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Starter Distillate-Volume 3 #86 7/12/74

VOLUME 3

GRAS MONOGRAPH SERIES

STARTER DISTILLATE

(COPIES OF ARTICLES CITED BUT NOT USED IN MONOGRAPH SUMMARY)

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

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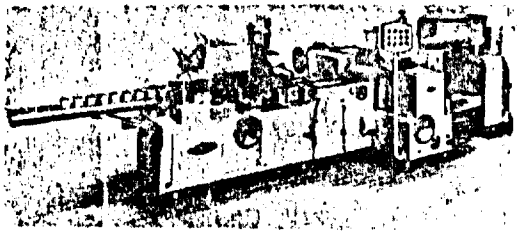

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THE INSTITUTE OF BREWING ANALYSIS COMMITTEE
THE DETERMINATION OF VICINAL DIKETONES IN BEER

Submitted by R. G. Ault for the Analysis Committee.

Received 30th August, 1967

J. Inst. Brew. (1968) 74 (2), 196-9

A survey of a number of methods for the determination of the diacetyl content of beer resulted in the recommendation for routine use of a colorimetric technique based on the method of Owades, Jakovac & Vigilante. The method is both simple and rapid in operation but is not specific for diacetyl, estimating also other related diketones. It was felt, however, that the estimation of vicinal diketones was advantageous since the results were more in line with aroma assessment.

The Analysis Committee appointed the following Sub-Committee to recommend a method for the determination of diacetyl in beer:—

R. G. Ault (Chairman), M. O. Jones, J. R. Hudson, G. A. Howard, P. A. Martin, P. F. Bayles, L. R. Bishop, R. N. Greenshields, L. Taylor, A. Sinclair (co-opted). The findings of the Sub-Committee are given in this paper.

Of the numerous published methods available for the determination of diacetyl, the Sub-Committee selected four for trial purposes *viz.*

(I) Owades, J. L., Jakovac, J. A., & Vigilante, C., *Amer. Brewer*, January 1961, **42**, 23–25.

(II) Owades, J. L., & Jakovac, J. A., *Proc. A.M. Amer. Soc. Brew. Chem.*, 1963, 22.

(III) Brenner, M. W., Blick, S. R., Frenkel, G., & Siebenberg, J., *Proc. Eur. Brew. Conv. Brussels*, 1963, 233.

(IV) Method (III) as modified by A. D. Portno, this *Journal*, 1965, 9.

Whereas Method I is a non-specific method measuring vicinal diketones, Methods II, III, IV are stated to be specific for diacetyl.

Five collaborative tests in all were carried out by the Sub-Committee but preliminary trials suggested that Method II was difficult to operate and too time-consuming for routine laboratory analysis while the modification of Method III as proposed by A. D. Portno was preferred to the original method. The use of a 100-ml. Markham still in Methods I and IV was considered to be an improvement and further work was continued with these two methods. Experiments with added diacetyl showed that Method I gave somewhat better recoveries than Method IV. (See Table I).

With Method I the recovery was 90–92%, whereas in Method IV the recovery was 66–80%, and, since it is known that there is an actual loss of diacetyl during distillation, the recoveries recorded for Method I were considered to be most satisfactory.

In view of the heterogeneous character of the diacetyl fraction in beer, the Committee felt that there was some doubt about the specificity of Method IV for the determination of diacetyl in beer. Moreover, since the "diacetyl flavour" is a summation of flavours from all the closely related diketones (*e.g.*, 2,3-pentane dione, etc.) comprising the diacetyl fraction, the view was taken that a non-specific method such as that of Owades, Jakovac & Vigilante would give results more in line with aroma assessment. Further collaborative work on the determination of vicinal diketones (V.D.K.) in beer by Method I showed that there was good agreement between replicates within laboratories and satisfactory agreement on the recorded average values between laboratories. This is clearly shown in Table II where the results for a range of beers with differing diacetyl contents are tabulated.

On the basis of the above results and other confirmatory work not here recorded, the Committee felt justified in recommending the use of Method I for beers with diacetyl contents up to approx. 1·5 p.p.m. This method differed from that originally described by Owades, Jakovac & Vigilante only on

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TABLE I

RECOVERY OF ADDED DIACETYL

Laboratory	Method I			Method IV		
	Diacetyl (p.p.m.)			Diacetyl (p.p.m.)		
	Control	Control + 0·5 p.p.m. diacetyl	Control + 1·0 p.p.m. diacetyl	Control	Control + 0·5 p.p.m. diacetyl	Control + 1·0 p.p.m. diacetyl
A	0·40	0·96	1·49	0·22	0·59	0·97
B	0·24	0·72	1·22	0·16	0·50	1·02
D	0·25	0·60	1·09	0·16	0·42	0·91
E	0·26	0·72	1·20	0·16	0·47	0·99
F	0·28	0·72	1·27	0·15	0·47	0·95
G	0·15	0·52	0·88	0·16	0·47	0·88
H	0·18	0·64	1·08	0·15	0·59	1·04
Average	0·25	0·70	1·17	0·17	0·50	0·97
Recovery	90–92%			66–80%		

minor points, *e.g.*, the use of the 100-ml. Markham still, no decarbonation of samples, the limitation of the distillation time to 5–10 min. and the use of a distilled water blank instead of a reagent blank In making the recommendation, the Committee had also in mind the simplicity and rapidity of the actual estimation and the fact that, apart from a spectrophotometer, only inexpensive apparatus was required.

TABLE II

VICINAL DIKETONE CONTENT OF VARIOUS BEERS BY METHOD I

Laboratory	Vicinal diketones (p.p.m.)									
	Sample I		Sample II		Sample III		Sample IV		Sample V	
	Result	Average	Result	Average	Result	Average	Result	Average	Result	Average
A	0·11 0·14 0·13	0·13	0·26 0·28 0·27	0·27	0·71 0·70 0·70	0·70	0·73 0·73 0·73	0·73	1·56 1·56 1·57	1·56
B	0·07 0·09	0·08	0·10 0·11	0·11	0·69 0·57	0·63	0·71 0·63	0·67	1·60 1·54	1·57
C	0·13	0·13	0·15	0·15	0·90 0·90	0·90	1·0 0·90 0·90	0·93	2·20 2·10	2·15
D	0·18 0·10 0·20 0·13	0·15	0·23 0·21 0·18 0·25	0·22	0·91 0·91	0·91	1·13 1·13	1·13	2·00 1·95	1·98
E	0·12 0·13	0·13	0·22 0·23	0·23	0·87 0·88	0·88	0·92 0·89	0·91	1·84 1·85	1·85
F	0·07	0·07	0·24	0·24	0·58 0·46	0·52	0·80 0·84	0·82	1·10 1·17	1·14
G	0·12 0·13 0·11 0·11	0·11	0·18 0·19 0·20	0·19	0·58 0·57	0·58	0·64 0·62	0·63	1·24 1·21	1·23
H	0·09 0·08 0·08	0·08	0·16 0·16 0·14	0·15	0·74 0·76	0·75	0·84 0·84	0·84	1·82 1·70	1·76
Average		0·11		0·20		0·73		0·83		1·65
Std. Dev.		0·03		0·05		0·14		0·15		0·33

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Nevertheless, it must be emphasized that the method is not specific for diacetyl since 2,3-pentane dione and other related diketones react to give a colour with α-naphthol and are hence included in the value obtained. The details of the recommended procedure are set out below.

Determination of Vicinal Diketones in Beer

Reagents

(*a*) Alpha-naphthol solution.

Dissolve 4·0 g. of pure α-naphthol in 100 ml. of 99·6% re-distilled isopropanol.

The solution is decolorized with activated charcoal (approx. 0·5 gms.) and may be stored in the dark in an amber glass bottle for periods up to two weeks.

(*b*) KOH-Creatine solution.

Dissolve 0·3 g. of creatine in 80 ml. of 40% potassium hydroxide and filter. Store cold, preferably in a polyethylene container.

(*c*) Diacetyl stock solution.

Dissolve 0·5 g. of pure diacetyl (or equivalent amount of commercial diacetyl, the purity of which has been previously determined) in 1000 ml. of distilled water and store cold in an amber glass bottle.

Immediately before use, dilute 1 ml. of the stock solution to 100 ml. with distilled water. One ml. of this solution contains 5·0 μg. of diacetyl.

(*d*) Silicone antifoam agent.

Apparatus

(*a*) Glass distillation apparatus, consisting of:

(i) Markham still, 100 ml. capacity (manufactured by Med. Lab. Ltd., 2–6 Agard Street, Derby).

(ii) Graduated cylinder (25 ml. capacity).

(*b*) Volumetric flasks, 10 ml. and 25 ml.

(*c*) Spectrophotometer suitable for measuring absorbance at 230 mμ and 530 mμ.

Method

100 ml. of the sample (beer, not decarbonated, or diacetyl solution of known concentration) is distilled in the Markham still, the distillate being collected in a 25-ml. graduated cylinder containing 3 ml. of distilled water. During distillation the tip of the condenser must be under the surface of the liquid in the cylinder. The rate of distillation should be such that 15–20 ml. is collected in a time of 5–10 min. Frothing can be prevented by the use of a silicone antifoam agent. The volume is then made up to the 25-ml. mark with distilled water and a 5-ml. aliquot taken for colour development.

To the 5-ml. aliquot is added 1 ml. of the decolorized α-naphthol solution and 0·5 ml. of the alkaline creatine solution with shaking after each addition. The mixture is made up as rapidly as possible to a volume of 10 ml. with distilled water and shaken vigorously for 1 min. The O.D. is then read at 530 mμ. against a distilled water blank between five and six minutes from the end of shaking.

Results are calculated by reference to a standard curve constructed by submitting freshly-prepared diacetyl solutions of known concentration to the same distillation and colour development procedures and plotting optical densities against concentration. The response is linear over the range 0–2 p.p.m. diacetyl. A method for determining the purity of commercial diacetyl preparations is described in the Appendix.

APPENDIX

Determination of the Purity of Diacetyl Preparations

Commercially-available diacetyl preparations vary in their content of diacetyl and it is necessary to make the appropriate correction when preparing the standard curve in the recommended method. The purity of the diacetyl solution used as standard may be determined by calibration against pure dimethylglyoxime according to the method of Brenner, Blick, Frenkel & Siebenberg (*i.e.*, Method III).

Reagents

(i) Hydroxylamine hydrochloride, A.R. grade. 10% aqueous solution.

(ii) Sodium tetraborate, A.R. grade. 1·91% aqueous solution with freshly boiled distilled water (final pH 9·1).

(iii) Standard dimethylglyoxime solution. Dissolve 0·1348 g. of pure dimethylglyoxime in 3–5 ml. of ethanol and make up to 1000 ml. with pure distilled water. One ml. of this solution contains the equivalent of 100 μg. of diacetyl.

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Preparation of a standard curve for dimethylglyoxime

Using a microburette, a range of solutions is prepared such that 20-ml. portions contain the equivalent of 0·00–100·0 μg. of diacetyl. For example, 0·5 ml. of standard solution +2 ml. of borate buffer +17·5 ml. water (total volume 20 ml.) contain the equivalent of 50 μg. diacetyl. Each final 20-ml. portion is read against a blank of distilled water at 230 mμ. A standard curve is then constructed by plotting O.D. against diacetyl concentration.

Method A (without distillation)

The diacetyl preparation is freshly distilled and a solution is accurately made up containing theoretically 5 p.p.m. diacetyl (on the basis of 100% purity). Two 10-ml. portions are pipetted into two 25-ml. conical flasks and designated sample and blank. 1 ml. of 10% hydroxylamine hydrochloride solution is added to the sample and both flasks are maintained for 10–15 min. in a water-bath at 80° C. The bath temperature is then raised to 103–105° C. and the flasks are not removed until the volume in each flask is 3–4 ml. The flasks are cooled and 2·0 ml. of borate buffer are pipetted into each flask. One ml. of 10% hydroxylamine hydrochloride solution is now added to the blank and the volume adjusted in each flask to 20 ml. The optical density of the sample against the blank is read at 230 mμ.

The concentration of diacetyl in the sample can be determined by reference to the standard dimethylglyoxime curve. The value must be doubled to give the true diacetyl concentration in the original diacetyl solution and from this the purity of the commercial preparation can be readily calculated.

