of 4-hydroxy-2-pentenoic acid is noteworthy, because of the implication of unsaturated gamma lactones in anti-

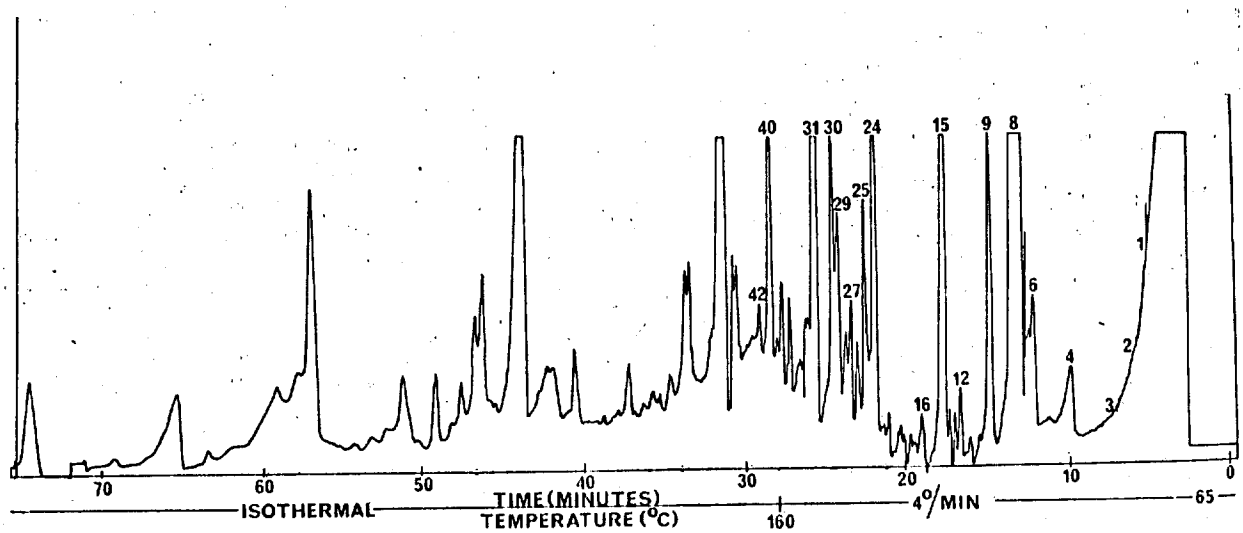

Fig. 2. Typical gas chromatogram of the volatiles obtained from the distillation of glucose at 250°C for 30 min.

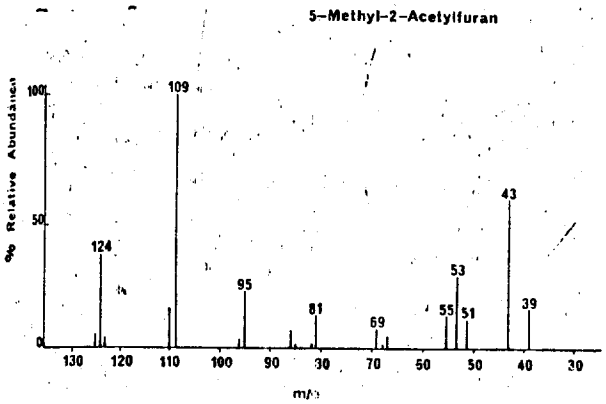

Fig. 3. Mass spectra of some compounds of intermediate volatility identified in the distillate of glucose heated at 250°C for 30 min.

biosis (Oxford 1945). Also, an enolic isomer of 3-methylcyclopentane-1,2-dione is reported as being used to impart a maple flavor and as a flavor potentiator by Filipic *et al.* (1965).

The mechanism of formation of 4-, 5-, and 6-carbon furan structures from monosaccharides has already been proposed Newth (1951), Anet (1964), and Byrne *et al.* (1966), but the presence of 7-carbon furan compounds in the volatiles from heated glucose (propylfuran, methylacetylfuran and 1-(2′-furyl)-propane-1,2-dione leads to the suspicion that secondary reactions of primary decomposition products also influence the vapor phase composition. For example, acetic acid and furfural could conceivably react to form 1-(2′-furyl)-propane-1,2-dione. In previous works, this compound was obtained only as an oxidative product of 2,furylacetone (Cosgrove *et al.*, 1952) and one component of coffee volatiles (Gianturco *et al.*, 1964). Such a compound, however, could also originate from a polymer fragmentation process.

A common source of benzenoid and alicyclic compounds is the pyrolysis of carbonaceous matter (bituminous coal) at very high temperatures (1000–3000°C), in the absence of air. The presence of phenol and 3-methylcyclopentane-1, 2-dione at 250°C may therefore indicate a common reaction path, but the details of the mechanism of formation of these structures from heated glucose has yet to be elucidated.

Most of the compounds identified have a unique, mild aroma none of which could be described as that of caramel. It may therefore be that the caramel aroma itself arises from the mixture of volatiles present.

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Ms. accepted 2/14/68.

The authors are indebted to Mr. Henry A. Bondarovich of the Fundamental Research Department, The Coca-Cola Company, for his invaluable assistance in the interpretation of mass spectra.

The authors also wish to thank Dr. W. H. McFadden of the Western Regional Laboratory of the United States Department of Agriculture for his comments on the mass spectrum of 4-hydroxy-2-pentenoic acid, gamma lactone.

216

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Influence of Fermentation By-Products on Beer Quality

Brew. Dig. 42(1): 74-78, (1967) (Eng.)

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THE AROMA or flavor of beer represents a mixture of various components, such as, for example, volatile sulfur compounds, acetaldehyde, esters and higher alcohols, acetoin and diacetyl, etc. The detection and identification of the individual substances are frequently difficult, since the aroma components have many properties in common and some of them undergo transformations which are not as yet clearly understood.

Yeast produces during the main fermentation the so-called higher aliphatic alcohols as metabolic and fermentative by-products from the amino acids present in wort. These include n-propanol, iso-butanol, n-butanol, optically active amyl alcohol, and iso-amyl alcohol. Additional by-products formed include the aromatic alcohols tyrosol, tryptophol and beta-phenyl ethanol, of which the last is most clearly demonstrable in the finished beer.

Translated with permission from *Brauwelt 106*, No. 44, 813 (1966).

When yeast is stimulated by a rise in the fermentation temperature or excessive wort aeration and undergoes increased multiplication (accelerated growth rate), this generally also results in an increase in higher alcohols. Normally, the higher alcohols are present at a low enough level to be innocuous to beer flavor. When beers do show flavor deviations by possessing a pronounced harsh bitterness as well as increased levels of higher alcohols, it is well to isolate the higher alcohols by means of gas chromatography as this will often show that individual components, for example iso-butanol and iso-amyl alcohol, will be present in greater amounts due to faulty processing and hence adversely affect beer flavor.

Other metabolic products encountered include diacetyl and acetoin which at higher level are detrimental to beer flavor. In previous publications we have pointed out that the formation of diacetyl is not solely attributable to pediococcus infec-

Table I
Formation of Fermentation By-Products

Fermentation Time: Hrs.	Acetaldehyde mg/l	Acetoin mg/l	Diacetyl mg/l	Higher Alcohols mg/l
24	8.2	14.61	0.12	10.8
48	11.7	15.64	0.13	21.4
72	13.4	10.92	0.16	41.8
96	11.7	8.58	0.36	56.4
120	12.2	8.47	0.32	75.2
144	13.0	7.15	0.27	87.4
168	13.4	6.80	0.25	96.6
192	11.4	6.34	0.23	97.4

tions, but that every yeast produces more or less of this substance during fermentation. This fact indicates the importance of selecting a suitable yeast and of basing that selection on factors other than merely the fermentative strength.

The following discussions and experiments aim to provide a general survey of the formation of various metabolic by-products during the fermentation and aging of beer and to show to what extent beer flavor is thereby influenced.

By-Product Formation

In addition to alcohol and carbon dioxide, various by-products are formed during fermentation which, though present in only very small amounts, may be detrimental to the beer's taste and aroma. This may be specially so when, as a result of improper yeast selection or other adverse factors, the main and secondary fermentations fail to follow the desired course.

Table I illustrates the formation of the more important fermentative by-products. For this purpose, regular brewery wort of 12.99 per cent original extract was fermented by a flocculent yeast at a maximum temperature of 8° C.

The values for acetaldehyde are shown in the second column of the table. This substance is a normal by-product of fermentation; it is formed from pyruvic acid and is a precursor of ethyl alcohol, diacetyl and acetoin. It can be detected even at the outset of fermentation, the amount produced depending on the yeast strain and the fermentation method employed. Higher fermentation temperatures do not result in greater acetaldehyde formation. Thus, for instance, top fermented beers generally show only about half the acetaldehyde content of bottom fermented beers, and the beers fermented under pressure, as in the Wellhoener system, contain [illegible] acetaldehyde than do conventional fermented beers.

The most rapid formation of acetaldehyde occurs during the high [illegible] stage. In the subsequent [illegible] of fermentation there is a decline that continues during aging. Numerous analyses carried out by us [illegible] values ranging from 10.3 to [illegible] liter of acetaldehyde at the [illegible] the main fermentation. Although acetaldehyde has not so far been considered to be as dangerous [illegible] flavor as are its derivatives acetoin and diacetyl, Urion (1) has characterized aldehydes as typical [illegible] beer bouquet constituents which, if they do not undergo sufficient conversion during the secondary fermentation, may cause a young beer flavor.

The threshold value of acetaldehyde in finished beer appears to lie at 20 to 25 mg/liter according to our present observations.

In the third and fourth columns of **Table I** are shown the values for acetoin and diacetyl. These substances are also normal by-products of yeast metabolism, which is contrary to previously held views. The precursor of acetoin is acetaldehyde and oxidation of acetoin produces diacetyl. As is evident from **Table I**, acetoin is formed at the start of fermentation and then diminishes as the fermentation reaches its end. Diacetyl is produced throughout the course of fermentation. The highest diacetyl values coincide with the stage of most vigorous fermentation which, in the conventional process, corresponds to about the fifth or sixth day. As the fermentation rate declines, the diacetyl content is also reduced. Diacetyl, even at minimal concentrations, imparts an acidic and buttery aroma and a very poor flavor to beer, often designated as a pasteurization or infection taste.

Acetoin is not as detrimental from a flavor and aroma standpoint as is diacetyl. However, when it is present in abnormal amounts it will impart a musty, cellar-like taste to beer.

Both acetoin and diacetyl are extensively reduced in the course of secondary fermentation. A lagging secondary fermentation and access of air have an adverse effect and inhibit their desired reduction during beer storage.

The deleterious effect of air is clearly apparent from the fact that following the transfer of freshly fermented beer into storage tanks, which are normally filled with air, there is an increase in the diacetyl content for the first few days, caused by oxidation, which is again reduced only after the onset of a vigorous secondary fermentation. Excessive aeration of the wort, a wrong choice of yeast and an increased pitching rate, among other things, all have an adverse effect on the diacetyl content of beer. Warmer fermentation temperatures do not increase the diacetyl content, but bring about its more rapid reduction. The taste threshold in finished beer is about 0.2 to 0.3 mg/liter for diacetyl, and 6 to 7 mg/liter for acetoin.

The final column of **Table I** shows the total content of higher aliphatic alcohols, which constitute additional, and generally bitter, flavor components. They appear early in the fermentation and increase progressively until the end.

In general the higher alcohols may be thought of as products of the amino acid metabolism of yeast, except that amino acids are not really essential to their formation. We have observed that in contrast to the flavor components discussed so far, the higher alcohols continue to increase during secondary fermentation, thereby confirming previous findings by Glimm and Stenzel (2).

The level of higher alcohols in beer depends on wort composition, wort aeration, yeast strain, fermentation temperature and fermentation method. It appears worthy of mention that, as determined by gas chromatography, excessive wort aeration increases the iso-butanol content with its detrimental effect of the flavor and aroma of beer. Even though iso-butanol is organoleptically more acceptable than iso-amyl alcohol, we have found that when it is present in relatively high concentrations it will impart a harsh (more bitter and drier) taste to beer.

Top fermented beers in general contain greater amounts of higher alcohols than do bottom fermented beers. However, this holds true only when conventional brewing methods are used.

There exists a considerable difference of opinion concerning the quantities of higher alcohols present in beer, their acceptability, as well as their threshold values.

Numerous analyses that we have carried out in recent years lead us to conclude that German beers at least contain greater amounts of higher aliphatic alcohols than are reported in the literature. A level of 80 to 120 mg/liter of higher alcohol is about normal for German beers, including the top fermented types.

The acceptable quality of top fermented beers confirms to some degree the view that the higher alcohols make a contribution to the character of beer.

Influence of Yeast Strain

Each yeast strain exerts its own special influence on the aroma, flavor and character of beer. To demonstrate this fact, 20 different yeasts from our collection were used to ferment the same wort under identical conditions. The composition of the wort used in this experiment is indicated in **Tables IIa** and **IIb** and shows generally normal values, with a low pH and relatively extensive protein modification.

The analytical data of the carbohydrate composition obtained by means of paper chromatography indicate a favorable composition of the fermentable sugars, *i.e.*, a high pro-

2167

Table IIa
Analysis of Starting Wort

Original Extract, °Plato	12.99
Apparent Extract, %	2.90
Degree of Attenuation, %	77.7
pH	5.03
Bitter Value	29.4 mg/l
Total Protein, N/100 ml	125.3 mg
Coaguable Protein, N/100 ml	1.85 mg
Formol Nitrogen, N/100 ml	36.6 mg

Protein Fractionation according to Lundin

Adjusted to 12% Orig. Ext.	% of Total Protein
Fraction A 21.4 mg	18.8
B 16.2 mg	14.2
C 76.5 mg	67.0
$MgSO_4$-precip. N/100 ml	23.9 mg

Table IIb
Paper Chromatographic Analysis of the Carbohydrates

Total Carbohydrates	11.5 g	% of Total Carbohydrates
Fructose	0.13 g	1.1
Glucose	1.40 g	12.2
Saccharose	0.60 g	5.2
Maltose	6.13 g	53.3
Maltotriose	1.15 g	10.0
Maltotetraose and Dextrins	2.09 g	18.2

portion of readily fermentable sugars, with relatively low levels of maltotriose, maltotetraose and dextrins.

The yeasts included 12 flocculating types and eight powdery types. The pitching rates corresponded to those normally used in practice (0.5 liter/hl). The fermentation temperature was maintained at 8° C. Wort samples were analyzed for acetaldehyde, acetoin, diacetyl and higher alcohols after the first, fifth and ninth day of fermentation.

The by-products of fermentation have already been listed in **Table I** and discussed above. The fermentation tests were designed to disclose the differential influence of various yeast strains on the formation of the by-products. **Table III** shows the dependence of acetaldehyde formation on different yeast strains. Flocculating yeasts produced acetaldehyde contents of 11.4 to 20.8 mg/liter, while powdery yeasts gave levels of 12.1 to 21.6 mg/liter. Although no significant differences between flocculating and powdery yeasts were established, it is apparent that the acetaldehyde formation by individual yeasts differed considerably. This again points to the need for a careful selection of plant yeasts.

The values for acetoin formation are presented in **Table IV.** Here again there is evidence of distinct differences among the individual yeast strains, but not between flocculating and powdery yeasts.

Revealing values of the diacetyl levels found are reported in **Table V.** The negative influence exerted by diacetyl on beer flavor and aroma has been already pointed out. Twofold differences in diacetyl production were observed among individual yeast strains which, when translated into practice, indicate that improper yeast selection may create off-flavors and aromas, especially if no close check

Table III
Dependence of Acetaldehyde Formation on Yeast

Days of Fermentation	Flocculating Yeasts 509	520	531	547	554	556	559	562	564	566	568	548	Powdery Yeasts 504	521	525	555	558	560	561	570
1	7.8	6.7	5.7	9.2	7.4	6.7	4.9	7.6	5.4	7.8	8.4	8.2	5.3	8.6	8.5	7.5	6.6	7.4	7.1	6.4
5	14.3	17.2	15.9	16.7	14.7	15.1	15.2	16.7	13.5	16.2	14.2	12.2	14.0	13.2	14.6	18.2	10.6	14.8	15.7	11.9
9	12.9	16.3	15.3	20.8	14.9	13.6	14.9	15.2	14.6	16.3	11.4	11.4	12.1	17.8	21.6	19.8	12.6	21.0	21.6	12.7
											Range	11.4-20.8						Range	12.1-21.6	

Table IV
Dependence of Acetoin Formation on Yeast

Days of Fermentation	Flocculating Yeasts 509	520	531	547	554	556	559	562	564	566	568	548	Powdery Yeasts 504	521	525	555	558	560	561	570
1	11.69	10.12	7.34	12.08	10.13	13.64	9.50	9.98	7.94	10.69	11.10	14.61	9.64	13.17	12.31	8.92	10.69	13.78	11.53	12.47
5	10.76	16.92	15.36	11.58	11.63	14.78	11.80	10.43	10.58	15.53	11.83	8.47	12.48	14.71	10.50	16.70	6.25	10.40	11.05	17.54
9	8.42	12.15	13.82	9.50	11.42	10.20	11.47	7.86	11.54	12.12	11.54	6.34	5.84	10.62	8.94	14.24	5.36	8.97	10.64	10.53
											Range	6.34-13.82						Range	5.36-14.24	

Table V
Dependence of Diacetyl Formation on Yeast

Days of Fermentation	Flocculating Yeasts 509	520	531	547	554	556	559	562	564	566	568	548	Powdery Yeasts 504	521	525	555	558	560	561	570
1	0.12	0.11	0.09	0.10	0.10	0.11	0.12	0.10	0.10	0.15	0.10	0.12	0.10	0.10	0.11	0.21	0.15	0.15	0.10	0.07
5	0.56	0.37	0.18	0.23	0.30	0.14	0.13	0.16	0.14	0.18	0.22	0.23	0.18	0.15	0.21	0.17	0.32	0.31	0.21	0.21
9	0.34	0.17	0.17	0.12	0.27	0.15	0.34	0.18	0.15	0.24	0.21	0.23	0.27	0.22	0.31	0.19	0.24	0.16	0.19	0.18
											Range	0.12-0.34						Range	0.16-0.31	

Table VI
Dependence of Higher Alcohol Formation on Yeast

Days of Fermentation	Flocculating Yeasts												Powdery Yeasts							
	509	520	531	547	554	556	559	562	564	566	568	548	504	521	525	555	558	560	561	570
1	10.6	32.2	13.4	35.4	19.6	35.6	18.4	31.6	21.0	25.2	34.4	18.4	40.2	15.8	37.8	17.6	22.0	37.2	21.2	40.4
5	45.0	57.6	64.0	78.6	92.0	67.0	75.4	93.4	95.2	70.4	92.0	118.6	58.0	80.4	61.8	49.8	43.4	63.6	61.2	84.4
9	49.4	100.2	73.2	81.4	90.6	79.4	82.4	93.2	95.2	99.0	93.6	121.6	79.6	79.6	75.8	85.4	62.0	83.0	72.8	93.8
										Range		49.4-121.6							Range	62.0-93.8

Table VII
Secondary Fermentation and Storage (Export Beer)

	Freshly Fermented Beer	Storage Time					
		1st week	2nd week	3rd week	4th week	5th week	6th week
Apparent Extract, %	4.05	3.80	3.76	3.67	3.65	3.63	3.62
Acetaldehyde, mg/l	18.4	11.0	10.3	11.2	10.9	10.8	11.1
Acetoin, mg/l	4.48	4.74	3.54	3.60	3.30	3.81	3.76
Diacetyl, mg/l	0.14	0.29	0.12	0.11	0.16	0.15	0.13
Higher Alcohols, mg/l	98.6	91.4	106.2	116.0	115.8	117.4	121.2
Phenylethanol, mg/l	17.0	—	—	—	—	—	16.7

Table VIII
Secondary Fermentation and Storage (Pilsen Type Beer)

	Freshly Fermented Beer	Storage Time				
		1st week	2nd week	3rd week	4th week	5th week
Apparent Extract, %	3.39	3.33	3.24	3.17	3.16	3.15
Acetaldehyde, mg/l	10.3	8.7	8.7	9.0	9.5	9.8
Acetoin, mg/l	5.38	4.05	3.93	3.20	3.56	3.68
Diacetyl, mg/l	0.25	0.35	0.29	0.26	0.11	0.09
Higher Alcohols, mg/l	103.6	104.0	103.8	104.0	105.8	109.6
Phenylethanol, mg/l	16.9	—	—	—	—	15.2

is exercised over the diacetyl content of the beer.

The diacetyl content is further adversely affected by a lagging or incomplete secondary fermentation, as this fails to achieve a sufficient reduction of the diacetyl. The result is a deterioration in beer flavor of undetected origin.

It was further shown that diacetyl formation is strongly dependent on the yeast strain. While no distinct quantitative differences in diacetyl formation by flocculent and powdery yeasts could be established, it does appear as though the powdery yeasts yielded somewhat higher values than the flocculent yeasts, which may be due to their more active metabolic rate.

Table VI shows that the total content of higher alcohols is also dependent on the yeast strain. However, the fluctuations among the different strains are not as pronounced here as in the case of the other fermentation by-products. On the other hand, the difference between flocculating and powdery yeasts is quite distinct. Thus, the total content of higher alcohols in freshly fermented beer is greater with flocculating yeasts than with powdery yeasts, pointing to the conclusion that the higher alcohol content of finished beer that has been fermented with a powdery yeast will also be lower. The make-up of the higher alcohols in powdery yeast beers must nevertheless be less favorable than in flocculating yeast beers as we know from practice that the former beers are often characterized by a harsh flavor. Only gas chromatographic separation of the individual components can clarify this problem. In the experimental fermentations the content of higher alcohols of freshly fermented beers fluctuated between 49.4 to 121.6 mg/liter for the flocculating yeasts, and 62.0 to 93.8 mg/liter for the powdery yeasts.

Secondary Fermentation/Aging

Our consulting work with various breweries has frequently involved a study of the secondary fermentation and aging of beer, especially with reference to changes in fermentative by-products.

The analytical data obtained in a brewery on changes occurring in organoleptically important constituents in an export beer and a Pilsener type beer from the time of transfer to the end of storage are given in **Tables VII and VIII**. The original extract of the export beer (**Table VII**) was about 13.4 per cent and that of the Pilsener beer 11.9 per cent. The worts received normal treatment, *i.e.*, they were freed of the hot trub and fermented for eight days with a flocculating yeast at a maximum temperature of 8.1° C. Storage was at 1.0° C. for the first two weeks, and then at 0.5 to 0.2° C., with a bung pressure of 0.39 atm. (See **Table VIII.**)

The freshly fermented beer had an apparent extract of 4.05 per cent which decreased to 3.62 per cent after six weeks of storage. The sharpest decline in extract occurred shortly after beer transfer, *i.e.*, during the first week of storage. We continually find this in storage. This rapid loss in extract is explained by the agitation and air uptake by the beer during transfer. After three weeks of storage there was practically no decrease in extract and the difference from the end of fermentation was only 0.6 per cent.

The acetaldehyde content also exhibited its sharpest decline during the first week of storage, *i.e.*, shortly after transfer, and remained practically constant thereafter. Since acetaldehyde represents a typical green-beer bouquet substance, its marked decrease at the beginning of storage

Table IX
Comparison of Freshly Fermented and Finished Beers of Export and Pilsener Types

	Export Type Beer		Pilsener Type Beer	
	Freshly Fermented	Packaged	Freshly Fermented	Packaged
Acetaldehyde, mg/l	18.4	16.4	10.3	9.8
Acetoin, mg/l	4.48	2.66	5.38	2.63
Diacetyl, mg/l	0.14	0.13	0.25	0.16
Higher Alcohols mg/l	98.6	122.4	103.6	111.0
Phenylethanol, mg/l	17.0	16.9	16.9	13.0

may be attributed to its being swept from the beer by evolving carbon dioxide gas.

Acetoin decreased perceptibly during the first four weeks, and then maintained a constant level during the remaining two weeks. The reduction in diacetyl was quite small, which is probably attributable to the relatively feeble secondary fermentation as exemplified by an apparent extract loss of only about 0.2 per cent. Typical again was the oxidation-induced increase in diacetyl shortly after beer transfer.

The higher alcohols showed an increase of 22.6 mg/liter throughout the storage period. Their increase during secondary fermentation and aging varied with the two beers, but the basic tendency was a rising one.

Among the individual higher alcohols in the beers under discussion, n-propanol, an organically harmless component increased by 2.3 mg/liter, and isobutanol, which has a detrimental effect on flavor, by 2.4 mg/liter. n-Butanol occurred only in trace amounts at both the start and finish of the secondary fermentation. A more marked increase of 4.7 mg liter was noted with optically active amyl alcohol, while iso-amyl alcohol showed the greatest increase with 13.2 mg/liter. Increased levels of iso-amyl alcohol generally exert a detrimental effect on beer quality.

Beta-phenyl ethanol was the only aromatic alcohol we measured and its value remained essentially unchanged throughout the post-fermentation period.

An interpretive evaluation of the tabulated data relating to the aging of beer indicates that practically no fermentation took place during the final three weeks, but that there was an undesirable increase in the fermentation by-products. From this it can be concluded that the optimum period of storage was exceeded and that the duration of lagering cannot serve as a criterion of beer quality. On the contrary, excessively extended storage of beer may lead to a deterioration of its flavor and of its quality.

The data presented for the Pilsener beer (**Table VIII**) follow a course similar to that for the export beer. Here again, however, the secondary fermentation was rather feeble as evidenced by an extract loss of only 0.24 per cent over a five week period. The acetaldehyde showed a very minor decrease, whereas both acetoin and diacetyl showed a distinct decline, though only up to the third and fourth weeks.

The higher alcohols were present in somewhat greater amounts in freshly fermented Pilsener beer than in the export beer. On the other hand, the increase during storage amounted to only 6.0 mg/liter for the Pilsener beer as against 22.6 mg/liter for the export beer. For the same storage period, i.e., five weeks in this instance, the export beer showed an increase of 18.8 mg/liter. The value for beta-phenyl ethanol in the Pilsener beer also declined only slightly during storage.

Comparison of Beers

Table IX presents a comparison of freshly fermented and the corresponding finished beers of both the export and Pilsener types.

The acetaldehyde content decreased more markedly in the export than in the Pilsener beer. In the bottled export beer the acetaldehyde level was slightly on the high side, while in the Pilsener beer it was normal, when compared to the average value of 9.4 mg/liter for German bottled beer found by us in numerous analyses. Bärwald (3) has reported a value of 10 to 11 mg/liter for bottom fermented German beers which corresponds to our findings.

Acetoin declined during storage by 1.82 mg liter in the export beer, and by 2.75 mg in the Pilsener beer. It remained constant in bottled beers, and may quantitatively be considered as normal and even favorable, since we established an average value of 3.01 mg in 120 assays carried out on bottom fermented beers with an original extract of 12 to 13 per cent.

The diacetyl content decreased during storage, in this instance more extensively in the Pilsener than in the export beer. The levels of 0.13 and 0.16 mg liter respectively, of the bottled beers lie somewhat above the average value of 0.11 mg which we have found in bottom fermented beers. The latter value corresponds with the findings of Gjertsen, Undstrup and Trolle (4). In the beers under discussion the higher diacetyl content may have been due to the somewhat lagging secondary fermentation.

Our findings are in disagreement with the conclusion of Drews, Specht and Trenel (5) that an extended storage period will produce the most favorable diacetyl values since our observations are that a low diacetyl content in conventional brewing is dependent primarily on the selection of suitable yeast strains, proper fermentation temperatures and a vigorous secondary fermentation, but is independent of the storage time. This is further supported by the fact, for example, that beers produced by fermentations which are carried out under pressure and consequently attain more closely the limit of attenuation nearly always show lower diacetyl levels than do conventionally produced beers. This obviates a continuing check of diacetyl content.

The higher alcohols showed in part a distinct increase during storage, with the final levels being higher in the export beer than in the Pilsener beer. Normal values, as pointed out earlier, have been shown by our investigations to lie within the range of 80 to 120 mg/liter.

Beta-phenyl ethanol remained unchanged during the secondary fermentation. The values of 16.9 and 13.0 mg/liter found, respectively, in the export and Pilsener beers are low and favorable. In 20 bottom fermented beers which we have analyzed, levels of 13.0 to 36.0 mg/liter were encountered. These values correspond to those found by Ayräpää (6) who reported a range of 10 to 40 mg/liter for various beers.

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2175

J. Biol. Chem. 161: 495-502, (1945)

A COLORIMETRIC DETERMINATION OF BLOOD ACETOIN*

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(Received for publication, September 11, 1945)

Most of the methods described for the determination of acetoin and biacetyl are based on the original Lemoigne-van Niel procedure (1, 2), which depends upon the formation of insoluble nickel dimethylglyoxime when biacetyl is treated with hydroxylamine in the presence of a nickel salt; acetoin is similarly determined after oxidation to biacetyl by heating with ferric chloride. In a recent modification Stotz and Raborg (3) have employed a colorimetric determination of the nickel in the precipitate instead of the usual gravimetric analysis, and the sensitivity of the method has thereby been greatly increased. A polarographic method for the determination of acetoin and biacetyl has also been described by Greenberg (4).

The method herein described was developed because none of the previous methods was sufficiently sensitive for our purpose; its chief advantages are simplicity and sensitivity. It is based on the color reaction originally discovered by Voges and Proskauer (5), and later shown by Harden and Norris (6, 7) to be due to the reaction between biacetyl and a guanidino group in the presence of alkali. Attempts to increase the sensitivity of this reaction led to the addition of creatine by O'Meara (8) and α-naphthol by Barritt (9). While the original Voges-Proskauer reaction could not be developed into a satisfactory quantitative method, the Barritt modification was found to be suitable. In this laboratory it was first tried by W. E. Knox, and a similar method has recently been described by Eggleton *et al.* (10). The chemical reactions involved in the development of the red color have been studied (10–12) but not fully elucidated.

Details of the procedure for color development and the method for determining acetoin in blood are described.

Colorimetric Determination of Acetoin and Biacetyl

Reagents—

0.5 per cent creatine; 1 gm. of creatine dissolved in 200 cc. of water.

5 per cent α-naphthol; 1 gm. of powdered colorless α-naphthol (redistilled under nitrogen) dissolved in 20 cc. of 2.5 N NaOH. The solution is prepared *immediately* before using.

Method—To 5 cc. of solution containing between 1 and 12 γ of acetoin or

* This study was supported by a grant from the Nutrition Foundation, Inc.

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biacetyl are added consecutively 1 cc. of 0.5 per cent creatine and 1 cc. of the 5 per cent α-naphthol solution. The latter reagent should not be prepared until after the creatine has been added to the test solution, and it is used as soon as the α-naphthol dissolves. The color is allowed to develop at room temperature for exactly 10 minutes when biacetyl is being determined, and 1 hour for the determination of acetoin. The amount of color is then read in an Evelyn photoelectric colorimeter with a 540 filter; a reagent blank prepared simultaneously with 5 cc. of water is used to adjust the galvanometer to 100, and a standard solution containing 5 γ of acetoin or biacetyl can be included in each series of analysis.

The colors obtained by this method were reproducible but somewhat unstable. The maximum color was obtained with biacetyl between 5 and 10 minutes after the addition of the alkaline α-naphthol; fading was barely perceptible on the galvanometer at 15 minutes, and it amounted to a 10 per cent error at 30 minutes. The color obtained with acetoin was more stable; the maximum intensity was reached in 60 minutes after the addition of the alkaline α-naphthol; fading could not be detected at 75 minutes, and was barely perceptible at 90 minutes. The absorption spectra of the colors obtained with biacetyl and acetoin were quite similar. The peak of absorption occurred at 530 mμ, and both curves were relatively flat between 520 and 540 mμ.

Maximum color was obtained with creatine, but other compounds containing the guanidino group (arginine, creatinine, guanidine carbonate, methylguanidine sulfate) gave similar colors in the presence of biacetyl, α-naphthol, and alkali. α-Naphthol could not be replaced advantageously by any one of a large number of substances tested, and it was without effect if added to the test solution of creatine and biacetyl after the alkali.

It was found convenient, but not essential, to dissolve the α-naphthol in the alkali under a stream of nitrogen, since the reagent became colored readily in air. Only freshly prepared reagent, containing no more than a faint yellow color, was used. Less consistent results were obtained when the α-naphthol was dissolved in 95 per cent alcohol and added separately from the alkali.

Standard Curves—A standard solution of biacetyl containing approximately 1 mg. per cc. was prepared from the fraction boiling sharply at 88°. A similar solution of acetoin was prepared from the crystalline polymer that was equally well purified by thorough washing with anhydrous ether or by fractional distillation. Both standards remained unchanged for several months at 6°, after which the biacetyl solution deteriorated. Suitable dilutions were prepared from each of these standards, so that 5

* The acetoin and biacetyl were obtained from the Loudol Corporation, Buffalo, New York.

2175

cc. contained from 1 to 12 γ of acetoin or biacetyl. Colors were developed in a series of such dilutions and read against the reagent blank in the photoelectric colorimeter after 10 minutes for the biacetyl determination and 1 hour for acetoin. The standard curves for both acetoin and biacetyl, shown in Fig. 1, were linear for amounts of acetoin and biacetyl below 5 to 6 γ, but departed slightly from this relationship with larger amounts.

Oxidation of Acetoin to Biacetyl

Reagents—

$FeSO_4 \cdot 7\ H_2O$ (solid).

50 per cent $FeCl_3 \cdot 6\ H_2O$; 500 gm. of $FeCl_3 \cdot 6\ H_2O$ dissolved in water and diluted to 1 liter.

10 N H_2SO_4; 280 cc. of concentrated H_2SO_4 diluted to 1 liter.

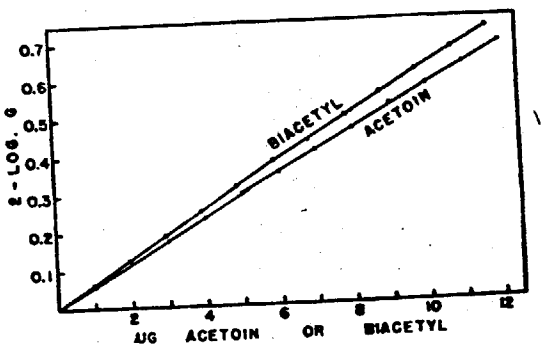

FIG. 1. The relationship of color intensity at 540 mμ to the amount of biacetyl or acetoin tested.

When acetoin cannot be determined directly because of interfering substances, a preliminary oxidation to biacetyl and distillation of the latter can be employed; smaller amounts can also be determined by this procedure, since the biacetyl can be concentrated during the distillation. The determination of total acetoin plus biacetyl in mixtures of the two likewise depends upon this preliminary oxidation.

The usual method of carrying out this oxidation with ferric chloride gave 85 to 90 per cent of the theoretical amount of biacetyl; this has been the common experience (13–15). By using a mixture of ferric chloride and ferrous sulfate, as suggested by Kniphorst and Kruisheer (15), the yields were substantially improved, and the following method was adopted.

The oxidizing reagent is prepared by dissolving 10 gm. of $FeSO_4 \cdot 7\ H_2O$ in 30 cc. of 50 per cent $FeCl_3$. 2 cc. of this solution and 1 cc. of 10 N H_2SO_4

are added to 7 cc. of the acetoin-containing solution. The tube is stoppered lightly with a clean rubber stopper and placed in a boiling water bath for 30 seconds to relieve excessive pressure before the stopper is inserted tightly. After heating for 30 minutes in a boiling water bath, the tube is cooled and the contents rinsed into a small distillation flask described by Stotz (16). A glass bead is added and the solution distilled carefully to avoid obtaining any trace of iron salt in the distillate by bumping or spraying. 5 cc. of distillate are collected in a 10 cc. graduated cylinder, and the contents made up to 5.5 or 10 cc., depending upon the amount of biacetyl expected. The color reaction for biacetyl is carried out with 5 cc. of this solution or an aliquot diluted to 5 cc.

TABLE I

Oxidation of Acetoin to Biacetyl

Acetoin oxidized	Biacetyl in aliquot analyzed		Recovery
	Recovered	Theory	
γ	γ	γ	per cent
2	1.82	1.78	102
4	3.66	3.56	103
6	5.22	5.34	98
8	6.86	7.12	96
10	8.72	8.90	98
15	7.20	7.34	98
25	4.83	4.89	99
35	6.67	6.84	98
50	4.60	4.89	94
80	7.50	7.83	96
100	9.35	9.78	96
140	5.30	5.48	97
250	4.87	4.89	100
500	4.83	4.89	99

The recovery of biacetyl from the oxidation of acetoin by this method is shown in Table I. 98 per cent of the theoretical amount was obtained from 2 to 500 γ of acetoin; larger amounts were not tested.