Alternative Method B (with distillation)

The diacetyl preparation is freshly distilled and a solution is accurately made up containing theoretically 1·0 p.p.m. diacetyl (on the basis of 100% purity). 100 ml. of this solution are distilled in the Markham still, the distillate being collected in a 25-ml. graduated cylinder containing 3 ml. of distilled water. During distillation, the tip of the condenser must dip below the level of the water in the cylinder. Approx. 15 ml. of distillate are collected making the total volume about 18 ml. The volume is made up to 20 ml. with distilled water and divided into two 10-ml. portions. These are transferred into two 25-ml. conical flasks and designated sample and blank. 1 ml. of 10% hydroxylamine hydrochloride solution is added to the sample and both flasks are maintained for 10–15 min. in a water-bath at 80° C. The bath temperature is then raised to 103–105° C. and the flasks are not removed until the volume in each flask is 3–4 ml. The flasks are cooled and 2·0 ml. of borate buffer are pipetted into each flask. One ml. of 10% hydroxylamine hydrochloride solution is now added to the blank and the volume adjusted in each flask to 20 ml. The optical density of the sample against the blank is read at 230 mμ.

The concentration of diacetyl in the sample can be determined by reference to the standard dimethylglyoxime curve. The value must be doubled to give the true diacetyl concentration in the original diacetyl solution and from this the purity of the commerical preparation can be readily calculated.

Tests have shown that Method (B) gives very slightly lower results than Method (A) as a result of distillation losses of 4–5% of diacetyl. Distillation of commercial diacetyl preparations was found to increase the purity significantly (*e.g.* by 15%).

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L. W. AURAND, J. A. SINGLETON, T. A. BELL, and J. L. ETCHELLS
Department of Food Science and U. S. Food Fermentation Laboratory
North Carolina Agricultural Experiment Station
Raleigh, North Carolina 27607

Volatile Components in the Vapors of Natural and Distilled Vinegars

J. Food Sci. 31(2): 172- 7, (1966)

SUMMARY

Volatile components in the vapors of natural and distilled vinegars were identified by gas chromatography by comparing their retention volumes with compounds of known composition and by functional group analysis. Twenty-five volatile components were identified from three natural vinegars (cider, 19 components; wine, 17 components; tarragon, 20 components), and 11 volatile components were identified from five samples of distilled vinegar. Four components were present in all vinegars tested: acetaldehyde, acetone, ethyl acetate, and ethyl alcohol. The natural vinegars had the largest number of volatile components. This was attributed to the nature of the fermentable carbohydrate material used for production of the alcohol subsequently used for vinegar manufacture (e.g. apples and grapes). The importance of the carbonyls, alcohols, and esters to vinegar flavor is discussed.

INTRODUCTION

Vinegar (meaning sour wine) has been used as a food ingredient for over a thousand years, and it is only in the last one hundred years that the chemical and microbiological nature has been realized (Prescott and Dunn, 1959). By definition, vinegars are condiments made from alcoholic fermentation of carbohydrates and then subsequent bacterial fermentation to acetic acid. Although most of the commercial vinegar is made by this two-step process, vinegar is also made by the acetic acid bacteria directly from ethyl alcohol.

Vinegar methods of manufacture fall into two general classes: 1) "distilled," "grain," or "spirit," which denotes that the ethyl alcohol was concentrated by distillation; and 2) "natural," which designates the carbohydrate sources for the alcoholic fermentation and is so described on the labels as "apple," "cider," "wine," "grape," and "malt." Vinegars are measured in strength by their acetic acid concentration (1 grain equals 0.1% acid), and the commercial products range from 50 to 120 grains. Aqueous solutions of glacial acetic acid have a sharp unpleasant taste, so the characteristic flavors of vinegar are dependent on substances other than acetic acid, i.e., the flavor of a vinegar depends on the constituents formed during the fermentation of the raw materials as well as the subsequent acetous fermentation. Little is known about the volatile constituents that give a particular vinegar its characteristic aroma. Earlier studies (Jacobs, 1951) on cider vinegar gave the range of volatile esters as 0.30 to 0.91 g/100 ml, measured as ethyl acetate, and of alcohol as 0.03 to 2.00 g/100 ml. In vinegar generator operations, residual alcohol concentration of 0.3–0.5% is considered satisfactory. Conner *et al.* (1959) demonstrated that as the acetic acid in the final product is increased, the cycle time lengthens, the rate at which oxygen is utilized decreases, and the residual alcohol increases (e.g., an increase from 108 to 120 grain increases cycle time from 1 to 2 weeks and residual alcohol from 0.26 to 0.56%).

Suomalainen and Kangasperko (1963), using gas chromatography, identified the volatile components of a wine vinegar as acetoin, isoamyl alcohol, optically active amyl alcohol, isobutyl alcohol, ethyl acetate, isobutyl acetate, sec. butyl alcohol, isoamyl acetate, and diacetyl. They also reported that a distilled vinegar sample contained only ethyl acetate as the volatile component.

The standards of the Food and Drug Administration define the natural vinegars as a condiment made by the alcoholic and subsequent acetous fermentation of the juices of apples, and to contain not less than 4 g acetic acid per 100 ml (20°C). The same standard applies for other vinegars except that source of carbohydrate is replaced with natural products as grape, corn, barley, rye, and malt. With distilled vinegar the standard does not indicate the carbohydrate source or method of ethyl alcohol manufacture but requires the product to be made from the ace-

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ous fermentation of dilute distilled ethyl alcohol and to contain not less than 4 g acetic acid per 100 ml (20°C) (Prescott and Dunn, 1959).

MATERIALS AND METHODS

Source and preparation of vinegar samples. Five commercial vinegars, purchased from the local markets, and three vinegar samples supplied from pickle companies which operate their own vinegar generators, were analyzed for volatile flavor constituents. The description of the vinegar samples, together with their percent acetic acid, is as follows: a) apple cider (5%); b) wine (6%) manufactured from California grape wine; c) tarragon flavored (5%), a mixture of distilled and malt vinegars with tarragon spices; d) distilled vinegar, sample No. 1, labeled "Crystal Distilled" (5%); e) distilled, sample No. 2 marked "Distilled White" (5%) and manufactured from corn, barley malt, and rye; f) distilled sample No. 3 (6%) manufactured by a pickle company located in Minnesota and using the "Frings Process"; g) distilled sample No. 4 (11.6%) from a pickle company in Michigan, made by "acetator process"; h) distilled sample No. 5 (11.6%) from a pickle company in Ohio, made by "Frings process." The above five commercial vinegars were diluted by the manufacturer to the acidities indicated for uniform pickling and table strength. Ten ml of each vinegar was added to a vial containing anhydrous sodium sulfate (1.2 g/ml). The vial was sealed with a rubber septum, shaken for 5 min, and then placed in a constant-temperature water bath (70°C) for a 3-min period to equilibrate the volatile vapors. A 5-ml vapor sample was withdrawn and subjected to gas chromatographic (GLC) analysis.

Separation and identification. A Barber-Colman gas chromatograph unit, model 10, equipped with a flame ionization detector, was used for the qualitative determination of the volatile compounds. The samples were chromatographed on two different stationary phases. The U-shaped columns were heavy-walled glass tubing, 5 mm

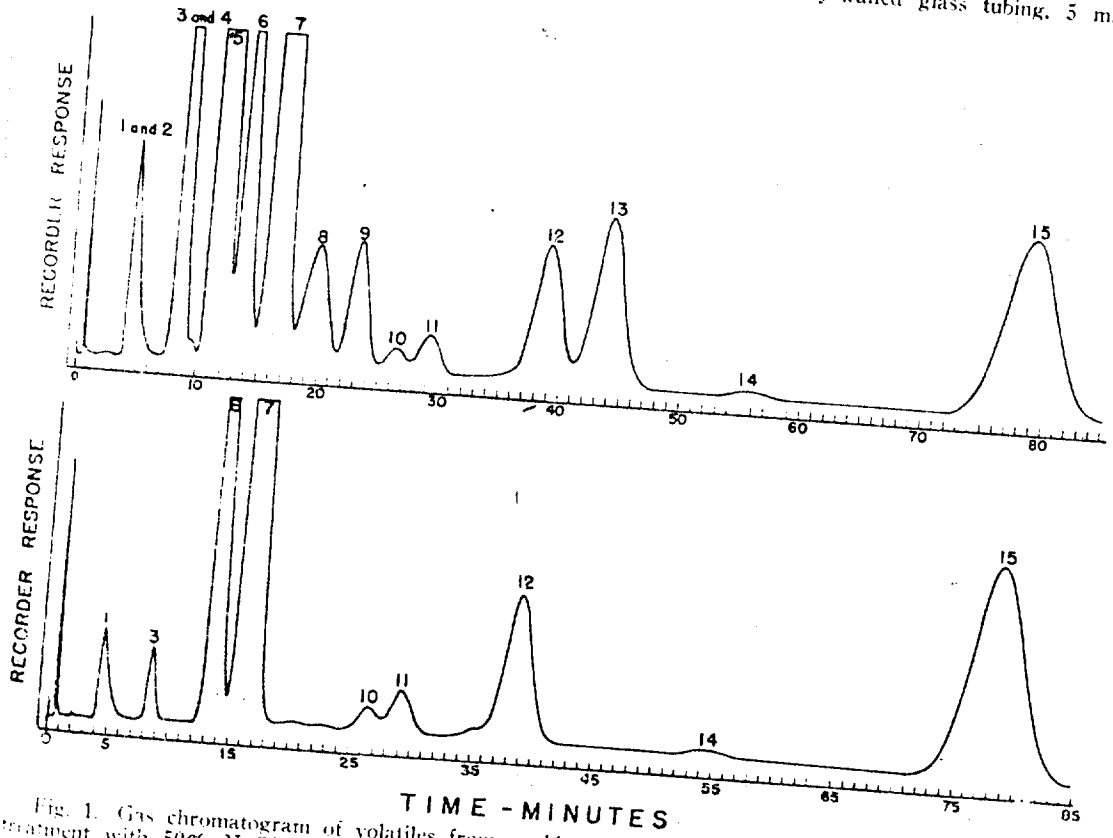

Fig. 1. Gas chromatogram of volatiles from a cider vinegar. Before (upper) and after (lower) treatment with 50% NaOH. The peaks represent 1) acetaldehyde, 2) methyl formate, 3) acetone, 4) ethyl formate, 5) ethyl acetate, 6) diacetyl, 7) ethyl alcohol, 8) sec-butyl acetate, 9) isobutyl acetate, 10) sec-butyl alcohol, 11) propyl alcohol, 12) isobutyl alcohol, 13) isoamyl acetate, 14) n-butyl alcohol, 15) pri-active amyl alcohol.

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1D and 6 ft long, packed with either polyethylene glycol 600 (15%) on 60–80-mesh GC-22 firebrick or with diisodecylphthalate (15%) on 60–80-mesh celite. The operating conditions were: column temperature, 105°C; injection port, 215°C; heater bath, 145°C; carrier gas (He), 18 psig; and sensitivity, 10^{-8} amps.

The resolved components were identified by comparing their retention volumes with compounds of known composition.

Functional group analysis. The syringe technique of Hoff and Feit (1964) was used for gas chromatographic analysis of functional groups of compounds in the vapor mixtures from various vinegar samples. Under the conditions used, saturated potassium permanganate in water removed aldehydes, leaving ketones; hydroxylamine hydrochloride (4 g/50 ml) in water removed carbonyl compounds; and sodium hydroxide removed esters. However, for the latter step it was necessary to modify the above technique in order to hydrolyze the esters effectively. Two ml of 50% sodium hydroxide was added to a vial containing 10 ml of the vinegar sample and allowed to stand for 1 hr prior to withdrawing the vapor sample for GLC analysis. The alcohols were determined by difference.