Specificity—The original Voges-Proskauer reaction is nearly specific for acetoin and biacetyl. The related 5-carbon ketol gives a somewhat similar color, while the 6-carbon ketol gives a light olive-green color within the usual time limit employed (17). In the test herein described, approximately 90 γ of methylglyoxal gave as much color as 1 γ of biacetyl, while 1 mg. of glyoxal gave no color. 1 mg. of benzil and benzoin in alcohol gave a slight color equivalent to approximately 1 γ of biacetyl and 0.5 γ of acetoin respectively.

2175

The nickel dimethylglyoxime precipitation is probably more specific for biacetyl than this color reaction. 1.8 mg. of methylglyoxal gave no precipitate in the procedure described by Stotz and Raborg (3). The introduction of α-naphthol into the reaction also increases the possibility of obtaining colors from unrelated substances that react with the α-naphthol alone. Such interference, as yet unknown, could readily be detected by omitting the creatine from the test.

Blood Acetoin

Method of Determination—A 1:5 tungstic acid filtrate of blood is prepared by adding 2 volumes of water, 1 volume of blood, 1 volume of 10 per cent

TABLE II

Recovery of Acetoin Added to Blood

Acetoin added	1 cc. blood	5 cc. blood	25 cc. blood
γ	*per cent*	*per cent*	*per cent*
1		101	95
2		100	92
3		105	96
5	102	95	92
7	102		89
10	100	93	88
15	97	97	85
25	96	95	
50	96	98	
100	95	101	
250	97	100	
500	95	99	

The acetoin was added to the water with which the blood was laked during the preparation of a protein-free filtrate. The filtrate was analyzed for acetoin by the method described. The percentage recovery includes a correction for the 0.012 to 0.015 mg. per cent of acetoin found in the blood to which the acetoin was added.

sodium tungstate, and 1 volume of $\frac{2}{3}$ N H_2SO_4; the precipitate is centrifuged off. An aliquot of the supernatant is added to solid NaCl (1 gm. per 3 cc.) and distilled from a Claisen flask practically to dryness *in vacuo* (150 to 200 mm. of Hg). The distillate is oxidized as previously described by adding approximately 2 cc. of the $FeCl_3$-$FeSO_4$ mixture and 1 cc. of 10 N H_2SO_4 to each 7 cc. of distillate, and heating in a stoppered tube for 30 minutes. The solution is then redistilled, and the biacetyl is collected in the first 5 cc. of distillate and estimated colorimetrically.

The recoveries of added acetoin obtained by this procedure are shown in Table II. For the accurate analysis of the amount of acetoin occurring in normal blood it is necessary to use the maximum amount of filtrate that can

be obtained from a relatively large sample of blood, *e.g.* 15 to 25 cc. When the blood acetoin is elevated slightly, a satisfactory analysis can be run on the filtrate obtained from 5 cc. of blood.

In developing this method for the determination of acetoin in blood, various procedures were tested for the completeness of recovery of added acetoin, and were accordingly developed further or discarded. Direct color development with a tungstic acid or zinc hydroxide filtrate of blood could not be employed because of the appearance of a yellow-brown color during the reaction. When the blood filtrate was oxidized directly with the iron salts previously described and the biacetyl obtained in the distillate was determined colorimetrically, low recoveries were obtained. This was due primarily to the presence of urea in the blood filtrate; urea reacted with the biacetyl, resulting from the oxidation of acetoin, to form a diureide (18, 19). In order to avoid this type of interference by urea (and to a lesser extent by

TABLE III

Formation of Biacetyl from Glucose by Acid Hydrolysis and Subsequent Oxidation

Heating prior to oxidation	Biacetyl formed		
	1.2 N H_2SO_4	2.2 N H_2SO_4	4.2 N H_2SO_4
hrs.	γ	γ	γ
0.5	0.44	0.97	3.14
1	0.82	1.87	4.35
2	1.63	3.89	8.30

9 mg. of glucose in approximately 15 cc. of the indicated acid were heated in a stoppered tube in boiling water for the indicated time. After cooling and adding 4 cc. of the $FeCl_3$-$FeSO_4$ solution, the tube was restoppered and heated an additional 30 minutes. The biacetyl was then distilled and determined colorimetrically.

uric acid and allantoin) the acetoin was first distilled from the filtrate and subsequently oxidized to biacetyl for the purpose of concentrating the latter.

Artifacts in Acetoin Analyses—During the studies incidental to developing the method for determining blood acetoin, it was observed that relatively large amounts of biacetyl could be obtained from a blood filtrate by subjecting the latter to an acid hydrolysis prior to oxidation with the $FeCl_3$-$FeSO_4$ mixture. Most of this newly formed biacetyl was found to be derived from glucose, and was unrelated to the preexisting blood acetoin. Although the existence of a bound form of acetoin in blood cannot be denied categorically, its existence in appreciable amounts is unlikely, since neither the blood filtrate nor glucose gave rise to any large amount of biacetyl when the oxidizing mixture of $FeCl_3$-$FeSO_4$ was present throughout the hydrolysis.

Table III shows the amount of biacetyl formed from crystalline gluco

2175

under various conditions of acid hydrolysis and subsequent oxidation with the iron salts. The identity of the biacetyl formed from glucose was established by the following experiments. (*a*) No color was obtained in the biacetyl analysis if either creatine or α-naphthol was omitted from the reaction (the small amounts of biacetyl tested could not give a Voges-Proskauer reaction in the absence of α-naphthol). (*b*) 750 γ of biacetyl were obtained by heating 1 gm. of glucose in 50 cc. of 10 N H_2SO_4 for 6 hours, diluting, and oxidizing with the $FeCl_3$-$FeSO_4$ solution; the biacetyl was concentrated to a 5 cc. volume by two distillations, and a copious red precipitate characteristic of nickel dimethylglyoxime was obtained when this solution was treated with hydroxylamine in the presence of a nickel salt (3). (*c*) 960 γ of biacetyl were similarly obtained after heating 1 gm. of glucose in 200 cc. of 4 N H_2SO_4 for 6½ hours, and the concentrated solution of biacetyl was allowed to react with 2,4-dinitrophenylhydrazine in 2 N HCl. The precipitate was centrifuged, washed with water and alcohol, and recrystallized from nitrobenzene. The crystals were identical with the bis-2,4-dinitrophenylhydrazone of biacetyl in appearance and melting point (315°), and showed no depression on mixing with an authentic sample.

Pyruvate and lactate gave rise to small amounts of biacetyl when they were treated by the procedure used in oxidizing acetoin to biacetyl. A solution of 100 mg. of sodium pyruvate in 7 cc. of water, 1 cc. of 10 N H_2SO_4, and 2 cc. of the $FeCl_3$-$FeSO_4$ mixture was heated in a stoppered tube for 1 hour and then distilled; 75 γ of biacetyl were obtained in the distillate, and its identity substantiated by conversion to the red precipitate characteristic of nickel dimethylglyoxime. A similar treatment of 100 mg. of lactic acid yielded 2.4 γ of biacetyl, while 100 mg. of alanine gave none.

The preliminary distillation of acetoin from the blood filtrate was necessary in order to eliminate interference by substances that combined with biacetyl, as well as those that gave rise to it during the chemical reactions incidental to its determination. We cannot be certain that the small amount of acetoin found normally in blood was not an artifact due to the distillation of some other substance that gave biacetyl when treated with acid $FeCl_3$-$FeSO_4$. That the chromogenic material obtained from blood was actually biacetyl was indicated by the absence of color when creatine or α-naphthol was omitted from the test, and the formation of the characteristic red precipitate when the biacetyl obtained from 680 cc. of beef blood was treated with hydroxylamine and a nickel salt. Enrichment of the blood with 10 mg. per cent of sodium pyruvate, 10 mg. per cent of acetaldehyde, 300 mg. per cent of glucose, or 200 mg. per cent of alcohol did not alter the amount of biacetyl obtained in the analysis.

It is probable from these studies that the major part of the acetoin previously identified in normal blood (20) was actually formed from some other

constituent, such as pyruvate, during the chemical reactions involved in the isolation procedure. The relatively large amount of acetoin previously reported (21) in normal blood is undoubtedly an artifact for the same reason.

Normal Blood Acetoin. The acetoin concentration in freshly drawn blood from the rat, dog, cat, fasted rabbit, pig, and cow varied from 0.005 to 0.015 mg. per cent, with most of the values falling between 0.007 and 0.012 mg. per cent. Pigeon blood averaged about 0.03 mg. per cent. The concentration of acetoin in beef plasma was approximately 80 per cent of that observed in whole blood; when the acetoin concentration was elevated to 0.365 mg. per cent in rat blood by the administration of acetaldehyde, the plasma contained 0.113 mg. per cent.

SUMMARY

A method has been described for the colorimetric determination of acetoin in blood. Possible interference from several constituents of normal blood was described.

The normal blood concentration of acetoin in most of the species studied was approximately 0.01 mg. per cent.

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2175

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THE VOLATILE COMPONENTS OF WHITE BREAD PREPARED BY A PRE-FERMENT METHOD[1]

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Cereal Chem. 41 (4) 300-15 (1964)

ABSTRACT

Investigation of the volatile components of bread prepared by a pre-ferment technique resulted in rigorous identification of ethanol, n-propanol, isobutanol, isoamyl alcohol, acetoin, furfural, acetic acid. and ethyl acetate. On the basis of gas chromatographic retention data, propanal, 2-butenal, 2-ethylhexanal, acetone, diacetyl, and 2-methylbutanol-1 were tentatively identified. On the basis of retention data from both thin-layer and flash-exchange chromatography, formaldehyde, acetaldehyde, propanal, n-butanal, isobutanal, n-pentanal, 3-methylbutanal, acetone, 2-butanone, 2-pentanone, and 2-hexanone were tentatively identified. Evidence was obtained for the presence of approximately 17 completely unknown trace components in addition to those listed above. Modification of dough composition by addition of 1,000 p.p.m. of L-proline caused no detectable change in composition of volatile components, in spite of the fact that proline-treated bread was preferred on the basis of its aroma. Odor-concentrates prepared from aqueous distillates amounted (ethanol-free basis) to 3–5 p.p.m. of the control bread, and to 8–10 p.p.m. of the proline-treated bread. Components of the odor concentrates were in all cases those listed above. The five alcohols, acetoin, furfural, and acetic acid were by far the major components. Carbonyl compounds were trace components.

Identification of the volatile components of fresh bread has been the goal of many investigations (1–6). Volatile components of fresh white bread prepared by conventional procedures have been shown by Visser't Hooft and deLeeuw (7), Baker *et al.* (8), Wiseblatt and Kohn (9), and Ng, Reed, and Pence (10) to be ethanol, formaldehyde, acetaldehyde, 2-butenal, 2-methylbutanal, n-hexanal, 2-ethylhexanal, furfural, pyruvaldehyde, 2-propanone, 2-butanone, 2-hexanone, 3-heptanone, diacetyl, acetoin, ethyl pyruvate, ethyl levulinate, acetic acid, n-butanoic acid, and 3-methylbutanoic acid. n-Pentanal and 2-methylpropanal have been identified in oven vapors and are presumably volatile bread components. These results were obtained by isolation and separation of single groups of compounds, i.e., acids, alcohols, or 2,4-dinitrophenylhydrazones (2,4-DNPH's) of carbonyl compounds.

Much interest has also been shown in components (both nonvolatile and volatile) of pre-ferments (11–16) and of doughs prepared from pre-ferments (17). Acetic and lactic acids and ethyl acetate were measured

[1]Manuscript received May 13, 1963. Contribution No. 539 from Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge 39, Mass. The research on which this paper is based was conducted under contract with the U.S. Department of Agriculture and under the authority of the Research and Marketing Act of 1946. The contract was supervised by the Western Utilization Research and Development Division of the Agricultural Research Service.

[2]Kitchens of Sara Lee, Chicago, Ill.

(11), as were ethanol, total carbonyls, and total organic acids (14,15) in pre-ferments. With the exception of the work of Smith and Coffman (12), who isolated and identified a variety of neutral components of pre-ferments, all other investigations (13,16) were directed toward isolation and separation of carbonyl compounds by formation and separation of 2,4-DNPH's.

This paper describes an investigation of the volatile components of fresh white bread prepared by a pre-ferment method.

To determine the effect of modification of pre-ferment dough composition on the identity and quantity of these volatile components, bread to which L-proline had been added, and which was preferred on the basis of its aroma, was also studied. Since other investigators (2) of bread prepared by the pre-ferment method had isolated only the carbonyl components by 2,4-DNPH formation, it was considered of value to attempt to isolate all volatile bread components in an odor concentrate, to separate them by gas chromatography, and to identify the individual substances thus obtained.

Materials and Methods

Bread-Baking. Bread was prepared by standardized [illegible] procedure using the formulas (18) given in Table I. The pre-ferment

TABLE I

COMPOSITION OF PRE-FERMENT AND DOUGH

INGREDIENTS	QUANTITY	INGREDIENTS	QUANTITY
	g.		g.
Pre-ferment		Dough	
Water (sufficient for 1 liter final volume)		Flour[a]	700
Sucrose	66.0	Sucrose	42
Yeast	44.0	Shortening	21
Sodium chloride	11.0	Sodium chloride	10.5
Diammonium hydrogen phosphate	4.0	Yeast	7
Calcium carbonate	1.0	Pre-ferment solution (ml.)	220
Potassium sulfate	1.0	Water (ml.)	128[b]
Magnesium sulfate	0.5		
Potassium bromate	0.05	Total weight: 1,228.5 g.	
Potassium iodate	0.02		

[a] At 14% moisture.
[b] For 64% absorption.

liquid was stirred and maintained at 86° ± 1°F. for 4 hr. Appropriate aliquots of the pre-ferment broth and an aqueous suspension of yeast, and of solutions of sucrose and sodium chloride, were added in that order to the flour and mixed. The dough was proofed at 9[illegible]°F. and 90% relative humidity and baked at 425° ± 2°F. for 25 min.

2185

Dough composition was modified by addition of an arbitrarily chosen known amount of the following compounds. L-Arginine, gliadin, casein, and lactalbumin were added to the flour and mixed well before addition of the pre-ferment liquid. DL-Alanine, L-alanine, L-histidine, L-proline, L-leucine, DL-valine, L-valine, maltose, and lactose were added in solution after the pre-ferment was added. Preliminary work indicated that L-leucine added at a level of 500 p.p.m. or 50 mg./100 g. of dough weight could be detected by the panel. All other amino acids were arbitrarily added at 1,000 p.p.m. or 100 mg./100 g. dough weight. Maltose (1.79 g./100 g. dough), lactose (1.79 g./100 g. dough), casein (1.22 g./100 g. dough), lactalbumin (0.24 g./100 g. dough), and gliadin (1.22 g./100 g. dough) were added at levels approximating those which would be found if nonfat dry milk had been used in the dough formula. Amounts were calculated on the basis of the composition of nonfat dry milk (19) and amounts used in the ADMI Pre-ferment Formula (18).

Sensory Evaluation of Bread. Each batch was allowed to cool and was sliced, and the slices were separated into portions of crust and of crumb. Samples of crust and of crumb from control and additive-treated batches were compared in triangle tests. Evaluations were carried out as soon after completion of baking as possible (within 4 hr.). Panel members were students or staff members of the Department who were accustomed to taking part in sensory evaluations. The odor and taste of samples of crust and crumb were evaluated separately. Judges were asked which sample was (or samples were) preferred. Differences significant (20) at 5% level or less were noted.

Chemical Investigation of Volatile Components of Bread. Preparation of Flavor Distillates. Preparation of distillates which truly represented bread flavor and aroma depended upon removal under mild conditions of all volatile flavor and aroma components from as large a quantity of bread as possible. The procedure developed is similar to that described by Wiseblatt and Kohn (9).

Freshly baked warm bread (approximately 13 lb.) was sliced mechanically and placed on perforated aluminum trays inside an 18 by 18 by 1/8-in. pressure cooker. The top of the cooker was fitted with a vacuum gage and an outlet which led through Tygon[3] tubing and a condenser to a series of traps, a McLeod gage, a manostat, and finally to an efficient vacuum pump. The condenser was cooled with cold (30° ± 2°F.) ethanol. A large trap (3-liter) was immersed in a dry ice-ethanol bath, three additional traps (1-liter) were chilled in dry ice-

[3]Reference to a company or product by name does not imply approval or recommendation of the product by the Department of Agriculture.

ethanol, and three traps were chilled in liquid nitrogen. All connections were ball-and-socket glass joints except for the 6-in. length of Tygon tubing.

Immediately after the bread was placed in it, the cooker was closed, steam was passed through copper coils lining it, and distillation was carried out at 14–28 mm. Hg. Temperature within the cooker did not rise above 30°C. After about 8 hr. approximately 40% of the crumb had become dry. Pieces which still contained moisture were moved to the upper trays and distillation was continued for 8 more hr. until all the crumb was dry; it was ground and placed in the cooker, and distillation was carried out at 1 mm. Hg for approximately 1 hr. The resulting dry residue was odorless. However, typical bread odor could be regenerated in it by the addition of water.

The oxidizable carbon content (21) of the various distillates provided an index of the amount of organic matter present in each condensate and, in addition, allowed a rough comparison of the total yield of volatile substances obtained from each bread preparation. Almost no liquid condensate was collected in the liquid nitrogen traps during any of the distillations. The contents of each of these traps were treated with 10 ml. of 2*M* 2,4-dinitrophenylhydrazine hydrochloride.

The ethanol content of the distillates was determined by gas chromatography of 1-μliter samples on a Perkin-Elmer F. column (tetraethylene glycol dimethyl ether), 2 meters × 1/4 in., at 61°C. A standard curve prepared by plotting ethanol peak height against ethanol concentration for a number of known solutions of aqueous ethanol allowed analysis of the distillates. As noted by Wiseblatt and Kohn (9) in their work, colorimetric estimations of total carbonyls and esters in the distillates were not reliable.

Isolation of Odor Concentrates. Odor concentrates were obtained by saturation of the various distillates with sodium chloride, extraction with diethyl ether (1 liter ether:1 liter distillate) and concentration of the dried ether extract. Concentration was carried out in preparations 2, 3, and 4 by nitrogen entrainment and in subsequent preparations by fractional distillation. In the first case, the ether removed was collected in a solution of 2*M* 2,4-dinitrophenylhydrazine hydrochloride. Formation of a precipitate indicated that losses of carbonyls had occurred.

Concentration of ether extracts by distillation in a 0.5 × 9-in. column packed with glass helices was accomplished with minimal losses as determined by intermittent gas-chromatographic analysis of the ether recovered, on a column 2 m. × 2 mm. i.d., of 5% β,β'-oxy-

2185

dipropylnitrile on Gas Chrom A (100–120-mesh) at 46°C. in a Research Specialties Model 60-1 Flame Ionization chromatograph. The presence of ethanol was always detected. During the last stages of concentration trace amounts of components which had retention times in agreement with those of n-propanol, isobutanol, and isoamyl alcohol were detected in the ether distillate. These losses are not believed to have been significant.