RESULTS AND DISCUSSION

The fact that members of different homologous series of organic compounds may have similar retention volumes has limited the use of gas chromatography for qualitative analysis of complex mixtures. Consequently, retention volume data without a knowledge of compound type lacks sufficient specificity to permit positive identification. This problem can be readily resolved by means of functional group classification reagents (e.g. Hoff and Feit, 1964) and may be illustrated by the chromatograms in Fig. 1. The chromatogram of the cider vinegar (Fig. 1, upper) appears to be that of a mixture having 13 components. Functional group tests showed that the first peak was due to an ester and an aldehyde, and the second peak to an ester and a ketone. Thus, 15 components were present rather than the 13 indicated by the chromatogram. Furthermore, if one compares the two chromatograms (upper and lower) it will be noted that peaks 5, 8, 9, and 13 were absent (Fig. 1, lower) following treatment with the sodium hydroxide reagent, thereby indicating that the peaks represented esters. Sodium hydroxide effectively hydrolyzed the esters and produced peaks of the corresponding alcohols. However, the yield of these products was poor and it will be noted methanol was not detected (probably because of high solubility in the reagent). Following assignment of the functional group to each peak in a chromatogram, comparison with relative retention volumes of pure reference compounds permitted identification of all compounds.

Representative chromatograms of the volatiles present in several of the vinegar samples are shown in Fig. 2. Examination of the chromatograms shows qualitative differences between the vinegars made by the alcoholic and subsequent acetous fermentations (Figs. 2-A,B) as compared to the vinegars made by the acetous fermentation of dilute distilled alcohol (Figs. 2-C,D). A qualitative difference existed between the two dis-

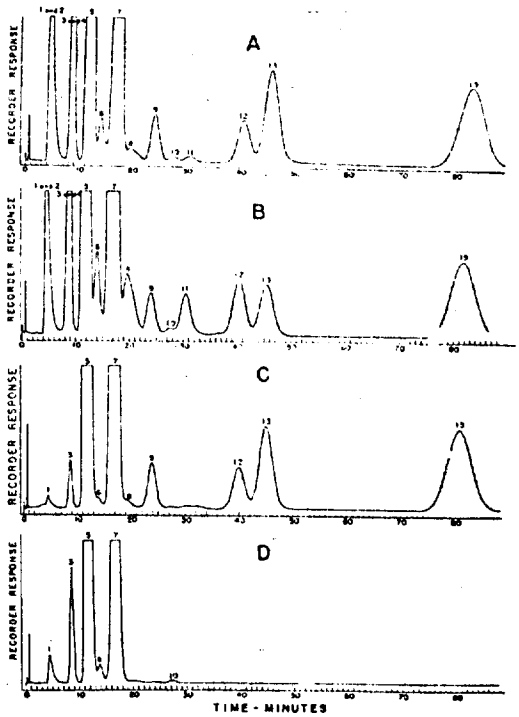

Fig. 2. Gas chromatograms of volatiles from several kinds of vinegar. Part *A*, wine vinegar; Part *B*, tarragon vinegar; Part *C*, distilled vinegar, sample No. 2; Part *D*, distilled vinegar, sample No. 1. These peaks represent: 1) acetaldehyde, 2) methyl formate, 3) acetone, 4) ethyl formate, 5) ethyl acetate, 6) diacetyl, 7) ethyl alcohol, 8) sec-butyl acetate, 9) isobutyl acetate, 10) sec-butyl alcohol, 11) propyl alcohol, 12) isobutyl alcohol, 13) isoamyl acetate, 14) n-butyl alcohol, 15) primary amyl alcohol.

tilled vinegars (Figs. 2-C,D). This difference resulted from the acetous fermentation of an alcohol (Fig. 2-D) prepared from fermentation of a mixture of corn, barley malt, and rye (distilled vinegar No. 2), whereas the vinegar (sample 1 in Fig. 2-C) was from the acetous fermentation of an alcohol whose source was not identified on the label. Similarly, close examination of the gas chromatograms reveals that there are quantitative differences among the various samples by comparing the peak heights of some of the components.

The importance to vinegar flavor of the 25 constituents in vinegar, as listed in Table 1, will require additional quantitative data. However, studies in other fermentation areas such as dairy products, wine, and beer, indicate that the flavor characteristics of these organic compounds are very important to the acceptability of the product.

Carbonyl compounds. Five of the eight vinegar samples contained acetaldehyde, acetone, and diacetyl. Amounts of diacetyl in the distilled vinegar samples were minute as compared with the natural vinegars (cider and wine). Diacetyl is undoubtedly one of the important flavor components of vinegar. In reviews on the flavor of beer (Stevens, 1960; Lawrence, 1964), diacetyl was re-

Table 1. Volatile compounds identified in the vapor mixtures from different vinegar samples.

	Natural[a] vinegars		Distilled[b] vinegars sample no.					Special[c] "mixed" vinegar tarragon
	Cider	wine	1	2	3	4	5	
Carbonyls								
acetaldehyde	+	+	+	+	+	+	+	+
acetone	+	+	+	+	+	+	+	+
diacetyl	+	+	+	+				+
acetoin						+		
isobutyraldehyde	+	+	+		+	+	+	+
isovaleraldehyde					+	+	+	
a-methyl valeraldehyde					+	+	+	
methyl isobutyl ketone						+		
Esters								
methyl formate	+	+						+
ethyl formate	+	+						+
ethyl acetate	+	+	+	+	+	+	+	+
ethyl propionate	+							+
isobutyl acetate	+	+		+				+
n-butyl acetate	+							+
ethyl butyrate				+				+
sec-butyl acetate	+	+		+				
isoamyl acetate	+	+		+				+
Alcohols								
ethyl alcohol	+	+	+	+	+	+	+	+
propyl alcohol	+	+						+
n-butyl alcohol	+							+
sec-butyl alcohol	+	+	+					
pri-active isobutyl alcohol	+	+		+				+
amyl alcohol	+	+		+				+
isoamyl alcohol	+	+						+
sec-active amyl alcohol		+						

[a] Natural vinegars: cider (5% acetic acid); wine (6%) from California grape wine.
[b] Distilled vinegars: Sample No. 1, crystal distilled 5% acetic; No. 2, distilled white (5% acetic) and made from corn, barley and rye; No. 3, 4 and 5 distilled vinegar samples from pickle companies which manufacture their own vinegar for pickle products. Samples No. 3 and 5 made by "Frings Process" (passed through shavings) and No. 4 by "The Acetator" (submerged fermentation).
[c] Special mixed vinegar: Tarragon flavored and a mixture of distilled and malt vinegar.

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ported as contributing to an objectionable off-flavor at levels of 0.5 ppm and above. In dairy products, diacetyl has been considered a desirable flavor component, with threshold levels ranging from 0.01 to 0.20 ppm (Bennett *et al.*, 1965). Diacetyl has been reported in red wine in quantities of 2–4 ppm (Prescott and Dunn, 1959). However, in the present study the possible flavor contribution of the other carbonyl compounds such as acetaldehyde, acetone, isobutyraldehyde, and acetoin has not been established. It has been suggested that acetoin may be further oxidized to diacetyl (Lawrence, 1964).

Alcohols. Seven higher alcohols were identified in addition to ethyl alcohol (Table 1). The natural fruit vinegars (cider and wine) contained six of these alcohols, and the five distilled vinegar samples contained two of these alcohols. The flavor and aroma of fruit vinegars, when compared to distilled vinegars, might be explained by the differences in their alcohol contents. Conceivably, these alcohols could form esters with the organic acids, particularly acetic acid, and thereby contribute to the aroma of the vinegars. The higher alcohols are considered the most important group of volatiles in beer flavor (Stevens, 1960; Lawrence, 1964), and they are the principal components of the fraction known as "fusel oil." The composition of fusel oil is influenced by two main factors: a) the nature of the organisms affecting fermentation; and b) the nature of the substrate. A standard fusel oil, 1 part isobutyl to 4 parts isoamyl alcohol (containing 20% of the optically active isomer), has been used for flavor studies (Hudson and Stevens, 1960). In beer, fusel oil content ranged from 39 to 323 ppm, and a sample of cider contained 98 ppm of fusel oil. In a lager beer, a panel of tasters was able to differentiate between samples containing 45 and 50 ppm of isoamyl alcohol (Hudson and Stevens, 1960). Isoamyl alcohol was identified in the natural vinegars (cider, wine and tarragon) and absent in the five distilled vinegar samples. Thus the composition and concentration of fusel oil may be partially responsible for the characteristic odor and flavor of the vinegar samples.

Esters. In the two-step fermentation (alcohol and acetic acid) of vinegars it is not surprising that esters make up the largest group of volatile flavor compounds, representing nine of the 25 identified (Table 1). Esters are known for their aromatic odors. It is plausible to assume that ethyl acetate is the predominating ester in vinegar since vinegar contains considerable amounts of acetic acid and ethyl alcohol. Also, ethyl acetate is used for denaturing industrial ethyl alcohol at the rate of 4.25 gal per 100 gal of alcohol. Suomalainen and Kangasperko (1963) showed that the ester content of a distilled vinegar (10% acid), measured as ethyl acetate, increased from 200 mg/L in a 60-day storage period to a content of 1500 mg/L and that most of the esters were formed in the first 30 days. Eight additional esters (Table 1) were identified in the volatiles from cider, wine, and tarragon vinegar samples. In four of the five distilled vinegar samples, ethyl acetate is the only ester present and may account for the contrast in odor between natural and distilled vinegars. The esters which have been identified are those of the eight alcohols and short-chain fatty acids (Table 1). They may be direct fermentation products or they may be formed during conditioning of the vinegar. The chief difference in chromatogram profiles between distilled and natural vinegars appears to be due to the higher alcohols and ester contents of the natural vinegars.

Rentschler (1942) and Jacobs (1951) reported that the presence of acetylmethylcarbinol (acetoin) can be used to distinguish a product of acetous fermentation from artificial vinegar. In the present studies, acetoin was found only in the tarragon vinegar and in distilled vinegar sample No. 4.

Toth (1941) reported that diacetyl was not detected in distilled (spirit) vinegar. In contrast, diacetyl was present in two of the five distilled vinegars used in this study.

In conclusion, it would appear that the determination of fermentation products by gas chromatography would provide an index of the genuineness of a vinegar. Similarly, it appears that the standards of identity of a particular vinegar could be determined by the relative concentration of the volatiles, especially the alcohols and esters.

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Studies on the Volatile Components of Different Varieties of Cocoa Beans

S. D. BAILEY, D. G. MITCHELL,[a] M. L. BAZINET, AND C. WEURMAN[b]
Pioneering Research Division, Quartermaster Research and Engineering Center
Natick, Massachusetts

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SUMMARY

Volatile compounds in the aroma of five varieties of roasted and unroasted (raw) cocoa beans have been identified by mass spectral analysis and gas chromatography. The five common varieties selected for this study all contain the following compounds usually in this order of abundance: isovaleraldehyde, isobutyraldehyde, propionaldehyde, methyl alcohol, acetaldehyde, methyl acetate, *n*-butyraldehyde, and diacetyl. An additional eight compounds appear in smaller amounts. As evidenced by gas chromatographic analysis, the raw bean aroma contains the same components but in lower concentrations. The principal differences between varieties are shown to be due to the ratios of these compounds rather than new compounds. The effect of roasting period on the concentration of four aldehydes in the aroma of the ground bean is shown.

Roelofsen (1958), in reviewing information on the fermentation, drying, and storage of cocoa beans, wrote that the "chocolate flavor and odor developed on roasting is by far the most important characteristic of cocoa beans." A search of the literature has revealed that the volatile components of the roasted cocoa bean have not been studied extensively.

Of the compounds previously identified in cocoa, few may be considered to be sufficiently volatile to be associated with the aroma characteristics of the roasted bean. α-Linalool (Bainbridge and Davies, 1912), the principal component of the essential oil in Ecuadorian cocoa bean (Arriba), is a C_{10} carbon ester. Theobromine, 3,7-dimethyl xanthrene, with an m.p. of 337°C, which changes very little during the roasting process (Chatt, 1953), was once believed to develop an aroma suggestive of chocolate, but this view is no longer held (Forsyth and Romboutsi, 1956 a, b). At least nine polyphenols were separated and identified by Forsyth (1957). These large molecules have long been supposed to be the precursors of aroma in tea, coffee, and cocoa. Organic acids known to occur in cocoa bean are well recognized in labeling beans according to acidity, astringency, and bitterness. Extraction of polyphenols and organic acids from the unroasted bean prevented development of the flavor on roasting (Roelofsen, 1958). Replacement of the extracted compounds did not restore the characteristic aroma. However, had these components been themselves carried through the roasting step, aroma enhancement might have occurred. The fermented and unfermented beans were shown by Maly (1955) to contain the amino acids aspartic and glutamic acids, alanine, cysteine, leucine, serine, threonine, methionine, and arginine.