Gas-Chromatographic Separation of the Components of Bread Odor Concentrates. A column 2 m. × 4 mm. i.d. of 20% Quadrol (tetrakis (2-hydroxypropyl) ethylene diamine) on firebrick (42–60-mesh), operated at 103°C., at a flow rate of 75 ml./min. and a helium pressure of 10 p.s.i.g., gave the best separation of the bread odor concentrates. The chromatograph used was constructed in our laboratory. Its detector contained matched 100,000-ohm thermistors.

Since the Quadrol column was alkaline and would irreversibly absorb acidic components, the odor concentrates were also separated on a column 2 m. × 8 mm. i.d. of 20% Ucon LB 1715 on chromosorb (30–60-mesh), operated at 101°C., flow rate 100 ml./min., helium pressure 4 p.s.i.g. A column 2 m. × 8 mm. i.d. of 20% DEGS (diethyleneglycol succinate) on acid-washed chromosorb (60–65-mesh), maintained at 60°C., flow rate 150 ml./min., helium gas pressure 10 p.s.i.g., was used to gain evidence for 2-methylbutanol-1 in the odor concentrates. It was known to separate this compound from isoamyl alcohol at these conditions. Separation of odor concentrates was also carried out on a column 2 m. × 2 mm. i.d. of 5% β,β'-oxydipropylnitrile (OPN) on Gas Chrom A (100–120-mesh) at 43°C. in a Research Specialties Model 60-1 Flame Ionization chromatograph.

Identification of the Components of Odor Concentrates. The fractions obtained by separation on the above three columns were trapped at the column exit in U-tubes chilled in liquid nitrogen. Their identification was accomplished if their infrared spectra were identical with spectra of known pure reference compounds and if their retention data were in agreement with those of the reference compounds. Retention times were in all cases calculated relative to isobutanol. Reference spectra were obtained from known compounds after purification by gas chromatography. To obtain a large enough quantity of any fraction (except the ethanol fraction) for spectrum analysis it was necessary to collect from at least three successive 40-μliter samples of concentrate. Even so, pure liquid spectra were not obtainable and it was necessary to add carbon tetrachloride (about 1 μliter) to fill the infrared cell. Spectra were obtained with a Beckman IR-5 spectrophotometer fitted with a 5X KBr beam condenser. Type D sodium chloride cells

(Connecticut Instrument Co.) having 0.05-mm. nominal path length were used.

The purity of individual trapped fractions was determined by rechromatography on either a column 2 m. × 2 mm. i.d. of 1% Ucon 50 HB 2000 on 100- to 120-mesh Gas Chrom A at about 36°C. or on the 5% β,β'-OPN column at 50°C. Flame ionization detectors in an Aerograph Model A-500B and a Research Specialties Model 60-1 were used. Columns and conditions were selected on the basis of their ability to separate compounds shown by other investigators (8,9,10) to occur in bread. With the exception of ethanol, which was easily trapped in large volumes, the carbon tetrachloride solutions used for infrared spectrum determinations of each fraction were rechromatographed.

Investigation of Acidic Constituents of Bread-Odor Distillates. Since acidic constituents might not have been completely removed by ether extraction, the odor distillates were lyophilized (to remove sodium chloride) and the resulting distillates made just alkaline. The alkaline solutions were lyophilized and the residue was isolated. Free acids were regenerated from sodium salts by the procedure of Hunter, Ng, and Pence (14) and separated on a column 2 m. × 4 mm. i.d. of 5% LAC IR-296 on acid-washed firebrick (60- to 80-mesh) at 125°C. The procedure was established by separation of a known mixture (normal C_2-C_8 and iso-C_4-C_5) of free acids and of the acids regenerated from their salts.

Investigation of Carbonyl Components of Bread-Odor Distillates. Immediately after completion of the distillations, 2,4-DNPH derivatives were prepared from the condensates collected in the traps chilled with dry ice-ethanol and liquid nitrogen. 2,4-DNPH's were also prepared from a 1-ml. aliquot of each of the ether-soluble odor concentrates and from the ether removed during concentration of the ether extracts, as were 2,4-DNPH derivatives of authentic samples of known bread components.

Separation by Thin-Layer Chromatography. The derivatives (5 μliters of a 0.1% (w/v) chloroform solution) of known carbonyls and of the unknown bread derivative mixtures were separated on a 0.01-in. layer of Silica Gel G (Brinkmann Instruments, Inc.) in water-saturated benzene (22). R_f values were determined relative to formaldehyde-2,4-DNPH. The reproducibility of separation of known derivatives varied from 2.43 to 5.01%, on the basis of 21 runs. Reproducibility of separation (17 runs) of bread mixtures varied from 1.52 to 4.38%.

Gas-Chromatographic Separation of Carbonyl Compounds Regenerated from 2,4-DNPH Derivatives. Carbonyls were regenerated according to Ralls' procedure (23,24) from selected spots isolated on the

2185

chromatoplates from bread fractions. A vial containing the reaction mixture was heated in an oil bath at 250°C. for 10–15 sec. and a 500-μliter vapor sample of its head gas was separated on a column 6 ft. × 1/8 in. of 1% Ucon 50 HB-2000 on 100- to 120-mesh Gas Chrom A at 34°C. An Aerograph A-500-B flame ionization detector was used. On the basis of relative peak areas, the carbonyl compounds could easily be selected from peaks caused by artifacts.

Results and Discussion

Sensory evaluation of control bread and of breads having modified dough composition showed that though differences were noted in a number of cases (Table II), only the addition of L-proline improved

TABLE II
SENSORY EVALUATION IN A TRIANGLE TEST OF BREAD PREPARED BY MODIFICATION OF PRE-FERMENT DOUGH COMPOSITION

Test No. and Additive[a]	Number of Correct Judgments	Number of Incorrect Judgments	Level of Significance	Preference
1. DL-alanine (100 mg.)				
Crust: Odor	26	12	<0.001	18 control 8 DL-alanine
Taste	26	12	<0.001	16 control 9 DL-alanine
2. L-arginine (100 mg.)				
Crumb: Odor	21	16	0.01	11 control 10 L-arginine
3. L-histidine (100 mg.)				
Crumb: Taste	16	14	0.05	8 control 5 L-histidine
4. L-proline (100 mg.)				
Crumb: Odor	19	19	0.05	4 control 13 L-proline
Crust: Odor	23	15	0.001	7 control 15 L-proline
Taste	23	15	0.001	12 control 11 L-proline
5. L-leucine (50 mg.)				
Crumb: Odor	22	21	0.03	2 control 1 L-leucine
6. L-valine (100 mg.)				
Crust: Odor	20	16	0.01	16 control 3 L-valine
Taste	19	17	0.03	15 control 2 L-valine
7. Casein (1.22 g.)				
Crumb: Odor	9	5	0.05	6 control 3 casein
8. Gliadin (1.22 g.)				
Crumb: Odor	10	5	0.01	9 control 1 gliadin
Taste	10	5	0.01	7 control 2 gliadin
Crust: Taste	9	6	0.05	5 control 4 gliadin

[a] Amount per 100 g. dough.

bread aroma. The amount of proline added (868 μmoles/100 g. or 100 mg./100 g. of dough) was very large, both from a practical standpoint and relative to the quantity of free proline normally found (9.60 μmoles/100 g. of dough) in fermented bread doughs (25). However, since a primary goal of the research was to determine whether improved flavor brought about by modification of dough composition would be reflected in the chemical composition of volatile components, the large quantity of proline used might be an advantage. Yields of volatile components might be increased.

Distillation of sliced fresh bread yielded aqueous distillates which exhibited characteristic (but not fresh) bread aroma. Table III gives

TABLE III
SUMMARY OF PREPARATIONS OF BREAD ODOR DISTILLATES AND CONCENTRATES

No.	Bread	Distillates: Volume	Distillates: Oxidizable Carbon Content	Distillates: Ethanol Concentration: Found	Distillates: Ethanol Concentration: Theory[a]	Odor Concentrates: Weight	Odor Concentrates: Weight[b]	Odor Concentrates: Yield[b]
	lb.	ml.	g./lb.	g./lb.	g./lb.	g.	mg.	p.p.m.
2	13.7	1,780	0.80	1.6	1.6	1.80	18	3
3	13.8	1,585	0.72	1.5	1.5	2.70	27	5
4	13.0	1,624	0.85	~1.8	1.8	2.22	22	3
5	12.6	1,730	0.87	2.0	1.8	3.54	35	~5
6[c]	12.8	1,780	1.06	2.7	2.3	6.08	60	~10
7[c]	12.6	1,704	1.03	2.2	2.1	4.80	47	~8

[a] The theoretical ethanol concentration is the amount of ethanol calculated as being present if all the oxidizable carbon found is assumed to be in the form of ethanol.
[b] Reported on an ethanol- and ether-free basis to emphasize the minute quantity of bread-flavor components present. Ether is present as a residue from ether extraction of the aqueous distillates.
[c] Proline added.

a summary of bread distillations. The oxidizable carbon content of the total distillates from identical control bread preparations (4 and 5, Table III) was essentially the same. Preparations 6 and 7 in which the dough had been modified by addition of L-proline at 1,000 p.p.m. of dough weight also yielded distillates having reproducible oxidizable carbon content. Their ethanol content in g. per lb. of bread is given in Table III, along with their theoretical ethanol content (calculated on the assumption that all the oxidizable carbon found was present in ethanol). In all cases the oxidizable material detected was accounted for as ethanol (Table III), indicating that the flavor components were present in minute quantities.

Gas-chromatographic analysis of the odor concentrates showed that ethanol and diethyl ether were by far the largest components present. On the basis of relative peak areas, these accounted for approximately 99% of each of the various concentrates. The weights of all odor isolates obtained (calculated on an ethanol- and ether-free basis) are

2185

shown in Table III, along with the yields in which they were obtained from bread. They represented from 3 to 5 p.p.m. of control bread (preparations 2 through 5) and 8 to 10 p.p.m. of bread to which proline had been added (preparations 6 and 7). Informal sensory evaluation indicated that they exhibited typical (but not fresh) bread aroma.

Gas-chromatographic separation of the odor concentrates on a 20% Quadrol column showed (Fig. 1) the presence of only six components

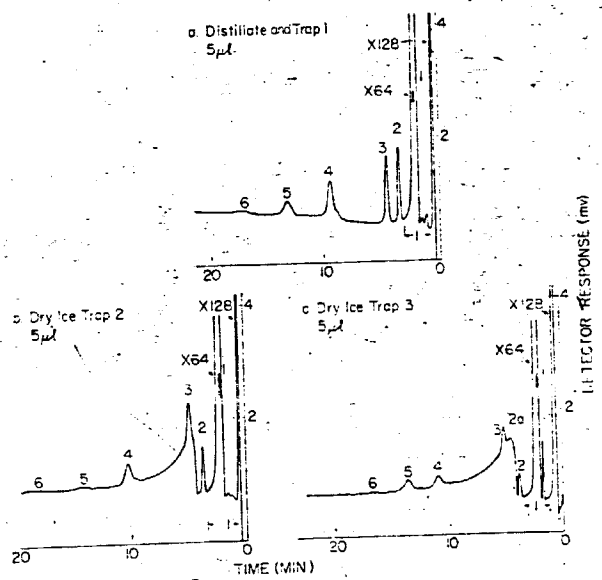

Fig. 1. Chromatograms of bread odor concentrates (prep. 5) on a 20% Quadrol column at 103°C. The unnumbered peak is diethyl ether. Peak 1, ethanol; 2, n-propanol; 2a, water; 3, isobutanol; 4, isoamyl alcohol; 5, acetoin; 6, furfural.

in addition to two trace substances detected in fraction 1. The isolates from the dry-ice traps (curves b and c, Fig. 1) contained an additional component (fraction 2a) which was later proved to be water. Similar chromatograms were obtained from analogous odor concentrates derived from all bread preparations. On the basis of the identity of their infrared spectra and retention data with those of authentic reference compounds, the peaks shown were proved in all the isolates to contain n-propanol (fraction 2), isobutanol (fraction 3), isoamyl alcohol and possibly 2-methylbutanol-1 (fraction 4), acetoin (fraction 5), and furfural (fraction 6). In all cases fraction 1 contained ethanol. Separation of the odor concentrates on a 20% Ucon LB 1715 column allowed isolation and identification of acetic acid which was not eluted from the alkaline Quadrol column. The presence of a shoulder on the isoamyl

alcohol fraction (number 4) on the Quadrol column indicated that this fraction was impure. Since its infrared spectrum was identical with the spectrum of known isoamyl alcohol, the impurity was concluded to be very similar in structure to isoamyl alcohol. This suggested the presence of 2-methylbutanol-1. The bread odor concentrates were therefore separated on a 20% DEGS column. On the basis of retention data, a fraction corresponding to 2-methylbutanol-1 was obtained, but not in large enough quantity to allow a definitive spectrum to be recorded. It is probable that 2-methylbutanol-1 was present. Use of the DEGS column also allowed identification of ethyl acetate, since the infrared spectrum of a small fraction eluted before ethanol was identical with that of authentic ethyl acetate.

The small number of components isolated from the above three columns was disappointing. However, it is not illogical if one reviews (Fig. 2) the separation obtained by means of the 5% β,β′-OPN column

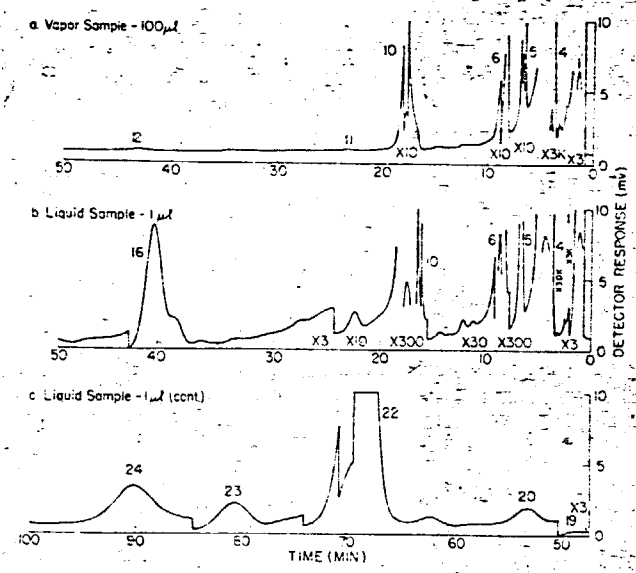

Fig. 2. Chromatograms of a bread odor concentrate (prep. 4) on a 5% β,β′-oxydipropylnitrile column at 43°C. (vapor vs. liquid sample). Peak 1, residual diethyl ether; 4, ethanol; 5, n-propanol; 6, isobutanol; 10, isoamyl alcohol; 22, acetoin (?).

and a flame ionization detector. This chromatogram, and the fact that all the odor concentrates, irrespective of the preparation or cold trap from which they were derived, gave a chromatogram qualitatively similar to it, showed that only six major components were present.

2185

Other substances were present in quantity too small to be detected by the conventional gas chromatographs necessarily used for separation and subsequent collection of individual fractions.

The major peaks (4, 5, 6, and 10 in both the vapor and liquid sample in Fig. 2) were ethanol, n-propanol, isobutanol, and isoamyl alcohol respectively. Of the approximately 11 additional substances detected in the liquid sample, only peak 22 could be tentatively identified as acetoin. Compounds identified by other investigators as components of bread would, with the exception of furfural, be eluted within the first 20 min. of the chromatograms. Furfural remains on the column at the conditions used. The chromatogram of the liquid sample shown in Fig. 2, b, thus provided evidence for the presence of at least 11 components not previously detected in bread. It is possible that additional substances would have been detected if the separation had been temperature-programmed. Though known to be a bread component, hydroxymethylfurfural was not expected to be found, since it is not volatile enough to have been distilled from the bread. Alcohols are by far the major constituents of the volatile components of bread. This suggests that man's odor receptors may be relatively insensitive to alcohols and very sensitive to the unknown and minor bread-odor components.

An example of the results of rechromatography of individual fractions is given in Fig. 3. The infrared spectrum of the fraction was

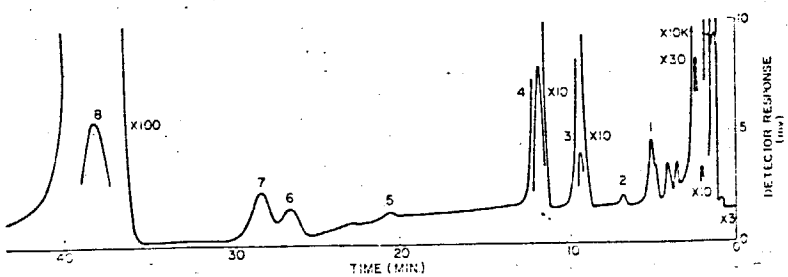

Fig. 3. Rechromatography of fraction 5 (prep. 4) trapped from a 20% Quadrol column on a 5% β,β'-oxydipropylnitrile column. The initial two large peaks are carbon tetrachloride (from the IR spectrum solution) and ethanol. Peak 1 is probably isobutanol; peak 3, isoamyl alcohol; peak 8, acetoin. The rest are unknown.

identical with that of an authentic sample of acetoin. However, rechromatography showed nine additional trace components. On the basis of retention data, the initial two peaks were carbon tetrachloride and ethanol. Peak 1 was apparently isobutanol and peak 3, isoamyl alcohol. Detection of trace quantities of preceding fractions was com-

mon. Peak 4 may be 2-ethylhexanal. The identity of the remaining constituents is unknown.

On the basis of results of this nature, tentative evidence was obtained for the presence of very small quantities of acetone, 2-butenal, diacetyl, propanal, 2-ethylhexanal, and approximately 17 completely unknown trace components. Acetic acid was the only acidic component detected, though the analytical method used (14) was capable (if the substances were present in sufficient quantity) of detecting the n-C_2-C_8 and iso-C_4-C_5 acids. Lactic and pyruvic acids, known bread constituents, were not expected to be found, since, owing to nonvolatility, they could not be present in the odor concentrates. No significant differences in composition of control and proline-treated bread odor concentrates were found.

Since direct gas chromatography of odor concentrates resulted in identification of only two carbonyl compounds, furfural and acetoin, and since numerous carbonyls were known to be bread components, it was clear that additional compounds should be detected and identified. Separation of 2,4-DNPH's from the odor concentrates, the contents of the traps cooled with dry ice-ethanol and liquid nitrogen, by thin layer chromatography is shown in Fig. 4. Qualitative variations were noted in the carbonyl content of analogous fractions within the control and proline-treated bread preparations as well as between these preparations. Regeneration from selected 2,4-DNPH spots and gas-

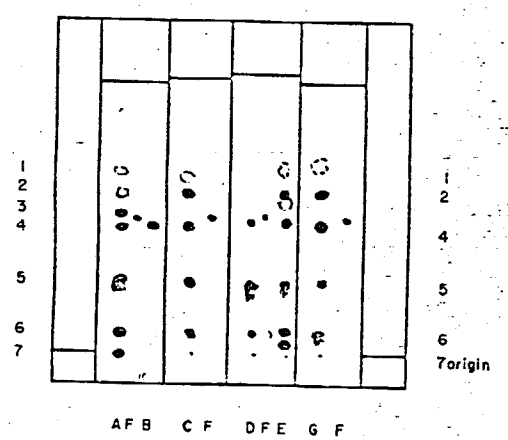

Fig. 4. Thin-layer chromatography of 2,4-DNPH's of bread isolates. A, odor concentrate; B, ether removed during concentration of ether extract; C,D,E, condensates in dry ice-ethanol traps; G, condensate in liquid-nitrogen trap; F, formaldehyde-2,4-DNPH. Spots 1, 2, 3, and 4 were regenerated and gas-chromatographed. Spot 5 is a mixture of acetoin and diacetyl-2,4-DNPH's. Spots 6 and 7 are believed due to 2,4-DNPH reagent.

2185

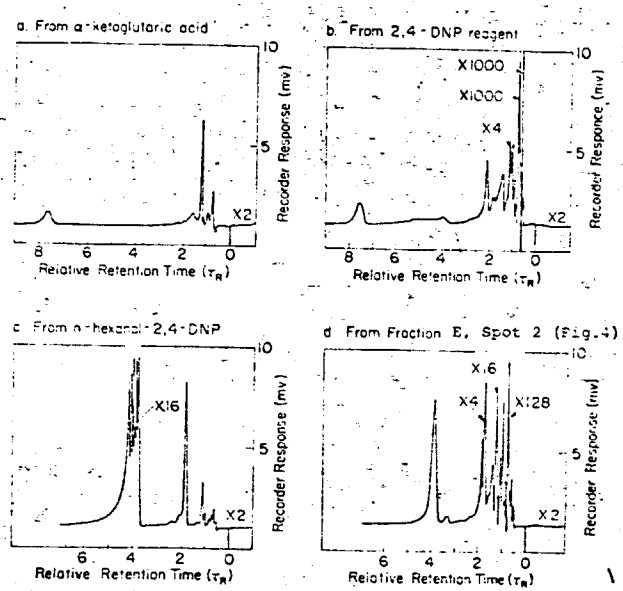

Fig. 5. Gas chromatograms obtained by regeneration of selected 2,4-DNPH's. Column: 1% Ucon 50 HB 2,000; column temperature 55°C.

chromatographic separation of the resulting free carbonyl compounds gave chromatograms typical of the one given in Fig. 5 (curve d). Only peaks which were clearly not derived from decomposition of alpha-ketoglutaric acid or of 2,4-DNPH reagent were considered. Thus the small peak at t_R 3.9 was not recorded as a carbonyl component of the spot. However, the attenuated peaks at t_R 0.811, 1.34, and 1.81 were believed to be bread components. On the basis of R_f values and retention data from thin-layer chromatography and flash-exchange gas chromatography (Table IV) of 2,4-DNPH's, formaldehyde, acetaldehyde, acetone, propanal, n-butanal, isobutanal, n-pentanal, 3-methylbutanal, 2-butanone, 2-pentanone, and 2-hexanone were tentatively identified.

Since chromatography is a separation procedure and not an identification method, the identities proposed above are tentative even though based on retention data from both thin-layer and gas chromatography. The fact that all the above compounds (with the exception of n-propanal, n-butanal, 2-pentanone, and 3-methylbutanal) have been shown by others (4,9,10) to be bread components gave needed support for their presence. The exceptions need further proof of their identity. It is quite probable that 2-methylbutanal was present as well as 3-methylbutanal. In view of the tentative nature of the identifications,

TABLE IV

THIN-LAYER AND FLASH-EXCHANGE CHROMATOGRAPHY OF CERTAIN UNKOWN 2,4-DNPH BREAD FRACTIONS

Fraction[a]	Thin-Layer Chromatography: Spot	R_f	Tentative Identity	Flash-Exchange Chromatography: Peak	t_R[b]	Conclusion
E	2	1.37	n-C_4al, iso-C_4al, 2-C_3one, n-C_3al	9	0.811	isobutanal
				8	1.34	?
				11	1.81	2-pentanone
	3	~1.11	C_3al, C_3al, 2-C_3one	1	0.520	?
				3	0.707	propanal
				4	0.747	?
				6	1.12	2-butanone
				7	1.31	?
G	2	1.43	iso-C_4al, n-C_4al, 2-C_5one, n-C_5al, 2-C_6one, 3-methylbutanal, furfural	2	0.614	?
				3	0.734	?
				4	0.827	isobutanal
				5	1.00	n-butanal
				6	1.06	?
				7	1.12	?
				8	1.33	3-methylbutanal
				11	1.83	n-pentanal
				16	3.94	2-hexanone
	4	0.995	C_2al, C_2al, C_3one	2	0.587	acetaldehyde
				3	0.676	formaldehyde
				4	0.787	acetone
				5	1.09	?
				6	1.35	?
				9	2.02	?

[a] From Fig. 4.
[b] Retention time relative to n-butanal.

no significance was attached to qualitative or quantitative differences observed between bread preparations. To our knowledge the presence of n-propanol, isobutanol, isoamyl alcohol, and ethyl acetate in bread has not previously been proved, nor has evidence for the presence of n-butanal, 2-pentanone, or 2-methylbutanol-1 been previously presented. It is logical that such compounds would be present.

Since previous knowledge of the volatile components of bread concerned primarily the nature of carbonyl compounds, it was initially surprising to find them present in such small quantity relative to other organic components. However, the situation is logical if one realizes that the carbonyl components had been isolated, separated, and identified as 2,4-DNPH derivatives. Noncarbonyl compounds were generally not studied in any detail. The fact that Wiseblatt and Kohn (9) did not report acetoin as a volatile bread component, whereas acetoin was found by us to be a major component, is interesting in this regard. The reason may be that the presence of excess 2,4-dinitrophenylhydrazine in Wiseblatt's distillate caused conversion of acetoin-2,4-DNPH to diacetyl-2,4-DNPH. If this did occur, no acetoin would be detected

and 2,4-dinitrophenylaniline would have been formed. Demonstration of its presence in the reaction mixture would support this postulate.

The possibility that 2-ethylhexanal may be artifact and not a bread component must be recognized. It is conceivable that Tygon tubing may have contaminated the condensates with 2-ethylhexanol, which in turn could have been converted to 2-ethylhexanal. It must also be recognized that oxygen from air may have been condensed in the liquid-nitrogen traps and have contributed to artifact production.

Much remains to be learned before the chemistry of bread flavor is understood. The many completely unknown trace components detected in this investigation may contain the missing information.

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21851

2186

STUDIES OF THE QUALITY AND FLAVOUR OF CEYLON TEA

By R. L. WICKREMASINGHE* and T. SWAIN

The amounts of total phenols, flavan-3-ols, leucoanthocyanins, theaflavins, thearubigins, and certain amino acids in methanolic extracts of Ceylon and other teas have been determined, together with the individual low-boiling volatile compounds which are produced on infusion of the teas in boiling water. The correlation between the quantities of phenolic compounds and commercial valuation, and the contribution of the volatiles to flavour, is discussed. N-Ethylasparagine has been tentatively identified in tea leaf.

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J. Sci. Fd Agric., 1965, Vol. 16, January

Introduction

The polyphenols present in fresh tea leaf juice and black tea have been extensively studied by Roberts and his co-workers,[1] whilst the polyphenols present in ethanolic extracts of a Ceylon tea test sample have been fractionated by Vinograz *et al.*[2] Both groups of workers showed the occurrence in the raw material of flavanols and their gallic acid esters, flavonols and their 3-glucosides, polyphenolic acids and leucoanthocyanins. Oxidation and polymerisation products were present only in small quantities in unprocessed green tea leaves, but formed the major polyphenolic fraction of black tea.[3] Among the products of fermentation of tea leaf, Roberts & Myers[4] isolated and characterised theaflavin, theaflavin gallate and a group of substances referred to as 'thearubigins'. The amounts of these were shown to determine the strength, colour and briskness of black tea liquors and to play an important part in the assessment of quality. Quality, however, is also dependent on other factors including the volatile constituents responsible for the flavour of tea. These substances were studied by Yamanishi and co-workers,[5] who separated and identified 27 components in the essential oil of green tea leaf. Gas-chromatographic methods were also used for the separation and identification of the volatile fatty acids of black tea,[6] and the earlier work on tea volatiles has been reviewed by Stahl.[7] Apart from the essential oil, amino-acids have also been shown to contribute to the aroma complex of black tea. Thus Bokuchava & Popov[8] showed that addition of different amino-acids to hot aqueous infusions of tea led to the production of different aromas, and that no aroma developed if a carbonyl-binding reagent was added. Based on these results, Popov[9] suggested a scheme for the formation of carbon dioxide, ammonia and aldehydes from amino-acids.

The present paper presents the results of comparative qualitative and quantitative studies of the polyphenols, amino-acids and low-boiling volatile compounds in two specially prepared samples of Ceylon tea, and those in 20 commercial samples of black tea, 8 of which were manufactured from Ceylon-grown tea. Two commercial brands of instant tea have also been examined. The correlation between commercial valuation and quality on the one hand and polyphenol content on the other, as well as the contribution of low-boiling volatiles to the flavour, is discussed.

Experimental

Materials

Green tea leaf.—As soon as possible after being plucked from selected bushes at two tea estates of the Tea Research Institute (Ceylon), samples of the two batches of fresh green leaf (the terminal bud with two leaves attached) were packed in sealed polythene bags and flown from Ceylon to London. The samples were kept at 3–5° during transit and used in the laboratory about 3 days after being plucked. Hence the term 'unprocessed leaf' used here (Table II) is not strictly applicable in view of the fact that 'chemical withering' begins immediately after the leaf is separated from the parent bush (G. W. Sanderson[92]).

'Fermented' tea.—'Fermented' tea was made in the laboratory after 'withering' a selected part of the green leaf samples at room temperature (21°) for 5 h. on a perforated tray through which air was passed continuously. The 'withered' leaf was then minced, the macerate spread on a tray, and allowed to 'ferment' at 25° for 2½ h. It was then kept at −20° until required.

Black tea.—Samples 1 and 2 (Table I) of black tea were manufactured in Ceylon from the remainder of two batches of green leaf mentioned above. Particulars regarding these and the 20 other black tea samples tested (numbered 3–22) are given in Table I.

Instant tea.—Two commercial brands of instant tea were examined, one produced in England and one in Ceylon.

Methods

Extracts.—For the qualitative and quantitative studies of *total phenols* and *total amino-acids* in unprocessed green leaf, bud, fermented leaf (leaf and bud together) and black tea, extracts were made first with boiling absolute methanol (3 × 25 ml./g.) and the residue was then re-extracted with boiling 80% (v/v) methanol (3 × 25 ml./g.). For the quantitative estimations

2186

of *individual amino-acids*, black tea samples were extracted only with boiling 80% methanol (5 × 25 ml. g.). *Ammonia* estimations were made on the infusion obtained by adding 25 ml. of boiling water to 2·5 g. of black tea in capped boiling tubes and leaving the tubes in a boiling water bath for 10 min.

Polyphenols.—Identification of polyphenols was made by two-dimensional paper chromatography.[10] Quantitative estimations of total phenols were made with the Folin Denis, vanillin and acidified butanol (leuco-anthocyanin) reagents,[11] and the sodium molybdate reagent.[12] Cacao leuco-anthocyanin was used as a standard for the acidified butanol reagent and epigallocatechin gallate for the other three. Theaflavins and thearubigins were estimated essentially as described by Roberts & Smith.[13]

Amino-acids.—Identification of the amino-acids was made by two-dimensional chromatography of the amino-acid fraction obtained by use of an ion-exchange column.[14] Quantitative estimations of total amino-acids were made according to Moore & Stein,[15] phenylalanine being used as standard. Determinations of leucine + isoleucine, valine, and theanine + γ-aminobutyric acid were made after separation by paper chromatography, by measuring the absorptivity of the individual spots after dipping in ninhydrin (0·2% in acetone) using the EEL Scanning Densitometer fitted with a green filter.[16]

Low-boiling volatile compounds.—*Ammonia* was estimated on 1-ml. aliquots of infusion by the microdiffusion method:[17] 1 ml. of 10% (w/v) sodium carbonate and 40% (w/v) sodium hydroxide respectively were used to liberate the ammonia (and amines), which was absorbed in boric acid containing indicator, and estimated by titration with 0·01N-hydrochloric acid. Other low-boiling volatile compounds obtained by head-space analysis from 0·5 g. samples of tea boiled in 3 ml. of freshly distilled water were separated by capillary column gas-chromatography[18] and identified by retention times, mixed chromatography and odour. From the top of the condenser 0·5-ml. samples of the head space vapour were collected into a stainless steel trap cooled in liquid oxygen. Three different capillary columns, viz., nylon (50 ft. long) coated with silicone oil, and two stainless steel (each 50 ft. long) coated with PEG 200 and di-2-cyanoethyl ether respectively), were used together with flame ionisation and micro-argon detectors. Quantitative estimations of some of the volatile compounds were made by the method described by Swain & Self.[19]

Table I

Particulars of samples of black tea

No.	Country of origin	Elevation*	Colour	Strength	Quality	Flavour	Valuation	Remarks‡
1	Ceylon	4500	Fair	Fair	Bright	—	5 3	B.O.P.
2	,,	300	Useful	Useful	Bright	—	4 4	Big bulk
3	,,	3398	Coloury brown	Little	Plain	—	3 11	B.O.P.
4	,,	600	Poor	Poor	Plain	—	3 8	B.O.P.F.
5	,,	4400	Good	Good	Bright	Attractive	5 6	Flowery B.O.P.
6	,,	4300	Good	Good	Little brightness	—	4 8	B.O.P.F.
7	,,	2500	Some	Little	Little brightness	—	3 6	Big bulk
8	,,	1000	Fair	Fair	Plain		3 4	B.O.P.
9	Assam	—	Good, bright	Fair	Bright	—	4 6 4 9	End of season tea
10	Dooars	—	Quite good, dull	Little	Plain		3 6 3 7	
11	Kenya	—	Quite good, dull	Quite good	Little	—	3 8 3 8½	
12	Uganda	—	Quite good, brightish	Fairly strong	Plain	—	3 6	—
13	Tanganyika	—	Fair, dull	Very little	Plain	—	3 2	—
14	Nyasaland	—	Fair	Fair	Little	—	2 10 2 11	—
15	Portuguese E. Africa	—	Fair	Fair	Fair	—	2 6½ 2 7½	
16	Rhodesia	—	Quite good	Quite good	Bright		3 6	
17	Mauritius	—	Fair, brightish	Fair	Little		3 2½	
18	Argentine	—	Coloury, dull	Little	Plain		[illegible]	
19	Vietnam	—	Not much	Not much	Bright	A little flavour	[illegible]	
20	S. India		Fair	Fair	Bright	Has flavour	[illegible]	
21	Malaya		Quite fair, bright	Quite good	Bright		[illegible]	
22	Kenya		Quite good, bright	Quite good	Bright	A little flavour	[illegible]	CTC manufacture, pungent

* Elevation in feet above mean sea level of estate in which tea was grown.
‡ B.O.P. = Broken Orange Pekoe; B.O.P.F. = B.O.P. Fannings; Big Bulk = Grade containing large particles of leaf.
CTC = Cutting, Tearing and Curling process.

2186

Results

Polyphenols

The main polyphenolic compounds described by Roberts[20] were identified in the green tea leaf and instant tea extracts—kaempferol 3-glucoside, isoquercitrin, myricetin-3-glucoside, rutin, epicatechin, epicatechin gallate, epi-gallocatechin, epi-gallocatechin gallate, chlorogenic acid, isochlorogenic acid, neochlorogenic acid, *p*-coumarylquinic acid and theogallin. On fermentation of the green tea leaf, the intensity of all the flavanol spots diminished and the streak corresponding to theaflavins and thearubigins appeared. It was also observed that the chlorophyll of green leaf underwent some change on fermentation. Chromatograms of black tea were qualitatively similar to those of fermented tea.

Table II shows the results of quantitative estimations of total polyphenols (measured by the Folin–Denis reagent) and of different phenolic nuclei (cf. King & White[21]), 5,7-dihydroxyflavans, measured by the vanillin method, *o*-dihydroxy groups by molybdate, and leuco-anthocyanins by the acid butanol reagents,[11, 12] the theaflavin and thearubigin contents, and the E_{380}/E_{460} ratios in the different tea samples. The results show that although there is considerable variation in the amounts of polyphenols in the *absolute* methanol extracts as measured by any of the reagents used, the quantities are remarkably constant in the *aqueous* methanol extracts of the 15 black tea samples tested. It is also evident that the polyphenol content of the unprocessed bud and stalk is higher than that of the leaf in both samples, and that fermentation of the leaf and subsequent fining leads to an observed decrease of methanol-extractable polyphenols, presumably due to polymerisation and condensation, since the relative proportion extracted by aqueous methanol as compared to absolute methanol increases (cf. Goldstein & Swain[12]). In the two samples of instant tea, this relative proportion is considerably less, indicating that either extraction of the polymeric polyphenols is far from complete in the preparations themselves or that the absence of other leaf components (e.g., cellulose and proteins) makes for easier extraction of the polymers in absolute methanol. The amounts of *theaflavin* in the black tea samples show no direct correlation to the amounts of polyphenols extracted by absolute methanol although they almost all fall on a relatively smooth curve (Fig. 1). The fact that the more or less linear relationship between theaflavins and total phenols ceases when the tea contains more than 25 mg./g. of total phenols is perhaps due to the increased formation of other polymers. The valuations of the black tea samples are indicated by the figures in brackets (Fig. 1) and are seen to depend mainly on the theaflavin content—as has already been shown by Roberts & Smith.[13] Table II shows that in the instant tea samples the proportion of thearubigins to theaflavin is considerably greater than in the black teas and this is surprising since little thearubigin-like material was observed by paper-chromatography. The values of the E_{380}/E_{460} readings, which Roberts & Smith[13] introduced as a measurement of both colour and thearubigin content, do not appear to correlate with the actual amount of thearubigins or the commercial evaluation of the teas examined here.