Those volatile components in cocoa which have been studied include diacetyl and acetylmethyl carbinol reported by Schmalfuss and Barthmeyer (1932) and Schmalfuss and Rethorn (1935). The presence of methyl, ethyl, isobutyl, isoamyl, and dimethyl amine

[a] Present address: Walter Baker Chocolate Co., General Foods Corp., Dorchester, Mass. Work initiated during active duty training under U. S. Army Reserve Program.

[b] Present address: Central Institute for Nutrition and Food Research, T.N.O., Utrecht, The Netherlands. NAS-NRC Visiting Scientist at the QM R&E Center from December 1959–April 1961.

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have been observed in cocoa powder by Weurman and De Rooy (1961). More recently, Mohr (1958) studied the composition of chocolate aroma and published initial results on the gas chromatography of chocolate conching. Mohr observed 23 compounds by gas chromatography in the solvent extract of the conched bean, but none were identified. The low order of concentration of the aroma components did not permit use of a direct sampling technique. In the present study, direct aroma sampling of the freshly ground bean was used extensively, thus permitting an examination of the cocoa bean aroma in a manner completely analogous to perception by the nose, i.e., there is no loss of components or change in ratios between components taking place in this direct sampling technique, as may occur when concentration is required. This type of gas chromatographic analysis has become possible through new, very sensitive detectors, and in this study the β-ray ionization type was used. For purposes of identification, analysis by mass spectrometry was used in accordance with procedures used in the study of volatile components of food (Merritt *et al.*, 1959).

EXPERIMENTAL

Sample. In order to study a practical method for the evaluation of aroma composition, some representative types of cocoa beans (*Theobroma cacao* L.) were selected that are well recognized in the chocolate industry for their particular characteristics. The following types of cocoa beans were used: *Accra*, a basic strong chocolate flavor, lacking the undesirable astringency and bitterness found in poorer-flavor varieties; *Arriba*, recognized by its unique and very perfumy aromatic character, though it generally has a moderate astringency and bitterness; *Bahia*, a relatively acid type of flavor, which has a basic chocolate taste but little or no aromatic quality; *Sanchez*, generally a harsh, spicy type of flavor that is masked by strong astringency and bitterness unless the beans are well-fermented; *Trinidad*, with a strong basic chocolate character similar to that found in Accra, and a typical "winey" type of aromatic flavor that is not found in other varieties.

The above are average characterizations. Actually, ranges of variation are wide from crop to crop and from bag to bag within a single shipment. This is caused by the difficulties in keeping the processing of the beans in the field under close control. Fermentation followed by air drying to a 5–7% moisture content is accomplished in the area in which the bean is grown.

The roasting process, which brings out the characteristic flavor of the cocoa bean, takes place in the chocolate manufacturer's plant. The final moisture content is of the order of 1.5–2.5%. The sample was roasted by placing the whole bean in a circulating hot air oven at 300°F. Unless otherwise indicated, the roasting period was 30 min.

Because of known differences within a given variety of bean due to curing operations, several samples were taken from each of six different commercial lots of the Accra variety. A comparison of the aroma composition of these samples showed only minor differences, which were in part due to limitations of the analytical method. For the other varieties two commercial lots from which several samples were selected were considered adequate.

Thirty grams of the roasted or unroasted (raw) bean were ground in a small laboratory blender and immediately placed in a 250-ml Erlenmeyer flask covered with a rubber serum cap. After 2 hr at room temperature to reach equilibrium, samples ranging from 1 to 5 ml of headspace over the samples were removed by glass syringe and introduced to the gas chromatography instrument. Initially, freezing and grinding with dry ice were used to prevent possible loss of heat-labile compounds or alteration in the ratio of volatile compounds. It was later found that this was not required, and, further, that excessive carbon dioxide in the headspace reduced the amount of cocoa bean volatiles in the sample taken for analysis. In the experiments below, only the first-mentioned method of preparing the samples was used.

Gas chromatographic analysis. Vapor samples from the headspace over the ground material were introduced into a Pye-Argon gas chromatograph equipped with a 20-mc strontium-90 ionization detector.

The 120-cm × 4-mm column was packed with 10% di-*n*-decyl-phthalate on firebrick. A flow of 95 ml/min of argon at a column temperature of 50°C was the standard experimental condition employed. Different column materials, at lower and higher temperatures and different flow rates, had been explored, and the above conditions selected for optimum results. A temperature programmed run from approx 5 to 75°C provided a greater separation of components, but with no increase in the number of peaks over those observed under isothermal conditions. When information on the identity had become available from mass spectrometric analysis, small quantities of the pure reference compound were added to the vapor of the cocoa bean samples to locate the

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position of the compound on the chromatograph. The increase, without distortion, of the unknown peak by the known compound provided further confirmation. The latter method was found to be of considerable value because of its very great sensitivity.

Mass spectral analysis. The mass spectral technique employed has been described (Bazinet and Merritt, 1959). Approx 1×10^{-6} moles of the vapor was transferred to a sampling bottle by high-vacuum low-temperature condensation. The gaseous sample was then fractionated directly into a Consolidated Engineering Model 21-103C Analytical Mass Spectrometer at low temperature and high vacuum. Separated components were identified by comparison of the fragmentation pattern with those of known compounds.

RESULTS

Fig. 1 shows a gas chromatogram of the volatile components released from a typical sample of roasted ground cocoa bean. Resolved peaks, identified by the methods described, are correspondingly labeled. Other varieties, as is subsequently shown, give quite similar gas chromatographic patterns. Table 1 shows the mass spectral analysis giving the mole percent computed on a water- and carbon-dioxide-free basis for a typical roasted sample. The base line of the chromatogram of Fig. 1 indicates compounds identified from mass spectral evidence but present in quantities too small for resolution by gas chromatography.

The several samples from a given lot and several lots of a given variety gave very reproducible results. Slight changes in the ratios of certain components were observed in some cases during reproducibility studies. Since the beans are an agricultural product it is not unusual to find such variation as a result of soil conditions, maturity differences, and variations in processing.

Table 1. Mass spectral analysis of roasted Bahia cocoa bean vapor.

	Mole %
Isovaleraldehyde	42.0
Isobutyraldehyde	15.4
Propionaldehyde	13.0
Methyl alcohol	9.1
Acetaldehyde	7.0
Methyl acetate	6.3
n-Butyraldehyde	3.0
Diacetyl	2.8
Dimethyl sulfide	0.3
Dimethyl disulfide	0.2
Ethyl alcohol	0.2
Furan	0.2
Toluene	0.1
Benzene	0.1
Methyl furan	Trace
Acetone	Trace

It was also considered to be of some interest to determine the effect of storage on a single lot of roasted cocoa beans. A sample held 4 months at room temperature was compared to an identical sample held 4 months at −15°C. The resulting gas chromatograms showed no significant differences.

In the chromatograms of the roasted samples, isovaleraldehyde (3-methyl butanal) is the most pronounced component in the varieties studied. The next major component is isobutyraldehyde, with propionaldehyde and acetaldehyde completing the four major aldehydes in the vapors of the roasted bean.

Five varieties of cocoa bean, raw, are compared in Figs. 2, 3, and 4. The importance of roasting

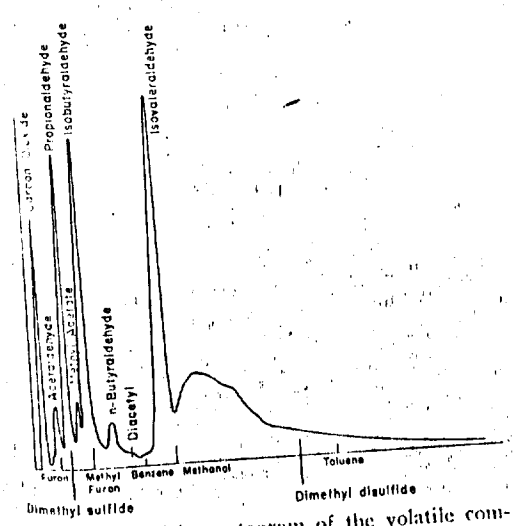

Fig. 1. Gas chromatogram of the volatile components of roasted ground cocoa. Typical chromatogram with peaks identified from mass spectral data and pure reference compounds.

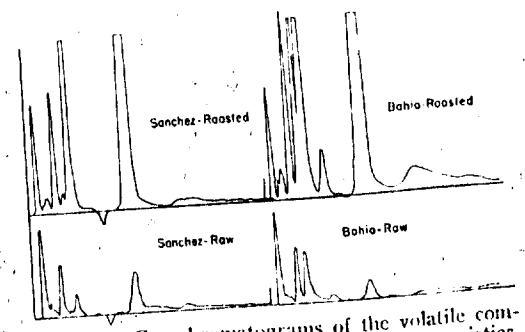

Fig. 2. Gas chromatograms of the volatile components of ground Sanchez and Bahia varieties, roasted and raw.

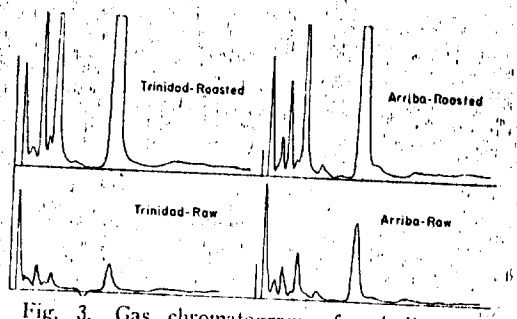

Fig. 3. Gas chromatogram of volatile components of ground Trinidad and Arriba cocoa beans, roasted and raw.

to enhancement of volatile components is observed in all varieties. A few of the minor components are not detectable in the raw bean.

The chromatograms of the unroasted Sanchez, Trinidad, Arriba, and Accra samples are very similar with respect to the relative amounts of three major components and a number of minor components. The Bahia variety, in both the raw and roasted samples, has a much higher relative amount of methyl acetate than do the other four varieties. These same five varieties were directly compared by measuring the peak areas of the four major aldehydes in chromatograms in which sample and sensitivity of the instrument permitted all peaks to be retained on the chart paper. The Accra roasted sample gave the largest total area for these four compounds, with the Bahia next and the Arriba the least. The Arriba cocoa bean is generally considered as having a high aromatic character, but this does not appear to be associated with the volatile components compared in this study.

Fig. 5 shows the effect of roasting period on the release of aldehydes. The peak heights of isovaleraldehyde, isobutyraldehyde, propionaldehyde, and acetaldehyde for roasting periods of 20, 30, 40, and 50 min are compared to another sample of the same lot that was not roasted. The release of higher aldehydes is markedly increased by 20 min of roasting. The propionaldehyde and acetaldehyde are not significantly affected by the roasting process. Roasting times of 30 and 40 min

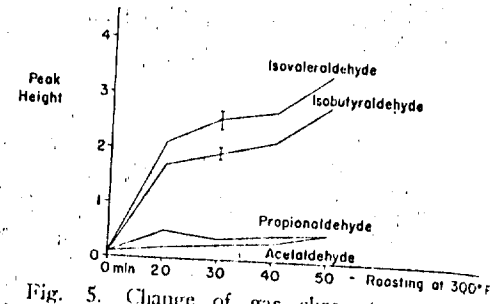

Fig. 5. Change of gas chromatogram peak heights of four volatile components with roasting period.

gave no significant increase over the 20-min roasting. Increased roasting time of 50 min, however, does appear to increase the release of the two higher aldehydes. From a flavor standpoint there was a preference by tasters for the 40- and 50-min samples, with five preferring the 40-min sample and three preferring the 50-min samples. One taster indicated no preference.

To obtain a general impression of the actual amounts of material represented by the chromatographic peaks, the amount of isovaleraldehyde in the chromatograms shown has been estimated by taking aliquot vapor samples from a 5.0-L spherical glass vessel in which μl amounts of the aldehyde were introduced. The quantity of aldehyde used permitted complete vaporization. It was therefore possible by taking out small gaseous samples to estimate rather accurately the quantity of isovaleraldehyde associated with a given peak height. At a detector voltage of 1750 V, a sensitivity of 1/3 maximum, and a flow rate of 95 ml/min on 10% di-*n*-decyl-phthalate, peaks covering the range of a 24-cm recording chart paper correspond to amounts up to 1.3 μg or 1.5×10^{-8} moles. In the chromatograms of Figs. 2, 3, and 4 the quantity of isovaleraldehyde is, therefore, of the order of 2 to 3×10^{-8} moles.