Amino-acids and ammonia

The following amino-acids were detected in the absolute methanol extracts of unprocessed tea leaf and bud, fermented tea and black tea (samples 1 and 2, Table I): α-alanine, β-alanine, γ-aminobutyric acid, asparagine, aspartic acid, cysteic acid, *N*-ethylasparagine, glutamic acid, glutamine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, theanine, threonine, tyrosine and valine. The intensity of the spots was observed to be less in fermented and black tea than in unprocessed tea, although the amino-acid pattern was the same. *N*-Ethylasparagine, which has not hitherto been detected in tea, was identified by its R_F values compared with those quoted by Gray & Fowden[22] and by the formation of aspartic acid only on elution and acid hydrolysis. The results of quantitative analysis of the total amino-acid content of unprocessed, fermented, black and instant tea are given in Table III. It will be seen that: (1) bud + stalk has a higher amino-acid content than green leaf; (2) fermentation leads to an appreciable decrease in the total amino-acid content; (3) black tea has an amino-acid content higher than that of fermented, but less than that of unprocessed, tea; and (4) the instant teas have a low amino-acid content.

Table II

Total polyphenols, theaflavin and thearubigin contents of tea samples

(All values expressed as mg./g. dry weight of tea; Folin-Denis, vanillin and molybdate values expressed in terms of epigallocatechin gallate; leuco-anthocyanin values in terms of cacao leuco-anthocyanin)

	Absolute methanol extracts				80% methanol extracts						
	Folin-Denis	Vanillin	Molybdate	Leuco-anthocyanins	Folin-Denis*	Vanillin	Molybdate	Leuco-anthocyanins	Theaflavin	Thearubigins	E_{380}/E_{460}
Unprocessed											
1. Leaf	68	70		5	16 (24)	15		5			
Bud + stalk	79	84		30	27 (34)	26		17			
2. Leaf	130	104		3	13 (10)	11		4			
Bud + stalk	218	143		10	51 (23)	30		20			
Fermented											
1	22	15		13	9 (41)	7		9			
2	57	45		3	17 (30)	16		20			
Black											
1	33	31	42	10	30 (91)	28	28	13	11·2	10·3	5·2
2	53	39	66	10	31 (59)	26	31	12	7·9	11·1	5·45
3	20	21	24	7	38	30	26	12	9·9	13·6	6·53
4	12	12	23	6	38	28	29	11	5·6	14·5	4·7
5	26	20	31	10	35	27	27	13	12·4	11·8	6·95
6	38	26	29	10	33	27	29	13	9·9	13·3	1·5
7	23	17	23	10	32	26	29	13	4·7	8·2	3·9
8	15	11	16	5	35	27	32	12	5·6	13·3	4·5
9	23	19			37	28			9·9	14	4·9
10	14	7			39	28			8·5	11·1	4·3
11	31	23			35	28			10·1	15·8	5·3
12	37	22			35	28			9·9	15·8	1·6
20	24	22			38	29			12·4	13·4	6·2
21	9	5			35	30			5·9	12	1·8
22	26	29			35	28			11·3	15·8	3·5
Instant											
A	127	100	88	38	13 (10)	11	12	7	2·7	12·5	4·1
B	65	61	51	18	12 (18)	13	11	6	7·9	33·8	9·2

* Values in brackets are % of the amount in absolute methanol extracts

The results of quantitative analyses of leucine and isoleucine, valine, and threonine [γ-aminobutyric acid and of ammonia are also shown in Table III. The figures for 'bound ammonia' include ethylamine which will be liberated from theanine and *N*-ethylasparagine by 40% sodium hydroxide. The variation in the amounts of amino-acids and ammonia may

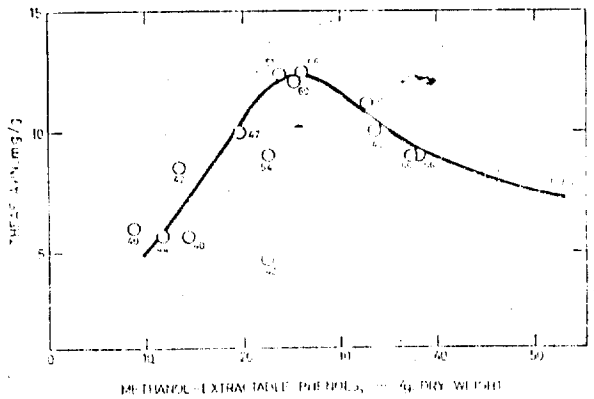

FIG. 1. *Relation between theaflavin, methanol-extractable phenols and valuation of black tea samples*

Numbers refer to valuations (pence/lb.)

2186

have some correlation with the flavour of tea as will be discussed later. The results of ammonia estimations on unprocessed green leaf were carried out on samples stored at −10° for about 5 months and are included only for purposes of comparison; it is evident that the amounts of both free and 'bound' ammonia are lower in unprocessed than black tea.

Gas liquid chromatography of low-boiling volatile compounds

Qualitative gas liquid chromatography of unprocessed tea leaf showed the presence of hydrogen sulphide, methanethiol, dimethyl sulphide, acetaldehyde, propionaldehyde, isobutyraldehyde and very large amounts of methanol and ethanol. On fermentation, the amounts of methanol and ethanol decreased and additional peaks corresponding to the formation of 2- and 3-methylbutyraldehyde, n-valeraldehyde and biacetyl appeared. In black tea, the amounts of methanol and ethanol were very small, and further peaks for acrolein, acetone, n-butyraldehyde and methyl ethyl ketone appeared. The results of quantitative analyses of some of the volatile compounds in the black tea samples are shown in Table IV. The concentrations of the methylbutyraldehydes were high and showed wide variations in the different tea samples, generally being low in the 'flavoury' teas and high in the teas without much flavour (Table I). There were also considerable variations in the absolute and relative amounts of the other compounds and this may contribute to the difference between the 'bouquets' of tea samples. The instant tea samples showed very low amounts of all volatiles except dimethyl sulphide, acetaldehyde and 3-methylbutyraldehyde.

Discussion

The *total* amount of extractable polyphenols was highest in the unprocessed leaf, decreased on fermentation, and rose slightly on 'firing' (Table II). The phenols reacting with vanillin,

Table III

Amino-acids and ammonia in tea samples

(All values expressed as μmoles/g. dry weight of tea; total amino-acids expressed in terms of β-phenylalanine)

Sample*	Total amino-acids: Absolute methanol extract	Total amino-acids: Aqueous methanol extract	Leucine isoleucine	Valine	Threonine + γ-amino-butyric acid	Ammonia: Free	Ammonia: 'Bound'
Unprocessed							
1. Leaf	145	218				1·4	2·7
Bud + stalk	382	170				0·8	2·1
2. Leaf	121	73					
Bud + stalk	212	108					
Fermented							
1	73	32					
2	85	18					
Black							
1	200	79	5·5	7·85	20·4	2·8	17·0
2	100	55	5·4	5·65	15·3	2·9	19·2
3	115	67	4·95	5·7	15·6	2·2	13·6
4	121	67	4·2	3·66	19·[illegible]	2·9	11·2
5	127	75	3·2	4·85	14·5	2·0	9·5
6	79	49	4·05	3·71	13·6	2·6	
7	85	73	6·55	8·95	20·4	3·5	20·8
8	91	91	6·0	6·3	11·4	5·5	14·9
9						3·7	11·0
10			3·42	5·35	25·1	3·0	11·5
11						3·4	11·9
12						2·9	15·1
20	103	85	2·52	5·[illegible]	17·5	1·6	7
21						2·5	11·[illegible]
22	39	103	3·12	6·35	20·6	1·5	9·6
Instant tea							
A	4·[illegible]5	7·[illegible]				4·55	19·[illegible]
B	5[illegible]	7·[illegible]				5·[illegible]5	17·3

* Numbers refer to sample numbers in Table I

on the other hand, decreased in the absolute methanol extract and increased in the aqueous methanol extract, and this reflects the effects of polymerisation.[12] A similar decrease in Lowenthal titre during the manufacture of tea was also observed by Roberts,[1] who suggested that this was due to the formation of insoluble products as well as the lower reactivity of theaflavins and thearubigins with permanganate. The changes in leuco-anthocyanins are not so marked, but they form a more substantial proportion of the total extractable phenols than other workers appear to have realised.[1] The observation that black tea has a significantly higher content of phenols extractable with aqueous methanol than does fermented tea may be due to lowered binding capacity of denatured protein and cell wall polysaccharides after the 'firing' process. In this connexion it may be significant that the level of amino-acids follows the same pattern as that of polyphenols (Table III) suggesting that the proteins may be partly broken down.

The results on the black tea samples (Table II) show that, whereas there are appreciable differences in the amounts of polyphenols extractable by absolute methanol, the amounts extractable by aqueous methanol are remarkably constant. The levels of polyphenols in the absolute methanol extract show some relationship to the theaflavin content and taster's valuation (Fig. 1). Samples containing between 25 and 30 mg. g. (on dry weight of tea) of polyphenols extractable by absolute methanol show both the highest theaflavin content and commercial value. Since the amount of polyphenols in black tea depends on the amount originally present in the unprocessed leaf (cf. samples 1 and 2, Table II), it may be possible to predict the quality of a black tea from an estimation of the polyphenol content before processing.

The relative constancy of the polyphenol content of the aqueous methanol extracts may indicate that the enzymic polymerisation of the polyphenols in different teas results in the formation of a relatively fixed quantity of a similar or closely related group of compounds, which is sufficient to inhibit the enzyme systems responsible for their production. Inhibition of tyrosinase activity by the condensation products obtained by the autoxidation of (+)-catechin has been demonstrated in other experiments (Wickremasinghe & Swain, to be published; cf.

Table IV

Low-boiling volatile compounds in tea

(All values expressed as μg./100 g. of tea)

Sample*	Dimethyl sulphide	Acetaldehyde	Propionaldehyde	Isobutyraldehyde	Acrolein	Methanol	Acetone	Ethanol	3-Methylbutyraldehyde	2-Methylbutyraldehyde	Diacetyl
Black tea											
1	7	23	0·4	15	0·2	[illegible]	0·4	15	15	18	2
2	5	28	[illegible]	10	[illegible]	10	2	38	15	18	13
3	7	21	10	18	3	[illegible]	2	1	0·2	90	[illegible]
4	10	27	12	26	0·2	[illegible]	1	1	20	25	3
5	8	25	[illegible]	20	3	15	1	16	0·5	25	10
6	8	[illegible]	9	26	3	[illegible]	0·6	Trace	120	141	3
7	7	[illegible]	[illegible]	28	0·2	[illegible]	2	2·5	140	150	6
8	7	28	16	27	13	Trace	Trace	Trace	43	[illegible]	[illegible]
9	7	22	14	18	3	[illegible]	Trace	Trace	52	60	6
10	6	10	13	18	0·1	3	1	16	27	33	6
11	7	20	14	24	0·2	[illegible]	1	23	108	129	6
12	7	28	11	26	5	17	2	Trace	22	25	6
13	[illegible]	21	26	16	[illegible]	[illegible]	1	Trace	[illegible]	[illegible]	10
14	[illegible]	[illegible]	15	25	0·2	[illegible]	[illegible]	Trace	88	10[illegible]	[illegible]
15	[illegible]	[illegible]	[illegible]	13	0·2	1	0·8	Trace	20	[illegible]	1
16	5	10	7	10	0·1	[illegible]	[illegible]	Trace	[illegible]	[illegible]	1
17	[illegible]	26	8	10	0·2	[illegible]	1	Trace	11	[illegible]	[illegible]
18	5	18	11	10	0·1	2	[illegible]	[illegible]	[illegible]	38	[illegible]
19	[illegible]	[illegible]	13	15	0·1	[illegible]	Trace	Trace	30	39	[illegible]
20	[illegible]	[illegible]	10	15	[illegible]	2	0·8	Trace	25	[illegible]	[illegible]
21	[illegible]	[illegible]	[illegible]	21	3	[illegible]	1	Trace	16	17	10
22	7	23	11	15	0·1	[illegible]					
Instant tea											
A	12	29	[illegible]	16	Trace	Trace	0·0	Trace	[illegible]	30	[illegible]
B	8	30	Trace	8	Trace	Trace	Trace	Trace	14	[illegible]	0

* Numbered as Table I

Byrde, Fielding & Williams[23]). This hypothesis would account for the observation that there is a linear relation between the level of polyphenols and theaflavin with the lower levels of phenols (see Fig. 1), since it can be assumed that the enzyme systems are not a limiting factor. Thus, a low initial substrate concentration would *per se* limit the theaflavin level, whilst a high concentration would favour the rapid formation of enzyme inhibiting compounds, which then interfere with the further formation of theaflavin.

Of the amino-acids, N-ethylasparagine (Gray & Fowden[22]), the aspartic acid analogue of theanine, is reported for the first time in tea. The total amino-acid content decreased during fermentation concomitant with the production of aldehydes, whilst appreciable amounts of ammonia were found in black tea.

Among the low-boiling volatile compounds, the presence of acrolein, n-valeraldehyde, methyl ethyl ketone, acetone and biacetyl is reported for the first time. The other compounds have been detected by Yamanishi *et al.*,[5] but no attempts have been made at comparing the amounts present in different tea samples. The amounts of the methylbutyraldehydes in the high-grown Ceylon teas were lower than those in low-grown Ceylon teas. Furthermore the leucine and isoleucine, free ammonia and methylbutyraldehydes were all relatively low in the flavoury teas (Nos. 5, 20 and 22) and provide some evidence that aldehydes in the bouquet of tea are derived by deamination of amino-acids.[8] However this correlation did not apply to the other tea samples tested, nor was there any quantitative agreement between the amounts of isobutyraldehyde and valine, although Roberts[1] has shown that the addition of valine to actively fermenting tea leaf leads to an increased production of isobutyraldehyde. The detection of n-butyraldehyde and n-valeraldehyde cannot be explained as being derived from amino-acids since norvaline and norleucine were not detected. Furthermore biacetyl, acetone and methyl ethyl ketone also probably arise from precursors other than amino-acids.

Acknowledgments

The authors are grateful to Messrs. Wm. Jas. & Hy. Thompson and Messrs. Kerr, Hope & Sons, both of London, for reporting on the tea samples and for supplying some of the commercial samples of black tea. They also express thanks to Messrs. R. Self and J. C. Casey for advice. Miss Isobel Jewson and Miss Kit Lutford carried out part of the analyses. One of them (R. L. W.) thanks the Director, Tea Research Institute of Ceylon, for permission to publish.

Low Temperature Research Station
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Received 9 June, 1964

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2215

2215

VARIATION IN THE MALTING OF LOW PROTEIN LARKER BARLEY AS IT AFFECTS BREWING QUALITY

Tech. Quarterly Masters Brewers Assoc. of America 7 (2): 105-10, (1970)

By PAUL R. WITT, Jr. and GEORGE S. ROBBINS

Introduction

A barley variety being readied for release to agriculture is required to reflect the overall components of quality including appearance and other physical characteristics. Furthermore, the selection of an approved variety of barley for malting is directed to the development of high extract and to ease of processing. It is accepted that the maltster requires a kernel size which precludes a minimum cleanout. Some brewers are reluctant to use malt prepared from the large kernel fraction of malting barleys. Since the desired qualities in a new variety will frequently include a kernel larger than its predecessor, it would behoove the breeder to assess factors other than plumpness to achieve this increase in extract.

One of the factors which usually results in higher extract is a low total protein content. While the terms "high" and "low" protein are arbitrary and nondefinitive, they do assume meaning whsen related to a particular barley variety and to the crop year and geographical origin.

Brewers in the United States have become accustomed to malts from Midwestern six-row barleys which have a total protein content of 12.5 - 13.5%. The laboratory worts from such malts generally reflect levels of soluble protein (N × 6.25) of 4.8 - 5.4%, based on dry weight. This amount of soluble protein in worts containing 45% of adjunct extract, has been deemed necessary for yeast nutrition anud a satisfactory attenuation.

Several questions confront both maltster and brewer when considering low protein barley as a source of malt. These include:

(a) Potential for enzyme development during malting. While the determination of diastatic power and alpha-amylase is commonplace, only scant attention has been given to the evaluation of proteolytic activity. Moreover, it is known that malt contains many other enzymes which are involved in mashing and contribute to wort coniposition.

(b) The quantity and quality of nitrogenous substances for yeast nutrition and for flavor. It may be generalized that the tendency to shorten the germination period tends to diminish wort soluble protein. While the level in laboratory wort can be controlled within a certain range, by the effective degree of kiln sulfuring, this may not always apply to adjunct-containing kettle wort.

Thus, when considering protein as a potential for enzymes and yeast nutrition, thought should be directed to *"how much," "what kind,"* and to *"how it is used."* It is well to remember that one of the parameters of enzyme efficiency is the nature or availability of the substrate. It is accepted that a high amylase activity does not necessarily mean a rapid mash conversion as tested with iodine.

The present investigation involved one "low protein" barley which had been converted into a series of malts reflecting a progression in wort soluble nitrogen. No sulfur or sulfur compounds were used at any stage in the malting process.

The purpose of the study was to determine the threshold, i.e., minimal level of soluble nitrogen necessary for yeast proliferation and a normal fermentation.

Materials and Methods

Barley: Larker, 1967 crop. Protein: 11.4 - 11.5% (dry basis).

Malting Procedure

Malt A. Steeped in well water (60° F.) with periodic overflow to 43.0 - 43.5% moisture. Germination for 72 hr. at 70 - 72° F. Final kilning-off: 1 hr. at 180° F.

Malt B. This procedure may be considered the "Control." Steeping as outlined with a 5-day germination period at 60° F. Kilning as above.

Malt C. Steeped in well water 4 hr., followed by draining and replacement with a well water (60° F.) containing 0.1 ppm. added gibberellin, for an additional 6 hr. Replacement with regular well water and steeping continued to 43.0 - 43.5% moisture. Germination for 5 days at 55 - 56° F. Kilning as above.

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Malt D. Steeping as in Malts A and B. Watered once with well water containing 4.0 ppm. gibberellin (approximately 0.3 ppm. based on barley weight). Total germination time—5 days. Germination temperature after gibberellin treatment was 53 - 54° F. Kilning as above.

Note: The acrospire length of all 4 malts was approximately the same.

Pilot Brewing

Materials: 70% malt and 30% yellow corn grits (as-is basis).

Water: Distilled water "corrected" with 400 ppm. Ceresalts.