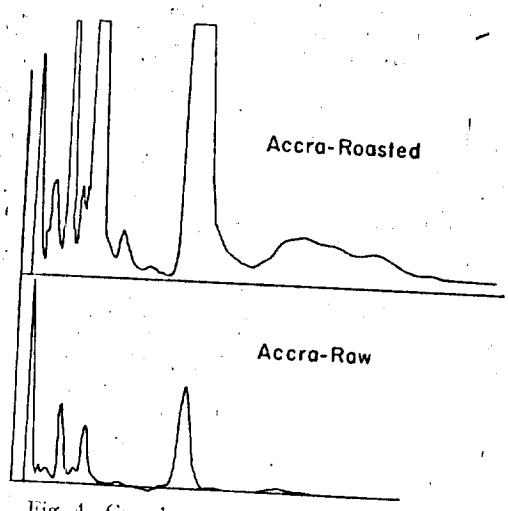

Fig. 4. Gas chromatograms of the volatile components of ground Accra cocoa bean, roasted and raw.

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The threshold perception of isovaleraldehyde was determined by sniffing dilute aqueous solutions of the aldehyde. Molal concentrations of 9.3×10^{-5}, 4.6×10^{-4}, and 2.3×10^{-3} respectively gave strong, faint, and very faint but detectable odor of the aldehyde. The same size of Erlenmeyer flask was used in this odor threshold study as was used in the sampling of ground cocoa beans; in this way, the headspace sampling may be considered analogous. This has given some indication of the levels of odor threshold perception, and the amount of isovaleraldehyde associated with the peak heights observed in the cocoa samples.

It then was considered desirable to see if the compounds responsible for the chromatogram, as shown in Fig. 1, could be related to the fresh aroma of the ground roasted cocoa bean. From previous chromatographic runs, the time required for elution at room temperature was determined precisely. A 20-ml headspace sample was then introduced, with the column disconnected to prevent overloading of the detector and to facilitate trapping. The volatile compounds were trapped at dry-ice temperature in a sand-filled spiral glass trap. Upon completion of trapping the trap was allowed to come to room temperature and the contents gently flushed out. The aroma emerging from the trap was found to be characteristic of the cocoa bean and essentially the same as the headspace sample introduced. The compounds identified, therefore, appear to be of importance in characterization of the roasted cocoa bean aroma.

To what extent these volatile components may continue to be observed in the next step of the chocolate manufacturing process, was determined by gas chromatographic analysis of a sample of chocolate liquor. This sample, prepared from Bahia roasted cocoa beans, was the same sample given the 30-min roast in the roasting-period study. The gas chromatogram of the headspace sample is shown in Fig. 6. Peak A does not appear in the roasted or raw samples. Peak B corresponds to acetaldehyde, C to propionaldehyde, D to methyl acetate, E to isobutyraldehyde; F and G are new; and H corresponds to isovaleraldehyde. The conditions for the analysis represented in this chromatogram correspond to conditions for the analysis resulting in the chromatograms of Figs. 1, 2, 3, and 4. The quantity of the volatile components is therefore markedly reduced in the formation of the chocolate liquor. A more detailed study is necessary to determine the aroma composition of the liquor. The chromatogram shown for the liquor would indicate the usefulness of this technique for such additional studies.

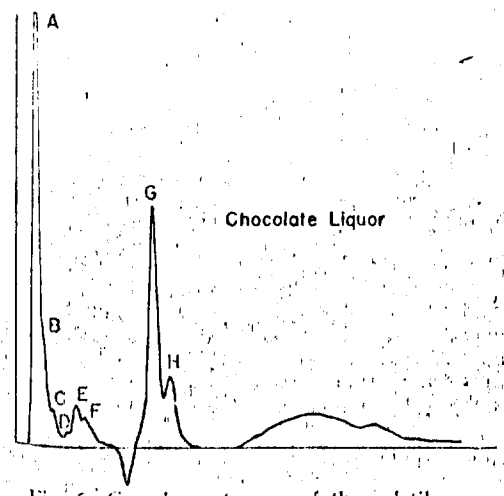
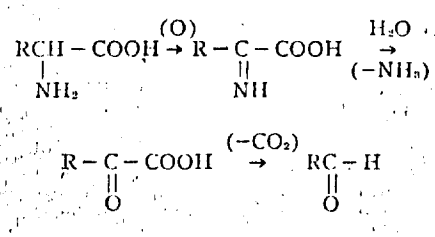


Fig. 6. Gas chromatogram of the volatile components of chocolate liquor.

DISCUSSION AND CONCLUSIONS

The aroma composition of these five varieties of roasted and raw cocoa bean was found to be very similar. The major differences were observed in the amounts of each component as shown by the gas chromatograms. A much larger concentration of methyl acetate was found in the Bahia variety, and *n*-butyraldehyde was present in the Bahia and Arriba bean to a greater extent than in other varieties.

It is generally recognized that fermentation produces a rapid breakdown of protein to produce soluble nitrogen such as amino acids (Becker and Stelling, 1952). The amino acids thus formed, through processes of oxidation, deamination, and decarboxylation during fermentation and on drying, may well serve as precursors to the aldehydes reported in this study. The general reaction for these processes, first established by Strecker in 1861, is given as follows:



The occurrence of isovaleraldehyde in cocoa bean, as reported in this study, may come from leucine through these reactions. Jack-

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son and Morgan (1954) attributed the malty aroma in dairy products to the conversion of leucine to isovaleraldehyde by *Streptococcus lactis* var. *maltigenes*. By similar reasoning the presence of isobutyraldehyde and other aldehydes reported here may be attributed to similar reactions. Further studies are indicated to substantiate this observation.

Roasting at 300°F increases the amount of isovaleraldehyde and isobutyraldehyde, the most pronounced effect occurring after 20 min at this temperature. Roasting periods of 30, 40, and 50 min gave somewhat smaller increases. In this connection the prolonged heating at the elevated temperature might be expected to result in the loss of the more volatile components. The failure of acetaldehyde and, to a lesser extent, propionaldehyde to show increased amounts on roasting may be accounted for in this way.

It remains as a future problem to attempt reconstitution of a cocoa aroma from these components as well as to fortify substandard beans with some of these to determine if acceptable aroma enhancement can be achieved.

While some evidence has been presented for the importance of these compounds in chocolate liquor, a greater attention must be given to this problem. The heating and grinding in the process of preparing the liquor would be expected to result in the loss of the more volatile components.

Finally it may be concluded that analytical techniques are now available for the study of fresh and processed foods in small quantities without the requirement for extraction and concentration of volatile components. This permits an assessment of aroma composition to a degree of reliability heretofore not possible.

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THE INTENSIFICATION OF THE VOGES-PROSKAUER REACTION BY THE ADDITION OF α-NAPHTHOL.

MAXWELL M. BARRITT.

Department of Bacteriology, City Laboratories, and University of Liverpool.

THE Voges-Proskauer (V-P.) test (Voges and Proskauer, 1898) is largely used as a means of differentiating between *B. coli* and *B. aerogenes* and is in effect, as shown by Harden and his colleagues (Harden and Walpole, 1905-06; Harden, 1905-06, 1930; Harden and Norris, 1911), a test for acetylmethylcarbinol ($CH_3.CH(OH).CO.CH_3$) which in the presence of caustic alkali and air becomes oxidised to diacetyl ($CH_3.CO.CO.CH_3$), which in turn reacts with a substance in the broth, probably arginine, possessed of a guanidine grouping ($NH : C(NH_2).NH.R$), to produce a pink fluorescent colouration.

The test as ordinarily performed (Ministry of Health Report, 1934) often gives faint and indefinite reactions and various modifications have from time to time been suggested. These have been chiefly directed towards enhancing the oxidation of acetylmethylcarbinol, to which end Levine, Weldin and Johnson (1917) employed hydrogen peroxide, barium peroxide and potassium dichromate, Bedford (1929) sodium peroxide, Werkman (1930) ferric chloride and Leifson (1932) a mixture of copper sulphate, ammonia and soda. None of these seems to have received wide acceptance but O'Meara's modification (1931), consisting in the addition of creatine to provide a greater amount of the reacting guanidine grouping, has been a definite improvement, as this addition considerably intensifies the colour change. Even with this some difficulty has been experienced in the form of indefinite reactions, particularly in connection with organisms of the "intermediate" group.

The present paper records experiments with a modification of the V-P. test by the addition of α-naphthol, which results in a great intensification of the colour change and a marked increase in sensitiveness, without, so far as can be ascertained, any loss in specificity. With this test it is found that certain organisms showing no reaction with the ordinary V-P. test or the O'Meara modification do in fact produce acetylmethylcarbinol. Experiments with certain coal tar derivatives showed that considerable

intensification of the colour change could be produced by the addition of an alcoholic solution of α-naphthol under suitable conditions. When diacetyl, peptone, KOH and α-naphthol were mixed in suitable proportions a deep red colour developed almost immediately. This colour did not occur in the absence of one or more of the other constituents, though in the absence of α-naphthol the ordinary salmon pink of the V-P. reaction was obtained.

After preliminary tests to determine optimal proportions a titration of diacetyl was made in which the following ingredients were added in the proportions indicated.

Diacetyl (B.D.H.), dilutions 1 : 100 to 1 : 1,000,000	1·00 c.c.
Distilled water	2·25 ,,
1 per cent. peptone water (Difco bactopeptone) .	1·00 ,,
α-naphthol (B.D.H.), 5 per cent. alcoholic solution .	0·50 ,,
Potassium hydroxide, 40 per cent. solution . .	0·25 ,,
	5·00 c.c.

The tubes were shaken to mix the contents and the colour reactions read after half an hour at room temperature. The colourations varied from a dark red with the 1 : 100 dilution of diacetyl to a pale pink with the 1 : 200,000 dilution. As the total volume in each tube was 5 c.c. this result indicates that the test was able to detect diacetyl in a concentration of 1 in a million. It was found that a slightly higher titre could be obtained by varying the amounts of the constituents, though excess KOH tended to mask the fainter reactions by giving a coppery colouration with the α-naphthol itself. If 1 c.c. of distilled water were substituted for the peptone water, and a knife-point of creatine added to each tube, the test was even more delicate, a trace of pink being just detectable with the 1 : 1,000,000 dilution of diacetyl, corresponding to a final concentration of 1 : 5,000,000.

If the α-naphthol were omitted and 1 c.c. of 40 per cent. KOH added instead of 0·25 c.c., diacetyl could be detected in a concentration of 1 : 50,000, and with the addition of creatine in 1 : 100,000. In the KOH test (without creatine) the colour reactions were more of a salmon pink and accompanied by fluorescence.

The nature of the α-naphthol reaction. I have no evidence as to the nature of this reaction, but it is not unlikely that α-naphthol acts by forming a compound with the diacetyl-peptone combination, the resulting product having a red colour. It appears that the α-naphthol must combine early in the reaction, for experiments have shown that when KOH was allowed to act on a mixture of peptone and diacetyl until a maximum salmon pink colour was obtained, the subsequent addition of α-naphthol caused no alteration

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in the colour. In conducting this test it is therefore advisable to add the α-naphthol before the KOH, and to avoid excess of the latter.

Determination of the chemical specificity of the α-naphthol test.

The α-naphthol test involves the use of three chief ingredients: (1) α-naphthol, (2) a nitrogenous substance (peptone) and (3) diacetyl. A series of tests was therefore carried out with a view to determining whether any of these could be replaced by substances of an allied nature, in order to see to what extent the test was chemically specific and how far this specificity agreed with that of the ordinary V-P. test.

A. *Tests for diacetyl with substances other than α-naphthol.*

The following substances were employed in aqueous or alcoholic solutions.

β-naphthol.	Guaiacol.	Naphthalin.
Resorcinol.	*o*-Cresol.	Toluene.
Orcinol.	*p*-Cresol.	Pyrogallol.
Salol.	Tricresol.	Catechol.
Phenol.	1 : 3 : 4 *m*-Xylenol.	Quinol.
Phloroglucinol.	1 : 3 : 5 *m*-Xylenol.	Benzidine.

None of these gave the red colour obtained with α-naphthol. With β-naphthol an orange colouration was produced in the stronger concentrations of diacetyl and a yellow in the weaker, a faint yellow being just detectable in a dilution of 1 : 100,000. Resorcinol and orcinol appeared to intensify slightly the salmon pink of the ordinary V-P. reaction. Pyrogallol, catechol and quinol darkened quickly on the addition of KOH and so were unsuitable for use in such a test. With phloroglucinol a faint yellow colour appeared only in strong concentrations of diacetyl. None of the other substances tested produced any effect. The power to produce this marked intensification of the red colour of the V-P. reaction seems therefore to be peculiar to α-naphthol.

B. *Tests for diacetyl with nitrogenous substances other than peptone.*

Harden and Norris, in order to ascertain which part of the protein molecule was concerned in the V-P. reaction, tested various proteins, protein derivatives and other substances in the presence of diacetyl and caustic alkali. Such of these substances as were obtainable, together with certain others, were tested by the KOH (ordinary V-P.) and α-naphthol tests, and comparison made where

possible with the results obtained by Harden and Norris. In the KOH test the amounts of the constituents varied, for, as Harden and Norris pointed out, each substance has its own optimal conditions. In the α-naphthol test, however, a knife-point of the substance tested was added to a solution containing 1 c.c. of diacetyl (1 : 1000), 3·25 c.c. of distilled water, 0·5 c.c. of α-naphthol (5 per cent. alcoholic solution) and 0·25 c.c. of KOH (40 per cent.). The fluid substances were added in quantities of 1 c.c., the total volume being adjusted to 5 c.c. by omitting 1 c.c. of distilled water. The colour reactions in the α-naphthol test varied from a rose or rosy magenta to a deep crimson or ruby, and were graded as + or ++ according to the intensity of the colour. As no reaction stronger than a pink or rose was obtained in the KOH test (ordinary V-P.) the positive results were recorded as +.

The following substances found positive by Harden and Norris gave + results in the KOH test and ++ results in the α-naphthol test.

Gelatin.	Pepsin.	Agmatine.
Egg albumin.	Meat extract.	Dicyandiamide.
Casein.	Broth.	Creatine.
Pancreatin.	Arginine.	Guanidine acetic acid.

In table I it will be seen that certain substances which Harden and Norris found to give doubtful or negative results yielded in my hands very similar results with the ordinary V-P. test but gave definitely positive reactions with the α-naphthol test. Certain substances closely allied to those used by Harden and Norris, but not tested by them, are included in the table for convenience of comparison.

Table I.

Results of the α-naphthol test with substances with which Harden and Norris obtained negative or doubtful results.

Substance.	Harden and Norris.	KOH test.	α-naphthol test.
Guanidine hydrochloride	?	?	++
Guanidine nitrate	?	?	++
Guanidine carbonate	?	+ slight	++
Guanidine thiocyanate	Not tested	?	++
Guanidine sulphate	Not tested	?	++
Methylguanidine	—	Not tested	Not tested
Methylguanidine hydrochloride	Not tested	+	++
Aminoguanidine	—	Not tested	Not tested
Aminoguanidine sulphate	Not tested	—	+
Aminoguanidine bicarbonate	Not tested	?	+ or ++
Urea	—	—	+
Biuret	—	—	+
Creatinine	—	+ slight	++
Normal urine	—	—	Varied + or ++

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Negative results were obtained with the following substances which Harden and Norris found negative.

Alanine.	Histidine.	Uric acid.
Phenyl-alanine.	Leucine.	Semicarbazide hydrochloride.
Asparagine.	Tryptophane.	Acetamide.
Cystine.	Tyrosine.	Succinimide.
Glutamic acid.	Valine.	Succinamide.
Glycine.	Glycocoll.	

Negative results were obtained with the following substances not tested by Harden and Norris.

Aspartic acid.	Caffeine.	Formamide.
Choline.	Theobromine.	Urethane.
Lysine.	Theophylline sodium acetate.	Malonamide.
Proline.	Adenine.	Oxamide.
Allantoin.	Xanthine.	Methyl urea.
Hydantoin.	Guanine.	Semicarbazide nitrate.
Oxaluric acid.		Agar-agar.
Hippuric acid.		

In no instance was the KOH test positive when the α-naphthol test was negative and all substances giving positive results in the KOH test gave positive results in the α-naphthol test. The α-naphthol test gave positive results with a few substances negative in the KOH test (*e.g.* urea and biuret).

The positive results obtained with creatinine (two specimens tested) may be attributed to conversion of some of this substance into creatine in the presence of KOH (Walpole, 1911), for when a saturated solution of sodium carbonate was substituted for KOH in the α-naphthol test a negative result was obtained with creatinine, though a positive with creatine.

Aminoguanidine sulphate was negative in the KOH test though positive in the α-naphthol test, but if the KOH were allowed to act for a few minutes before adding the α-naphthol, no reaction occurred. As KOH converts the sulphate and the bicarbonate of aminoguanidine into aminoguanidine it would seem that the base behaves differently from the salts.

From these results it is apparent that the α-naphthol test requires the presence of very similar nitrogenous substances to those reacting in the ordinary V-P. test, as determined by Harden and Norris, but that the reactions are much stronger and the test more delicate. If the positive results obtained with urea and biuret, which were not specially purified but used as obtained from a reliable firm, were due to these substances and not to a reactive contamination, and if we represent the formula of urea as

$$C{=}O\begin{cases}NH_2\\NH_2\end{cases}$$

and that of biuret as

$$C{=}O\begin{cases}NH_2\\NH.CO.NH_2\end{cases}$$

it suggests that

$$C{=}O\begin{cases}NH_2\\NH.R\end{cases}$$

may be a reactive grouping in addition to the guanidine nucleus

$$C{=}NH\begin{cases}NH_2\\NH.R\end{cases}$$

C. *Tests with substances other than diacetyl.*

The following substances gave negative results when added as such or in the form of solutions in place of diacetyl in the α-naphthol test.

Acetone.	Glycerol.	Tartaric acid.
Acetylacetone.	Acetaldehyde.	Ethyl alcohol (absolute).
Ethylacetone dicarboxylate.	Formaldehyde.	Methyl alcohol (acetone free).
Benzoylacetone.	Benzil.	Amyl alcohol.
Dihydroxyacetone.	Benzoin.	Inositol.
Glycerose.	Malic acid.	Dulcitol.
Acetoacetic ester.	Malonic acid.	Mannitol.
Acetic acid.	Maleic anhydride.	The commoner mono- and disaccharides, polysaccharides and glucosides.
Acetophenone.	Laevulinic acid.	Trimethylene glycol.
Acetonylacetone.	Succinic acid.	
Benzophenone.	Pyruvic acid.	
Dibenzoylmethane.	Formic acid.	
Glycollic acid.	Lactic acid.	
Glyoxal.	Citric acid.	

Positive results were obtained with acetylmethylcarbinol, 2 : 3 butylene glycol and ethylene glycol, and also with infusion broth and absolute industrial methylated spirit.

In titrations carried out as for diacetyl it was found that with the ordinary V-P. test acetylmethylcarbinol could be detected in concentrations of between 1 : 10,000 and 1 : 50,000; with addition of creatine it was detectable in a concentration of 1 : 50,000 but not in 1 : 100,000. The α-naphthol test was positive between 1 : 500,000 and 1 : 1,000,000, and if creatine were added, in 1 : 2,500,000. The reactions were slower than those with diacetyl and could be quickened by shaking, the colours being similar but limited to slightly lower concentrations.

With 2 : 3 butylene glycol and ethylene glycol positive reactions occurred in concentrations up to about 1 : 25. It is uncertain if these reactions were due to a direct reaction with the glycols themselves or to the presence of a slight impurity in the preparations, or if some oxidation to diacetyl occurred in the course of the test. The concentrations in which the reaction occurred are not of the order in which these substances are likely to occur in cultures, and

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therefore this possible fallacy would not affect the utility of the test in bacteriological work. Both substances gave negative reactions in the ordinary V-P. test.

Ordinary infusion broth was also found to give a positive reaction in the α-naphthol test. This is apparently associated with the meat extract present, for extracts of fresh ox, horse and guinea-pig skeletal muscle reacted similarly, especially when made by extraction at a low temperature followed by steaming. The reaction appeared to be quite independent of any bacterial action on the extract, and is apparently due to some substance similar to diacetyl or acetylmethylcarbinol extracted from the muscle. It is clear that infusion broth is not suitable for cultures for this test, and that some such medium as that generally used for the V-P. test is essential. (Ministry of Health, 1934).

A 5 per cent. solution of α-naphthol in absolute industrial methylated spirit gives a pink colour in the presence of peptone +KOH. This is probably due to the wood naphtha in the spirit, which is likely to contain traces of substances of a ketonic nature allied to or identical with diacetyl. It is therefore essential to use absolute ethyl alcohol as a solvent for α-naphthol in this test.

Application of the test to the study of cultures of bacteria.

Technique.

It has been found advisable, as suggested by O'Meara, to use cultures grown in wide tubes. I have therefore employed cultures in 6 × ⅝ in. tubes of glucose phosphate broth (Ministry of Health, 1934) made with Difco bactopeptone, which appears to be particularly suitable for this test. After 3 days' incubation at 37° C. 1 c.c. of culture was placed in a 6 × ¾ in. test tube, to which were added 0·6 c.c. of α-naphthol (5 per cent. alcoholic solution) and 0·2 c.c. of KOH (40 per cent. solution). Equally good results are obtainable with 0·5 c.c. of a 6 per cent. solution of α-naphthol and 0·5 c.c. of a 16 per cent. solution of KOH.

After shaking the tubes to mix the contents, positive reactions appear as a pink colour in 2-5 minutes. With well marked reactions this deepens to magenta or crimson in half an hour. A maximum colouration is reached within one hour when the stronger positives are deep crimson or ruby, and the weaker are magenta or a rosy pink. Negatives are colourless, but very occasionally show a faint trace of pink which is not usually noticeable unless the culture medium employed is colourless. After approximately one hour the negatives gradually develop a coppery colouration due to the action of KOH on the α-naphthol. The positives are readable for from 4 to 24 hours, depending on the strength of the reactions, assuming a coppery colouration on fading. The significance of the faint traces of pink observed in some cultures is uncertain. It is possible that they indicate minimal production of acetylmethylcarbinol under conditions which are not quite optimal. The question of the optimal conditions for the production of this substance is receiving attention. For the present such reactions are regarded as negative.

If the test is supplemented by the addition of creatine, there is a very slight intensification of all reactions. The reaction is appreciably quickened by heat.

Application of the α-naphthol test to the coli-aerogenes *group.*

Until recently a modified creatine (O'Meara) test was used at the Liverpool City Laboratories for the determination of acetylmethylcarbinol production by bacteria. A glucose phosphate broth culture (5 c.c.) was incubated for 3 days at 37° C. and a knife-point of creatine and 2·5 c.c. of 40 per cent. KOH added. The tube was shaken and a little of the mixture poured into a watch glass. In half-an-hour positives developed a pink to rose red colour which commenced to fade in 1 or 2 hours while negatives remained colourless. The reactions were controlled by the rest of the mixture in the tubes, in which a positive reaction was fainter and took longer to develop but, once developed, was more permanent.

This and the α-naphthol test were compared in the examination of 368 strains of the *coli-aerogenes* group as detailed below. The technique and media used were as described in the Ministry of Health Report no. 71 (1934), with the following exceptions. For the isolation of the organisms eosin-methylene blue agar was preferred to MacConkey's agar. The test for growth in citrate was examined after 3 days' incubation. Tests for gelatin liquefaction were not made; thus if any strain of *B. cloacæ* was encountered it was classified with *B. aerogenes*. The methyl red test was carried out after 3 days' incubation and a definite red considered positive, a definite yellow, negative. The first series examined consisted of 259 strains which fell into the groups *B. coli* fæcal types I and II, and 31 strains which belonged to *B. aerogenes* types I and II of the Ministry of Health Report (table II).

TABLE II.