Brewhouse: Cooker contained 8% malt. After cooker drop, mashing conducted 10 min. at 68° C., conversion at 72° C. and mashing-off at 78° C. Kettle boil: 30 min. at 5 lb. pressure. Hopping: 0.4 lbs./bbl.

Fermentation: Wort fermented for 8 days at 12° C. in 5-foot-deep fermenters. Pitching rate: 10^6 cells per ml. of wort.

All beers were chillproofed and pasteurized.

Results

The analysis of these four malts are given in Table I.

TABLE I

ANALYSES OF FOUR MALTS FROM LARKER BARLEY, 1967 CROP (11.4 - 11.5% protein)

	MALT A	B	C	D
Moisture, %	4.5	4.3	4.4	4.2
Extract, % dry wt.	75.8	77.2	78.3	78.6
Fine Difference Protein, Soluble, malt, dry, %	3.84	4.54	5.20	5.62
Soluble, Total Protein, %	32.5	38.4	44.0	47.7
Alpha-Amylase	25.2	30.5	44.7	64.0
Diastatic Power °L.	141.0	153.0	174.0	176.0

Since kilning was conducted in a single bed under direct ventilation at 80 cu. ft. bushel/min., drying was more rapid than experienced under conventional conditions where two-level kilns are frequently employed. Based on a physical evaluation of the green malt, it is estimated that conventional kilning would have produced fine-coarse grind extract difference of the following magnitudes:

A	B	C	D
2.3%	1.9%	1.7%	1.1%

The slower drying with some inherent "stewing" occasioned by the two-level kiln has also been known to give higher alpha-amylase activity.

Table II is of incidental importance. Attention is directed to the pH values and the buffering capacity of the unboiled laboratory worts. There seems to be no relationship with the pH of the beers, all of which are comparable.

TABLE II

COMPARISON OF THE BUFFERING CAPACITY OF THE LABORATORY WORT AND THE pH OF THE BEER

	MALT A	B	C	D
Lab. Wort				
pH	6.12	6.11	6.09	6.06
Buff. capacity*	6.4	7.5	8.0	8.1
Beer				
pH	4.40–4.40–4.50	4.30–4.35–4.35	4.40–4.45–4.40	4.45–4.40–4.40

*ml. N/10 H_2SO_4 required to adjust 100 ml. laboratory wort to pH 4.0.

Triplicate brews were made at 8-day intervals. Collection of the yeast after fermentation permitted the repitching of successive brews. The brewhouse data is given in table III.

It may be observed that, notwithstanding teh lower alpha-amylase activities of Malts A and B, the conversion times were normal and are characteristic of the majority of pilot brews conducted with commercial Larker malt from the 1967 crop. This would suggest a ready availability of substrate within the malt, per se, as well as an ample enzyme activity to convert the starch of the cooker adjunct.

TABLE III

BREWHOUSE PERFORMANCE OF THE FOUR MALTS FROM LOW PROTEIN LARKER.
(Three brews made at 8-day intervals)

Malts	A	A_1	A_2	B	B_1	B_2	C	C_1	C_2	D	D_1	D_2
Conversion min.	18	18	20	18	19	18	14	15	16	13	14	14
Run-off, min.	139	140	137	137	132	127	124	110	125	118	123	120
1st Wort °P	16.95	16.68	16.70	17.04	16.79	16.90	16.90	16.70	16.58	16.81	16.90	16.59
Last Wort °P	0.8	1.3	0.9	1.8	1.3	1.4	0.8	1.0	1.1	0.5	0.8	1.3
pH	5.37	5.43	5.45	5.41	5.43	5.41	ND	5.49	5.56	5.47	5.59	5.52
Protein (g./100ml.)	0.441	0.427	0.436	0.508	0.491	0.492	0.570	0.560	0.564	0.662	0.635	0.599
Color °L.	2.15	2.10	2.30	2.50	2.40	2.40	2.85	2.90	2.40	3.00	3.00	3.00
Total Extract, %, dry	79.9	79.0	80.1	80.8	80.5	80.7	80.9	80.2	80.6	82.2	82.2	81.8

2215

Such rationale is reinforced by an examination of the carbohydrate composition of the wort. In addition to the standard procedure for determining malto-dextrin, samples of the wort were incubated (35° C.) with 0.1% of a commercial preparation of gluco-amylase. The results are presented in Table IV.

TABLE IV

CARBOHYDRATE COMPOSITION OF THE KETTLE WORTS
(12.0 - 12.1° P., 70% malt × 30% corn grits)

	MALT			
	A	B	C	D
Maltose, %, wt.	8.16	8.09	8.24	8.39
Dextrose, % wt. acid hydrolysis	10.98	10.82	10.63	11.30
Gluco-amylase	10.30	10.16	10.00	10.50

It is to be noted that the maltose levels of the four worts (Table IV) are nearly comparable; that the total malto-dextrin complex, as evidenced by the gluco-amylase hydrolysis, is relatively large for Malt A, where the shorter germination period resulted in a smaller loss of carbohydrate utilized in respiration.

The wort carbohydrate levels also attest to the adaptability of the substrate to amylase degradation. It bears repetition that the alpha-amylase activity in Malt C was approximately 75% greater than in Malt A, which analyzed 25 units.

TABLE V

FREE AMINO ACIDS IN THE KETTLE WORTS.
(12 P., 70% malt × 30% corn grits)

MALT	AMINO ACIDS (mg./100 ml.)			
	A	B	C	D
Aspartic acid	5.27	5.20	4.66	4.97
Threonine, serine and amides (as serine)	16.54	20.47	23.06	24.56
Glutamic acid	2.68	3.44	4.00	3.72
Proline	14.61	23.80	35.36	38.02
Glycine	1.83	2.45	2.79	2.98
Alanine	5.72	7.84	7.96	9.42
Valine	5.54	8.65	11.10	12.29
Methionine	1.77	2.48	2.99	3.53
Isoleucine	3.15	5.13	6.72	7.74
Leucine	6.23	10.39	13.48	15.62
Tyrosine	4.54	6.48	8.72	9.49
Phenylalanine	5.48	8.22	11.82	12.15
Ganima-amino-butyric acid	6.32	6.16	7.91	8.49
Ammonia	2.11	3.13	3.61	5.45
Lysine	6.14	6.03	7.70	10.55
Histidine	3.81	3.49	4.41	5.79
Tryptophan	3.39	3.11	5.19	5.55
Arginine	10.24	11.10	14.46	16.83
Total	105.37	137.57	175.94	197.15
Total sol. protein (mg./100 ml.)	434	497	565	619

Amino Acid Utilization in Fermentation

The free amino acids content of the kettle worts, together with ammonia, is given in Table V. Also included for reference are the soluble protein (N × 6.25) values. Each is an average of the triplicate brews.

In an examination of data in Table V, the threshold level of amino nitrogen for yeast fermentation should be considered. The range of increase of some of the essential acids within the four malts is impressive. The group arginine, serine, valine, phenylalanine and leucine reflect an increase of 50-150%.

Contrasting the amino acid levels of the wort and the resulting beer does not necessarily reflect the amino acid utilization by the yeast since amino acids are released by the yeast cell during fermentation[1]. Yet, when assessing the probable minimum amino acid requirement, an estimate may be attempted. The values in Table VI express the arithmetic difference in the levels present in wort and in beer. The values, designated as "amino acid utilization", represents the beers from the second repitching. As will be shown later, the fermentation-rate curves for the beers become comparable during this phase of study.

TABLE VI

AMINO ACID "UTILIZATION" DURING FERMENTATION

MALT	AMINO ACIDS (mg./100 ml.)			
	A	B	C	D
Aspartic Acid	3.25	4.64	2.08	1.28
Threonine-Serine	15.85	19.91	20.45	20.45
Glutamic Acid	Nil	1.89	0.75	0.47
Glycine	Nil	0.40	0.17	0.36
Alanine	0.33	1.61	Nil	0.19
Valine	2.53	4.56	4.93	4.87
Methionine	0.23	1.23	1.62	2.06
Isoleucine	2.28	4.14	4.69	4.90
Leucine	4.78	8.83	10.17	11.01
Tyrosine	1.23	1.96	2.28	2.38
Phenylalanine	3.61	5.33	6.31	5.57
Ammonia	1.60	2.57	3.17	4.84
Lysine	5.41	5.84	7.54	9.99
Histidine	1.84	1.58	1.82	2.80
Arginine	7.02	8.98	9.60	10.45
Trytophan	1.81	1.53	2.96	3.21
Totals	50.77	75.00	78.54	84.83

It will be evident that the attenuation was deficient with Malt A, and normal with Malts B and C. From the data in Table VI, it is also apparent that Malt A produces a significantly lower level of threonine (serine), valine, methionine, isoleucine, leucine and arginine.

2215

Thorne[2] has investigated the metabolism of yeast and concludes that a correlation exists between yeast growth and the production of inhibitors. It is suggested that one third of the variability in fermentation behavior can be attributed to the inhibitor aspect and the remaining two thirds to the rate at which different amino acids undergo deamination. He also refers to the possible involvement of the Stickland mechanism in which a mutual oxidation-reduction of amino acids to produce assimilable ammonia occurs. Stickland designated specific amino acids as "hydrogen donators" and "hydrogen acceptors" and assigned enhanced nutritional values to the pairing of selected acids. When compared to the Ehrlich mechanisms[2] for release of ammonia from amino acids, the Stickland mechanism postulates an enhancement of 32% for the group of amino acids involved.

The question arises whether the Stickland mechanism could assist in establishing threshold nitrogen requirements, as pertain to the parameters of the present experiment. The alpha amino nitrogen of the "donator" and "acceptor" acids in the kettle worts is given in Table VII. Proline has been omitted because it does not contribute to the assimilable nitrogen. For purposes of comparison, the sums of the "acceptor" nitrogen has been subtracted from those of the "donator" and the remainder is reported as "surplus."

TABLE VII

KETTLE WORT NITROGEN "AVAILABLE" TO THE STICKLAND REACTION

	Malt A	Malt B	Malt C	Malt D
	Hydrogen donators			
	Alpha-amino nitrogen (mg./100 ml.)			
Aspartic acid	0.63	0.62	0.56	0.59
Threonine	1.10	1.37	1.54	1.64
Glutamic acid	0.26	0.33	0.38	0.36
Alanine	0.90	1.23	1.25	1.48
Valine	0.67	1.04	1.34	1.48
Leucine	0.67	1.11	1.44	1.67
Phenylalanine	0.50	0.75	1.08	1.11
Histidine	0.38	0.35	0.37	0.58
Total	5.11	6.80	7.96	8.91
	Hydrogen acceptors			
Glycine	0.34	0.46	0.52	0.56
Trytophan	0.23	0.21	0.22	0.38
Arginine	1.64	1.78	2.32	2.69
Total	2.21	2.45	3.06	3.63
"Surplus" donator	2.90	4.35	4.90	5.38

While possibly coincidental, it may be pointed out that the percentage difference between Malts A and B for Stickland "surplus donator" (Table VII) and the "amino acid utilization" during fermentation (Table VI) are comparable.

Yeast Population in the Fermenting Worts

The fermenters were sampled at pitching and after 24-hour intervals at a 2-ft. depth in the 5-ft. deep fermenting wort. Cell counts and wort gravity were recorded and the cell density plotted against the gravity. Figures 1, 2, and 3 illustrate the results obtained on pitching with "first generation" brewery yeast, and with two consecutive repitchings of the reclaimed yeast.

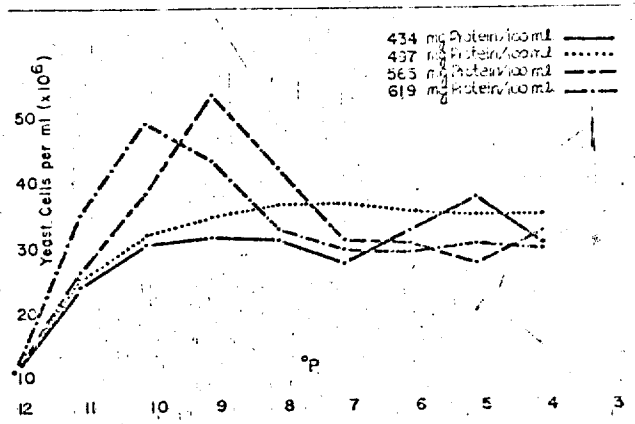

Figure 1. Yeast population of the four soluble protein levels.

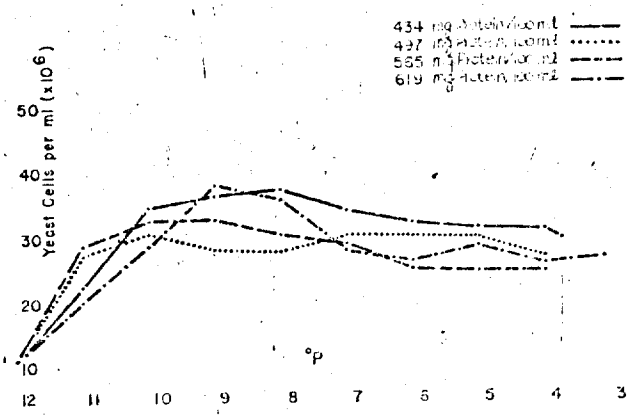

Figure 2. Yeast population of the four soluble protein levels, first repitching.

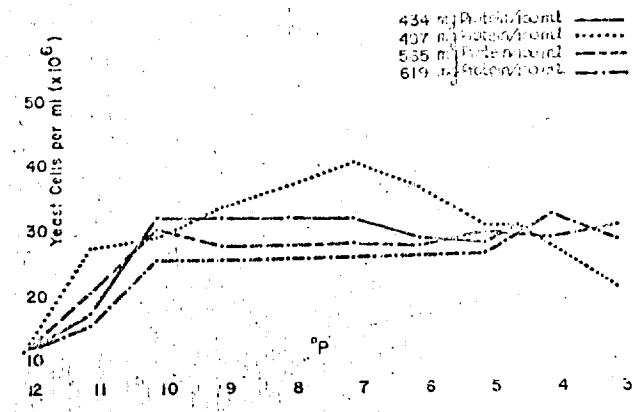

Figure 3. Yeast population of the four soluble protein levels, second repitching.

The peaks in yeast density for Malts C and D and the first pitching with fresh brewery yeast (Figure 1) level out after repitching with the yeast reclaimed from the initial brews (Figure 2). The curve for Malt B seems to reflect a comparable level for both fermentations. The possibility of a relationship to the amino acid level in the beer is suggested. The quantity of residual amino acids in the beers from two of the malts is shown in Table VIII.

TABLE VIII

RESIDUAL AMINO ACIDS IN BEER PRODUCED FROM MALT B AND MALT C
(Initial pitching and first repitching)

	Malt B		Malt C	
	Initial	Repitched	Initial	Repitched
Amino Acid* (mg./100 ml. beer)				
Aspartic Acid	1.34	2.19	4.55	3.12
Threonine	0.83	0.91	4.37	2.05
Serine	0.72	0.40	4.54	1.85
Glutamic Acid	2.14	2.73	4.27	3.78
Glycine	2.39	2.42	3.14	2.80
Alanine	7.39	8.16	9.82	9.01
Valine	4.24	5.22	7.70	6.50
Methionine	1.65	1.35	1.96	1.97
Isoleucine	1.45	1.62	3.51	2.55
Leucine	2.27	2.48	5.88	3.90
Tyrosine	4.70	4.85	7.28	6.60
Phenylalanine	3.35	3.71	7.46	5.93
Lysine	0.34	0.21	1.96	0.53
Histidine	2.33	2.19	2.98	2.58
Trytophan	1.55	1.70	2.59	2.07
Arginine	4.60	4.59	7.64	6.33
Totals	41.29	44.73	79.65	61.57

*Proline, gamma-aminobutyric acid and ammonia not included.

Diacetyl

Owades *et al.*[3] studied diacetyl synthesis by yeast and suggested that it may be a by-product of the synthesis of valine. It was demonstrated that valine, added to wort, dramatically suppressed diacetyl formation, presumably because the supply of a-acetolactate was reduced. Portno[4] confirmed these observations, showing that the time of onset of diacetyl formation was a function of the valine concentration in the wort.

A possible relationship between diacetyl formation and wort soluable nitrogen still has not been established. Since much of the present study is contingent on a large variation in malt soluble nitrogen, attention was given to the diacetyl content of the fermenting wort and the finished beer.

The 5-day fermentations were sampled at the 2-ft. depth, as in determining the yeast population. The fermenting wort was chilled to 0° C. and filtered through a Seitz filter under CO_2 pressure. All four beers were approximately 6.0° P. The filtered samples were held at 0° C. until analyzed. The results are presented in Table IX. The valine content of the wort is also included for reference.

TABLE IX

DIACETYL CONTENT OF FERMENTING WORTS AND FINISHED BEER

Malt	Valine	Fermenting Wort		Finished Beer
	(wort, ppm.)	Colorimetric A.S.B.C. (ppm.)	Gas Chromatography (ppm.)	Range of 3 brews (ppm.)
A	55.4	0.37	<0.10	0.00-0.11
B	86.5	0.27	<0.10	0.06-0.10
C	111.0	0.32	0.32	0.03-0.14
D	122.8	0.17	<0.10	0.04-0.10

With the exception of Malt C, the 5-day fermenter samples indicate a diacetyl level of about 0.1 ppm. when determined by the gas chromatographic method. The colorimetric method is now believed to be unreliable for determining diacetyl in fermenting worts, the values being too high.

It is of interest to note (Table IX) the diacetyl level is lower in the fermenting worts from the malts with the lowest soluble nitrogen and valine contents. Even though Malt D produced an unusually high level of soluble nitrogen, the resulting diacetyl level was not abnormal. The analytical data for Malt C does not seem to account for the higher level of diacetyl in the fermenting wort. Repeat analysis confirmed this high value.

Data pertinent to cellar behavior and some aspects of beer quality are given in Table X.

Primary filtration time is the time required to filter a definite volume of ruh beer under controlled conditions. It will be noted that a significantly longer time was required for the filtration of the beer produced from Malt A. A markedly higher first wort viscosity is indicated for this wort.

As stated, when comparing the amino acid contribution by the malts, Malt A reflects a definite decrease in attenuation. It should also be pointed out that such deficiency in attenuation is also reflected in the properties of the "repitched" beers.