Particulars of strains of the coli-aerogenes *group.*

Type.	No.	Reactions.			Sources.
		Indole.	M.R.	Citrate.	
B. coli type I . . .	250	+	+	−	Water 208, fæces 30, Lister Inst. 12
„ „ II. . .	9	−	+	−	Water 5, fæces 4
B. aerogenes type I . .	27	−	−	+	Water 24, fæces 2, Lister Inst. 1
„ „ II .	4	+	−	+	Water 4

With all these strains the two tests for acetylmethylcarbinol were in complete agreement, all the strains of *B. coli* giving negative reactions while those of *B. aerogenes* reacted positively.

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In addition to these, there were 7 strains which in their growth on eosin-methylene blue agar and in the methyl red and citrate tests behaved like *B. aerogenes* but gave negative reactions with the creatine test. All these gave positive reactions with α-naphthol.

So far as this series goes the conclusion seems warranted that the two tests are in reasonable agreement but that the α-naphthol test is the more delicate.

The so-called "intermediate" group of coliform organisms is usually described as giving positive reactions in the methyl red test, negative in the V-P. test and as growing in citrate (M.R.+ V-P.− citrate+). Indole production is variable and provides the basis for the Ministry of Health's (1934) division of the group into type I (indole −) and type II (indole +). Such combinations as M.R.+ V-P.+ or M.R.− V-P.− are regarded as irregular. It is in this "intermediate" group that the greatest discrepancies between the V-P. (creatine) and α-naphthol tests have been encountered. For the purposes of this discussion all strains giving the reactions M.R.+ citrate+ are regarded as being of the "intermediate" type. The additional reactions of 71 such strains are recorded in table III.

TABLE III.

Comparison of the α-naphthol and creatine tests in the examination of M.R.+ citrate+ strains.

No. of strains.	Indole.		Creatine test.		α-naphthol test.	
	+	−	+	−	+	−
45	0	45	13	32	28	17
26	26	0	8	18	26	0
71	26	45	21	50	54	17

From this it is seen that only 21 of the strains give positive reactions with the V-P. (creatine) test whereas 54 are positive with α-naphthol. The occurrence of the combination M.R.+ V-P.+ with the ordinary V-P. test without creatine is generally regarded as uncommon. Bardsley (1934) met with only 22 such strains in the course of isolating 581 intermediate strains from water. Assuming, as seems feasible, that the α-naphthol test is a more delicate V-P. reaction, then the combination of production of acetylmethylcarbinol with a positive methyl red test was observed in 54 strains out of 71 examined. One effect of this observation is to increase in this group the correlation between acetylmethylcarbinol production and power to use citrate which is so evident in the case of *B. coli* and *B. aerogenes*. On the basis of these tests it now becomes

possible to group the organisms concerned as in table IV, which is based on the report of the Ministry of Health. All strains producing indole and 28 out of 45 of the strains unable to produce indole are seen to be α-naphthol positive.

TABLE IV.

Re-classification of intermediate strains.

Type.	No. of strains.	Indole.		M.R.		Citrate.	α-naphthol.	
		+	−	+	−	+	+	−
Intermediate type IA .	17	0	17	17	0	17	0	17
Intermediate type IB .	28	0	28	28	0	28	28	0
Intermediate type II .	26	26	0	26	0	26	26	0

On the evidence of acetylmethylcarbinol production afforded by the α-naphthol test the intermediate type IA strains seem more closely allied to *B. coli*, and types IB and II to *B. aerogenes*. It has also been found that the 54 strains grouped as type IB and type II fermented sucrose with production of acid and gas, whereas 14 out of the 17 grouped as type IA failed to attack this sugar.

Application of the α-naphthol test to the Brucella *group.*

Heavy inocula from cultures on ascitic agar were made into glucose phosphate broth. All the strains tested grew in this medium at 37° C. without additional CO_2. The α-naphthol test was done on the cultures at intervals of a few days' incubation. No appreciable reaction was noted until about the 5th day, but thereafter there was with certain strains a gradual development of a positive reaction which slowly increased in intensity, generally up to about the 18th day. Table V gives the results of the α-naphthol test after 12-18 days' incubation at 37° C. Readings were taken after 1 hour at room temperature.

The positive reactions varied from a rose or distinct pink to a magenta or crimson, while the negative were colourless. Tests in which faint pink colours developed were considered doubtful.

The strains were of human, bovine, caprine, ovine and porcine origin. The results bore no relationship to the origin of the strains, with the striking exception that only 1 out of 23 porcine strains gave a positive reaction. As none of these strains were freshly isolated it is difficult to assess the value of these results. Nevertheless, it is evident that the majority of *melitensis* and *abortus bovis* strains produce acetylmethylcarbinol, though they

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have been classified as V-P. negative. On applying the ordinary V-P. test to these cultures, in no instance was a positive result obtained, though a few which gave the stronger α-naphthol reactions gave weak or doubtful results with the modified creatine test.

TABLE V.

Results of α-naphthol tests on organisms of the Brucella *group.*

Nature of strains.	No. of strains.	Donor of strains.	Result of α-naphthol test.		
			+	?	−
Br. melitensis	32	Prof. G. S. Wilson, London	29	0	3
" "	19	Dr R. M. Taylor, France	18	1	0
" "	4	Dr Axel Thomsen, Copenhagen	1	2	1
Totals	55		48	3	4
Br. abortus (bovine)	11	Prof. G. S. Wilson	9	0	2
" " "	4	Dr R. M. Taylor	4	0	0
" " "	8	Dr Axel Thomsen	8	0	0
Totals	23		21	0	2
Br. abortus (porcine)	7	Prof. G. S. Wilson	0	2	5
" " "	4	Dr Axel Thomsen	0	0	4
" " "	12	Dr I. F. Huddleson, Michigan	1	0	11
Totals	23		1	2	20
Grand totals	101		70	5	26

The α-naphthol test affords additional evidence of glucose utilisation by organisms of the *Brucella* group. McAlpine and Slanetz (1928), in an examination of 20 strains of *Br. melitensis*, 17 of *Br. abortus* (bovine) and 17 of *Br. abortus* (porcine), found that when they were grown in 1 per cent. glucose peptone (Fairchild) water for 7 days the amount of glucose utilised was as follows :—

Br. melitensis	4 to 18 per cent.	
Br. abortus (bovine)	2 "	(approx.).
Br. abortus (porcine)	4 to 18 "	

Other workers have not obtained the same or such clear-cut results (Wilson, 1933), but it is interesting to find that *Br. melitensis* and *Br. abortus* (porcine type) differ so markedly in their ability to form acetylmethylcarbinol. As the only satisfactory way at present to differentiate the porcine *abortus* (*Br. suis*) from the bovine *abortus* strains seems to be the dye test (Huddleson, 1929, 1931 ; Wilson, 1933), the negative α-naphthol results obtained with the porcine strains are of some interest.

Notes from donors regarding *Brucella* strains.

Professor G. S. Wilson's strains. Three of the *melitensis* strains belonged to the Lisbonne-Taylor group (*vide infra*) and were serologically *Br. abortus* but biochemically *Br. melitensis*. Two of the *melitensis* strains were too rough for serological classification and very old, but were otherwise *Br. melitensis*. These were both α-naphthol negative. Four of the porcine strains were of American, two of Danish, and one of Australian origin. Two of the American strains gave doubtful results in the α-naphthol test.

Dr R. M. Taylor's strains. These came from the south-east, east and north-east of France, and belong to the Lisbonne-Taylor group. Their chief divergence from the main group of strains is that many of them appear to behave like *Br. melitensis* in the dye and H_2S tests, but are serologically like *Br. abortus*, and to a less extent the converse holds true. Dr Taylor has classified them on their behaviour on basic fuchsin and thionin media and production of H_2S, and not on their serological behaviour.

Dr Axel Thomsen's strains. All these with the exception of the *Br. melitensis* were isolated in Denmark.

Dr I. F. Huddleson's strains. These strains of *Br. abortus* (porcine type) were isolated between 1920 and 1934 from the blood of human beings, cows' milk, swine and infected chickens.

Application of the α-naphthol test to other bacteria.

Tests were made on certain stock laboratory cultures in glucose phosphate broth at varying periods of incubation. Positive results were obtained with *Staphylococcus aureus*, *albus* and *citreus*, *Streptococcus fæcalis*, *V. metchnikovi*, the Finckler-Prior vibrio and some strains of *V. choleræ* and *C. diphtheriæ*.

DISCUSSION.

Many attempts have been made to increase the sensitiveness of the Voges-Proskauer test, most of which have involved the use of oxidising agents or catalysts to oxidise or hasten the oxidation of acetylmethylcarbinol to diacetyl, which is the essential substance giving the reaction with KOH and peptone. These modifications have not been generally successful because the effect of catalysts has been too slight and that of oxidising agents too transient. With oxidising agents the colour reaction tends to disappear rapidly, probably because the reacting diacetyl-peptone combination is quickly oxidised to a colourless compound.

By the addition of creatine to the V-P. test O'Meara considerably increased its sensitiveness and was able to detect acetylmethylcarbinol in a concentration of 1 in 50,000. By the α-naphthol method, however, the writer was able to detect 1 part in 2,500,000 when creatine was added. The findings described herein sufficiently indicate the chemical and bacteriological specificity of the α-naphthol test. Though Harden and Norris (1911) suggested that the guanidine nucleus was an integral part of the substance giving the V-P. reaction with diacetyl and KOH, this conclusion may

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require revision, as urea and biuret have been found to react in the α-naphthol test.

The results obtained with the intermediate types in the *coli-aerogenes* group show that a great many are α-naphthol positive, though V-P. (creatine modification) negative. This finding brings these organisms into closer relationship with *B. aerogenes* and suggests that many if not all of those strains which are M.R.+ V-P.+ should no longer be classified as irregular, but as intermediates which produce just sufficient acetylmethylcarbinol to be detectable by the ordinary methods. The results obtained with the *coli-aerogenes* group as a whole suggest that the method is very delicate but suitable for the study of the metabolism of bacteria. The application of this test to the *Brucella* group shows that many strains produce acetylmethylcarbinol, though they are V-P. negative with the ordinary test. The production of acetylmethylcarbinol by organisms of this group provides additional evidence of their glucose utilisation. The fact that only 1 out of 23 porcine *abortus* strains was α-naphthol positive may assist in their differentiation from bovine *abortus* strains (21 out of 23 α-naphthol positive) and supplement the dye test. The value of the α-naphthol test may be increased by the examination of freshly isolated strains, as some of the *Brucella* strains here examined may have lost their power of acetylmethylcarbinol production.

The positive results obtained with certain strains of *V. choleræ*, *C. diphtheriæ*, staphylococci and streptococci call for further examination of these and other bacteria. Finally, it seems clear that the ordinary V-P. test has no real value as *negative* evidence of acetylmethylcarbinol production, although it, or the creatine modification, may still be of some value in routine work.

Summary.

1. A new test is described involving the use of α-naphthol for the detection of diacetyl or acetylmethylcarbinol.

2. The test is shown to be very delicate and chemically specific, and to be essentially an intensification of the Voges-Proskauer reaction.

3. The test has been applied to the study of the *coli-aerogenes* group of bacteria, members of the *Brucella* group and certain other organisms.

4. It has been found that acetylmethylcarbinol is produced by many organisms usually considered unable to do so.

I am indebted to Professors A. Robertson and H. J. Channon for certain chemicals used in this investigation, and to Professor Robertson and Dr E. R. Jones for assistance with the chemical problems involved. I wish also to thank Professor G. S. Wilson, Dr R. M. Taylor, Dr Axel

Thomsen and Dr I. F. Huddleson for the cultures of *Brucella* strains which they provided and for information concerning them, and Professor H. D. Wright for criticism and advice during the investigation.

Other chemicals used were supplied by the British Drug Houses Ltd., Messrs Harrington Bros., and Drs Fraenkel and Landau (manufacturing chemists), Berlin.