Beer stability was determined with a Helm Radiometer at 0° C. after incubating at 43° C. for 2 weeks followed by chilling at 0° C. for 5 days. The clarity is reported in Nephelose units. Foam was determined by pouring and noting the collapse rate. While a definition of flavor may be considered subjective, it is imperative that this aspect not be overlooked—especially when beer

2215

TABLE X

ANALYSES OF THE BEERS PRODUCED FROM THE FOUR MALTS

MALT	A	A_1	A_2	B	B_1	B_2	C	C_1	C_2	D	D_1	D_2
Prim. Filt. Time (min.)	155	150	180	80	100	95	107	100	90	76	45	75
Protein %	0.315	0.298	0.305	0.336	0.332	0.332	0.405	0.402	0.408	0.463	0.470	0.464
Real Deg. Atten., %	55.6	57.8	55.0	61.4	59.5	61.3	59.5	60.2	60.8	62.4	63.4	62.8
Beer Stability (Neph. 32° F.)												
2 wk. 110° F.	51.5	58.0	54.0	70.0	67.0	66.0	59.5	51.0	54.5	66.0	63.0	60.5
Foam	27.1	33.1	25.2	24.7	17.4	16.0	26.8	15.1	18.9	20.4	18.3	18.6
Flavor												
Fresh Beer	13.4	14.7	13.9	14.9	15.3	13.6	13.9	13.6	13.0	12.0	11.6	12.2

Foam: <20-Excellent, 21-30-Very Good, 31-40-Good

Flavor: 10-11-Fair, 12-Fair to Good, 13-15-Good

has been prepared from a diversity of worts. Beers produced from Malts A, B, and C were considered desirable; that from Malt D was characterized by a "sharper" or "aldehydic" flavor.

SUMMARY

Four unsulfured malts with a wide range of soluble nitrogen were produced from "low protein" Larker malt. Acrospire development was comparable.

The malt containing the lowest level of soluble nitrogen also showed the least alpha-amylase activity, the lowest extract, and the greatest fine-coarse extract difference.

In the pilot brewery, mechanical brewhouse performance was comparable for the lots. While not included in the data presented, Malt A, which produced the lowest soluble nitrogen, gave a high first wort viscosity which was ultimately reflected in a slower rate of primary beer filtration.

Comparable quantities of reducing sugars and dextrins were found in the kettle worts. However, a significantly lower degree of attenuation was observed for the initial pitching as well as the two repitched beers from Malt A.

Yeast population during fermentation was estimated at 24-hour intervals. Malts C and D, treated with gibberellic acid and producing the larger quantities of soluble nitrogen, reflected high population peaks in the initial pitching. However, these "flattened out" upon repitching and generally paralleled the curves from the lower nitrogen worts.

Speculation as to the yeast population in the initially pitched and in the repitched brews led to a determination of the residual beer amino acids for Malts B and C ("flat" and "peaked" curves, respectively, in the initial pitching). It was noted that the residual beer amino acid content was comparable for Malt B, while a large reduction was observed when Malt C was repitched. While not included in the data presented, similar results were obtained with Malt D.

Amino acid "utilization" during fermentation was considered and Malts B and C were found to give comparable results. Malt A gave a lower utilization of the order of 35%.

Since the Stickland mechanism postulates that the pairing of specific amino acids results in a greater ammonia utilization by the yeast, the wort amino acids were examined in this connection. Here, too, it was revealed that Malt A produced much less "donator" acid than Malts B, C, and D.

The wort valine levels for the four malts ranged from a low to greater than the high reported in the literature. Fermenting worts (at about 6° P.) and the finished beers were analyzed for diacetyl content and values approximating 0.1 ppm. were found for all samples except for the fermenting wort for Malt C, an intermediate "high" soluble nitrogen wort. Within the parameters of this study, the lower soluble nitrogen malts were not associated with abnormally high diacetyl formation.

Based on the standards for pilot brewing, the clarity stability was good for all of the beers. The foam values, although somewhat variable, were also good. Flavor was thought to be good for the beer from Malts A, B, and C; that from Malt D was considered less desirable.

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2216

120

DIACETYL PRODUCTION IN ORANGE JUICE BY ORGANISMS GROWN IN A CONTINUOUS CULTURE SYSTEM

Florida State Hort Soc. 69: 120-5, (1956)

(2216)

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INTRODUCTION

The development of an off-flavor and odor during the manufacture of frozen orange juice concentrate has resulted in severe economic losses to a number of citrus concentrate packers. This off-flavor is reminiscent of "buttermilk" and is a result of the accumulation of diacetyl, a metabolic product of certain bacteria. Other than the work by Kilburn and Tuthill (11), former studies on the growth characteristics of these organisms and their production of diacetyl in orange juice has been limited to static batchwise cultures. Kilburn and Tuthill (11) used the continuous culture method to show the relationship between data obtained by plate count, microsopic count, and diacetyl analysis, and thereby validated the use of the latter analysis as a quality control tool in the citrus industry.

It was considered appropriate to supplement this applied investigation with a more basic approach to the characteristics involved in the formation of diacetyl in orange juice in a continuous culture system. This technique manages a unique separation of the rate of diacetyl production by a given organism from that organism's rate of growth. Batchwise investigations are necessarily a summation of growth and metabolic product formation and the latter cannot be studied separately.

In the continuous culture system the vessel or fermentor in which the test organisms are growing and forming metabolic products is supplied with fresh sterile medium at a constant rate. A constant volume is maintained in the fermentor by having an overflow rate that is constant as well as equal to the input rate of the fresh medium. By appropriate adjustment of the flow rate through the fermentor, a constant microbial population density of actively growing organisms can be maintained. At this constant population the rate of product formation can be studied without being affected by variations in the number of organisms.

THEORY OF PRODUCT FORMATION IN A CONTINUOUS CULTURE SYSTEM

To assist the reader, the following nomenclature will be used in the development of equations:

- a = diacetyl concentration at time t (in p.p.m.)
- a_o = diacetyl concentration at zero time (in p.p.m.)
- k = growth rate constant (in reciprocal hours)
- k' = reaction rate constant for diacetyl formation (in p.p.m. formed per hour by one million organisms per ml or a population density of one.)
- R = medium flow rate (in ml per hour)
- t = time (in hours)
- V = capacity of the fermentor (in ml)
- x = bacterial population density at time t (in million organisms per ml)

2216

x_0 = bacterial population density at zero time

Equations applicable to the behavior of a population density with time in a continuous culture system were derived by Monod (14) using a differential material balance of the system. By a slightly different approach, Golle (8) arrived at the same basic equation of

$$\frac{dx}{dt} = kx - \frac{Rx}{V}$$

Equation (1) states that the rate of change of bacterial population in the fermentor with time is equal to the rate of bacterial density increase less the rate at which the bacteria are being washed from the fermentor. Integration of equation (1) yields,

$$\frac{1}{t}\ln\frac{x}{x_0} = k - \frac{R}{V} \quad \text{or} \quad x = x_0 e^{(k-R/V)t}$$

Hence, when the rate of medium flow (R) divided by the capacity (V) of the fermentor is equal to growth rate constant (k), the population density of the bacteria in the fermentor will remain constant. Monod (14) presented a rigorous proof of this fact. Under these conditions the bacteria were being washed from the fermentor in the overflow at a rate commensurate with their rate of reproduction in the fermentor.

Although the characteristics of a bacterial population in a continuous culture system, as discussed above, have received a good deal of attention (6, 16), only a limited description of product formation by bacteria in this system has been made. Maxon (13) in his microbiological process report on continuous fermentation presented a detailed treatment of product formation in cases where the substrate concentration was rate-limiting. However, in this investigation of the rate of diacetyl production in orange juice, as in many other fermentations where the substrate concentration might be excessive, the rate-limiting factor in the system would be the enzyme concentration or bacterial population density.

In attempting to develop an expression for the product concentration and rate of product formation in a continuous culture system, it was assumed that the rate of product formation was proportional to the bacterial population density and that this was rate-limiting. Further, it was assumed that product concentration in the overflow from the fermentor was representative of the instantaneous product concentration within the fermentor, which from a practical standpoint, as shown by MacDonald and Piret (12), was not too difficult to realize. With these assumptions, a differential material balance of the fermentor gives the following expression:

$$da = k'x\,dt - \frac{Ra}{V}dt$$

In equation (3) the terms, R, V, and k' are constants, but the population density, x, in a general solution is a variable with time. However, the variation of the population density with time is described by the exponential form of equation (2). Hence, substituting for x in equation (3) from equation (2) and rearranging gives the basic expression

$$\frac{da}{dt} + \frac{R}{V}a = k'x_0 e^{(k-R/V)t}$$

This differential equation is first order and linear with respect to a, and has the solution:

$$a = e^{-\int\frac{R}{V}dt}\left[\int k'x_0 e^{(k-R/V)t} \cdot e^{\int\frac{R}{V}dt}\,dt + C\right]$$

which upon integration and simplification becomes

$$a = \frac{k'x_0}{k}e^{(k-R/V)t} + Ce^{-Rt/V}$$

When t=0, then a=a_0 and the constant of integration becomes

$$C = a_0 - \frac{k'x_0}{k}$$

Substituting into equation (6) for the constant of integration, C, as given in equation (7), the final expression obtained is:

$$a = \frac{k'x_0 e^{-Rt/V}}{k}(e^{kt} - 1) + a_0 e^{-Rt/V}$$

When equation (8) was used in the calculation of experimental results the unknown was the reaction rate constant for diacetyl formation, k'. Implicit in this constant are the experimental conditions such as temperature, medium, etc., but most important, the organism being tested. A comparison of the k' values of two different organisms tested under essentially the same conditions was a comparison of their ability to produce diacetyl

2216

under these conditions. Further, if the k' value of an organism was known, the level of diacetyl concentration for any bacterial population density in the continuous culture system could be estimated.

Determination of k': Under experimental conditions the population density was held essentially constant with x_n equal to x. Also under these conditions the diacetyl concentration is constant and a_n is equal to a. To obtain constant x and a values, conditions were adjusted such that k is equal to R/V. Hence, substituting into equation (8) for x_n, a_n, and k gives

$$a = \frac{k'xe^{-Rt/V}}{R/V}(e^{Rt/V} - 1) + ae^{-Rt/V}$$

which simplified and solved for k' gives

$$k' = \frac{aR}{xV}$$

The units of k' are expressed as p.p.m. of diacetyl formed per hour by a population density of one (a million organisms per ml).

Experimental Methods

The continuous culture system employed did not vary appreciably in general set-up from those already described in the literature (2, 4, 6, 7, 15), except for possible simplification. The fermentor consisted of a 500 ml flask with a side arm overflow. Both the fermentor and a sterile medium supply flask were immersed in a 30° C. constant temperature bath and equipped with magnetic stirrers to insure adequate agitation. The flow rate of sterile orange juice was controlled by a Sigmamotor pump which delivered by positive displacement as low as 4.7 ml per hour.

Growth in the fermentor was followed by plate counts and direct counts. A number of direct counting methods have been developed specifically for the determination of organisms in orange juice (9, 18, 21) and these along with some 25 other staining procedures were examined for possible use. The procedure finally adopted was as follows: With the use of a standard loop, 0.01 ml of the sample to be counted was evenly spread over an area of 1 sq. cm. that had previously been ruled off on a clean slide. The smear was air dried and lightly fixed with heat. A drop (approximately 0.01 ml) of stain solution (consisting of 1 ml 10% tannic acid, 2 ml 0.001% aqueous methylene blue, and 1 drop of acid alcohol) was placed over the fixed smear and examined through a coverslip which is sealed to the slide with wax. This wax seal prevents the evaporation of the stain solution. The medium dark contrast oil immersion objective of a phase microscope and 15X oculars were used in counting. The oculars contained a grid on which a field corresponding to 1/400th of a sq. mm. on the smear was ruled off by previously viewing a haemocytometer under the same conditions. The dilution factor for counting was 4×10^7.

From the number of different methods available in the literature for the quantitative determination of diacetyl (5, 17, 19), several of which were specifically used for the detection of diacetyl in orange juice (3, 10), the procedure of Westerfeld (20) was finally chosen and used without modification. To prepare the sample of orange juice for the diacetyl test, a distillation was performed. Distillation was not carried out for the purpose of concentration as done by Hill, Wenzel and Barreto (10) and by Byer (3), but to physically separate the diacetyl from the orange juice. The single strength juice used for this study contained an unknown substance which inhibited the development of the diacetyl test. It was shown by a series of experiments that this inhibition was not due to the juice masking the color of the test, to the presence of an unknown constituent in the juice competing with diacetyl for the guanidinyl groups of creatine, or to the juice having sufficient buffering capacity to prevent obtaining the required alkalinity. An attempt to remove the inhibiting factor by treatment with Norite charcoal was unsuccessful, hence, distillation was employed.

A standard curve relating diacetyl concentration and optical density readings was constructed using known amounts of purified diacetyl.

The two organisms used in this investigation, *Lactobacillus* No. 802 and *Lactobacillus* No. 805, were both originally isolated from orange juice and for neither organism was the species established. Both organisms were capable of growth in orange juice with the produc-

2216

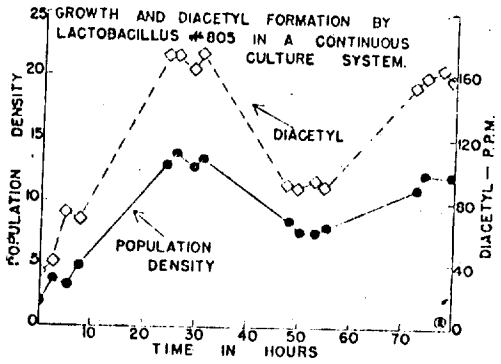
tion of a typical "buttermilk" off-odor and off-flavor.

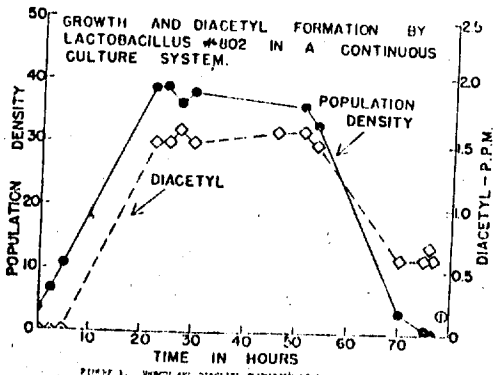
To start a continuous culture system experiment, the fermentor was inoculated with an actively growing culture of the organism to be tested. This culture was allowed to grow batchwise in the fermentor for five to eight hours before starting to add fresh sterile single strength orange juice. The fresh juice was added continuously and at a constant rate to the fermentor, although the rate was sometimes purposely changed during the course of an experiment to some other constant value. While a run was in progress, direct counts, plate counts, and diacetyl tests were made. The overflow of juice was collected and the volume measured at various times to determine the flow rate, R. When the run was completed—generally from 50 to 80 hours after the start—the population density and diacetyl concentration were plotted against time to give a graphical picture of the experiment. The rate of diacetyl production was also calculated. Each experiment differed slightly in detail, but all of them followed the general pattern described.

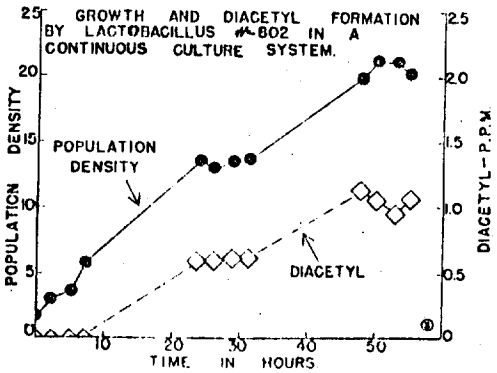
Results and Discussion

Three representative graphs of three typical experiments are given in Figs. 1, 2, and 3. In these three experiments, as well as in all other experiments performed during the course of this investigation, when the rate of delivery of fresh juice was altered, an alteration in the population density and the diacetyl concentration was experienced. Both the population density and the diacetyl concentration under conditions of slightly changed flow rate would seek new equilibrium concentrations and remain constant at these new levels as long as the new flow rate was kept constant. In seeking this new level the diacetyl concentration paralleled the population density as was observed by Kilburn and Tuthill (11) in their investigation. Adams and Hungate (1) discussed this leveling off phenomenon at some length and devised a system of predicting the leveling off population density at different flow rates.

In Fig. 1 over the course of the experiment the flow rate gradually increased until at about 50 hours the flow rate had increased







2216

sufficiently to dilute the organisms from the fermentor at a rate greater than they were being reproduced and both the population density and the diacetyl concentration dropped. However, it is important to note that these variables were kept constant for something over 30 hours. In Figs. 2 and 3, the flow rate was purposely changed and the curves responded as would be expected.

For those portions of these experiments where the population density and the diacetyl concentration remained constant, values of k′ were calculated using equation (10). Using all of the data available, k′ values were calculated and averaged for both test organisms. The average k′ value for *Lactobacillus* No. 805 was 2.5 and that of *Lactobacillus* No. 802 was 0.015. The reaction rate constant k′ is the proportionality factor which relates the rate of diacetyl production by a given organism to its population density, while the constant ratio of k′/k relates the concentration of diacetyl to the population density under equilibrium conditions. The average k′ values given merely express an order of magnitude of diacetyl formation, since at the high population densities used experimentally, the value of k varies slightly with the population density, as discussed by Adams and Hungate (1). Since k was considered a constant in the integration of equation (4), a true variation in k in turn caused a pseudo variation in k′, although the magnitude of the error was not great. At the lower population densities that would be met in commercial operations (less than one million organisms per ml), both k and k′ would be constant. Experimentally, however, it was very difficult to adequately balance the continuous culture system at these lower levels.

The results of these experiments again emphasize the lack of validity in using either plate or direct counts as the sole criterion for predicting potential off-flavor in concentrated orange juice. Organism No. 805 produced diacetyl at about 167 times the rate of organism No. 802, while growing at a rate that is roughly 67% the rate of the latter. Disregarding dilution, ten thousand No. 805 organisms per ml would produce 0.1 p.p.m. diacetyl (an unacceptable level) in four hours while it would require one million No. 802 organisms per ml a period of eight hours to produce this same level of diacetyl concentration.

Summary

Two organisms previously implicated in the spoilage of orange juice were grown in the continuous culture system and their rates of diacetyl production were determined. One of the test organisms, *Lactobacillus* No. 802, produced diacetyl at the rate of 0.015 p.p.m. per hour, per one million organisms per ml. The other test organism, *Lactobacillus* No. 805, produced diacetyl at a rate 167 times as great as the first, or 2.5 p.p.m. of diacetyl per hour, per one million organisms per ml.

The relationship of product formation to population density in a continuous culture system was mathematically defined and a method for calculating the diacetyl concentration and rate of diacetyl formation was derived.

Acknowledgment

The author wishes to thank V. S. Troy, J. M. Berry, and J. F. Folinazzo for their assistance and helpful suggestions and other members of the research staff who reviewed this manuscript and assisted in its preparation for publication.

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