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J. Dairy Res. 34, 109

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Acetaldehyde and diacetyl production by *Streptococcus thermophilus* and other lactic streptococci

By VITTORIO BOTTAZZI and FRANCO DELLAGLIO
Istituto di Microbiologia, Università Cattolica del S. Cuore, Piacenza, Italy

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SUMMARY. The quantities of acetaldehyde and diacetyl produced in skim-milk and MRS media are reported for strains of *Streptococcus lactis*, *Str. cremoris*, *Str. diacetilactis*, *Str. faecalis* and *Str. thermophilus* obtained from culture collections or freshly isolated from natural milk cultures used in cheese manufacture. The diacetyl:acetaldehyde ratio in skim-milk medium was found to be approximately 0·1:1 for *Str. lactis* and *Str. cremoris*, 0·5:1 for *Str. diacetilactis* (5:1 for *Str. diacetilactis* PIM 13 and K2 strains) and for *Str. thermophilus* 0·3:1, in skim-milk medium, and 0·8:1, in MRS medium; in the latter species cultured in MRS medium the ratio varied from 3·5:1 to 0·04:1 and when cultured in milk from 1·2:1 to 0·03:1.

In both media all strains of *Str. thermophilus* formed more acetaldehyde and diacetyl than other homofermentative lactic streptococci.

The ratio diacetyl:acetaldehyde in 9 strains of *Str. diacetilactis* was found to be unsuitable for a good culture flavour.

It is well known that products other than lactic acid may be elaborated by mesophilic homofermentative lactic acid streptococci. In recent years attention has been given to small quantities of formic, acetic, propionic and butyric acid, ethanol, carbon dioxide, acetoin, acetaldehyde and diacetyl produced by these organisms. Of these components, however, acetaldehyde and diacetyl have been recognized as especially important flavour components of dairy products. Diacetyl has long been regarded as one of the more important compounds found in butter aroma, and acetaldehyde has been shown by Bading & Galesloot (1962) to be responsible for a flavour defect of lactic cultures.

Whereas low concentrations of acetaldehyde are desirable in mixed-strain butter cultures and in yoghurt cultures (Pette & Lolkema, 1950) excessive quantities of acetaldehyde have been shown to be correlated with 'green flavour' (Lindsay, Day & Sandine, 1965). Lindsay & Day (1965) found that the concentrations of diacetyl and acetaldehyde in lactic cultures determine the intensity of the flavour but that the relative amounts are of great importance for the flavour quality; the diacetyl:acetaldehyde ratio of 4:1 occurred in normal flavoured cultures and when the ratio was lower than 3:1 a 'green flavour' was observed.

The quantitative production of diacetyl and acetaldehyde in cultures of *Str. lactis*, *Str. lactis* var. *maltigenes*, *Str. cremoris*, *Str. diacetilactis* and *Leuconostoc citrovorum*

has already been studied (Lindsay *et al.* 1965; Lindsay & Day, 1965; Pack, Sandine, Elliker, Day & Lindsay, 1964; Keenan, Lindsay, Morgan & Day, 1966; Morgan, Lindsay, Libbey & Pereira, 1966; Vedamuthu, 1966). The present investigation was made to determine the diacetyl:acetaldehyde ratio in *Str. thermophilus* cultures in comparison with cultures of *Str. lactis*, *Str. cremoris* and *Str. diacetilactis* and some *Str. faecalis* strains in skim-milk and in medium (MRS) in which the growth of many strains was better than in sterilized milk.

EXPERIMENTAL PROCEDURES

Cultures

Authenticated strains of *Str. thermophilus* and strains of other streptococci are listed in Table 1.

Other strains of *Str. thermophilus* were isolated from natural milk cultures commonly used in the manufacture of many types of cheese in the north of Italy (Table 2).

Table 1. *Authenticated strains of streptococci examined*

Str. thermophilus	P 15[1], 23 A[1], T 4[1], 26[1] 3203[2] Z 17[3]
Str. faecalis	NCDO 610, ATCC 11420 B 65[1], F 1[4]
Str. lactis	NCDO 275, NCDO 605, NCDO 606 M 87[1], T 2017[1]
Str. diacetilactis	NCDO 176, NCDO 188, NCDO 615 2[1] DRC 1[3], DRC 3[3], DRC 4[3] PIM 13[5], PIM K2[5], PIM 704[5], PIM 710[5]
Str. cremoris	3141[2] C 3[3], C 13[3]
Streptococcus group N	NCDO 617

NCDO = National Collection of Dairy Organisms.
ATCC = American Type Culture Collection.
Other strains received from :
1. National Institute for Research in Dairying, Shinfield, Reading, England.
2. Nederlands Instituut voor Zuivelonderzoek, Ede, Holland.
3. Dairy Research Institute Laboratory, Department of Agriculture, Victoria, Australia.
4. Istituto di Microbiologia, Università Cattolica, Piacenza, Italy.
5. University of Lomonosov, Moscow, U.S.S.R.

The cultures were transferred every 2 days in MRS medium and incubated at 45 °C for 18 h (*Str. thermophilus*) or at 30 °C for 18 h (other species).

For determination of acetaldehyde and diacetyl production in milk, the [illegible]ial strains were cultured in autoclaved skim-milk medium incubated at 30° [illegible] 5°C depending on the type of micro-organism.

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Determination of the acetaldehyde and diacetyl content

Each of the cultures after two 1-day transfers for activity restoration in the appropriate medium, were analysed for acetaldehyde, diacetyl and pH after 24- or 28-h incubation in skim-milk or MRS medium.

Acetaldehyde was determined by the 3-methyl-2-benzothiazolone hydrazone hydrochloride method described by Lindsay & Day (1965).

The Owades & Jakovac method, as modified by Pack *et al.* (1964), was used for diacetyl determination.

All analyses were made in duplicate.

Table 2. *Diacetyl and acetaldehyde production by freshly isolated strains of* Str. thermophilus *in skim-milk and in MRS media*

Medium . . .	Diacetyl (A) and acetaldehyde (B), μg/ml culture*						
	MRS			Skim-milk			
Strain	A	B	A/B	pH	A	B	A/B
S 215	9·60	2·80	3·40	4·72	0·90	2·90	0·30
S 112	6·20	4·60	1·40	4·50	1·50	6·50	0·20
S 127	6·10	2·70	2·50	4·60	3·80	6·20	0·60
S 152	3·60	3·50	1·00	4·57	1·20	6·50	0·20
S 61	5·20	2·60	2·00	4·60	1·10	5·60	0·20
S 221	10·00	3·70	2·70	4·60	1·90	6·30	0·30
S 222	4·30	4·20	1·00	4·65	0·70	4·80	0·20
S 120	2·10	5·70	0·30	4·40	0·50	8·30	0·06
S 164	3·80	6·10	0·60	4·45	0·10	7·40	0·01
S 188	6·40	6·50	1·00	4·40	0·40	7·50	0·05
S 18	3·00	2·60	1·10	5·02	0·10	1·60	0·06
S 26	1·10	4·30	0·30	4·50	0·30	1·80	0·15
S 82	4·30	4·60	0·90	4·52	1·70	6·50	0·25
S 128	1·80	4·80	0·30	4·45	1·70	5·90	0·25
S 132	0·85	3·70	0·20	4·50	1·85	4·60	0·40
S 53	6·40	4·00	1·60	4·60	2·30	6·70	0·35
S 88	10·00	4·60	2·20	4·45	1·65	7·10	0·25
S 97	8·50	3·10	2·80	4·70	1·45	6·70	0·20
S 130	8·20	3·80	2·20	4·50	2·45	8·30	0·30
S 133	5·70	5·00	1·10	4·60	1·75	7·70	0·25
S 142	6·30	2·70	2·30	4·50	2·00	4·00	0·50
S 155	8·80	2·70	3·00	4·26	3·10	2·60	1·20
S 190	2·00	3·30	0·70	5·39	1·20	2·80	0·40

* Average of duplicate analyses; blank values estimated on uninoculated media have been deducted.

RESULTS AND DISCUSSION

Table 3 shows the production of acetaldehyde and diacetyl in MRS and skim-milk media, by the authentic strains of *Str. cremoris*, *Str. lactis*, *Str. diacetilactis*, *Str. thermophilus* and *Str. faecalis;* whereas, in Table 2, figures are reported concerning the strains of *Str. thermophilus* isolated from natural milk cultures.

After 24-h incubation all the lactic homofermentative mesophilic streptococcal cultures were found to produce diacetyl and acetaldehyde in MRS and in autoclaved skim-milk media; some strains of *Str. cremoris*, and all strains of *Str. lactis*, produced very small amounts of diacetyl, and all cultures formed appreciably more acetaldehyde; similar results were obtained by Harvey (1960), Lindsay & Day (1965), Lindsay *et al.* (1965) and Keenan *et al.* (1966).

Table 3. *Diacetyl and acetaldehyde production by lactic acid streptococci in skim-milk and in MRS media*

Medium . . .		Diacetyl (A) and acetaldehyde (B), μg/ml culture*						
		MRS			Skim-milk			
Species and strain		A	B	A/B	pH	A	B	A/B
Str. diacetilactis	DRC1	3·07	7·40	0·40	4·42	3·40	5·20	0·65
	No. 2	3·20	8·90	0·30	4·55	4·10	6·40	0·65
	PIM13	22·30	8·60	2·60	5·15	30·10	7·20	4·10
	614	3·45	5·40	0·65	4·50	6·00	8·30	0·70
	PIMK2	39·20	11·20	3·50	5·02	55·00	11·40	4·80
	PIM704	3·90	4·60	0·90	4·72	1·50	9·70	0·15
	PIM710	3·60	6·40	0·60	4·71	1·20	10·30	0·10
	DRC3	2·90	10·20	0·30	4·35	1·40	2·20	0·65
	DRC4	2·30	2·30	1·00	5·20	0·60	5·50	0·10
	188	2·05	9·70	0·20	5·10	1·30	4·10	0·30
	176	5·70	7·30	0·80	4·30	3·70	7·50	0·50
Str. lactis	MS7	0·50	4·70	0·10	4·45	0·10	2·30	0·05
	275	0·60	4·70	0·15	4·40	0·05	0·70	0·07
	605	0·30	3·70	0·10	4·50	0·10	1·10	0·10
	606	0·30	3·50	0·10	4·40	0·10	0·80	0·10
	T2017	0·25	6·50	0·04	4·50	0·10	2·00	0·05
Str. cremoris	C3	0·25	8·00	0·03	4·90	0·05	1·10	0·05
	C13	0·20	2·90	0·07	4·80	0·10	0·30	0·30
	3141	1·90	7·20	0·25	4·80	2·30	3·70	0·60
Str. Group N (*lactis-cremoris* intermediate)	617	2·15	7·80	0·30	4·80	1·60	1·80	0·90
Str. thermophilus	P15	0·70	1·60	0·40	4·60	0·10	0·40	0·25
	Z17	1·20	2·50	0·50	5·10	1·00	1·30	0·75
	T4	2·80	4·55	0·60	4·15	0·50	6·20	0·08
	23A	3·20	2·65	1·30	4·35	1·20	4·50	0·25
	26	2·30	4·60	0·50	4·15	2·10	6·40	0·35
	3203	8·50	5·10	1·70	4·55	1·40	1·00	1·40
Str. faecalis	F1	3·90	3·90	1·00	4·95	0·00	0·00	—
	B65	1·20	11·20	0·10	5·20	0·00	3·50	—
	610	1·70	13·60	0·10	4·95	0·00	5·30	—
	11420	0·60	12·60	0·06	5·60	0·00	4·10	—

* Average of duplicate analyses; blank values estimated on uninoculated media have been deducted.

In general, *Str. diacetilactis* formed considerably higher quantities of both compounds than did *Str. cremoris* or *Str. lactis* either in MRS medium or in autoclaved skim-milk medium; the acetaldehyde production, however, except for the strains PIM, was somewhat lower than those reported by Harvey (1960) (mean 7 ppm. as compared to 12 ppm. reported by Harvey). The acetaldehyde production by the PIM strains was similar to that found by Harvey (1960) but the diacety c-tion reached exceptionally higher levels (30–50 ppm).

As indicated in Table 2, milk cultures of all our isolates of *Str. thermop* on-tained acetaldehyde and diacetyl: mean 3·0 and 1·0 ppm. in skim-milk o le-hyde and diacetyl, respectively, compared with 3·5 and 3·0 ppm. in MR m.

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This is consistent with our observation (Bottazzi, 1966) that strains of *Str. thermophilus* give positive Voges-Proskauer reaction.

Str. faecalis did not produce diacetyl in milk but produced about 5 ppm. of acetaldehyde.

Of more interest is the ratio diacetyl:acetaldehyde and its variations under the different experimental conditions: the mean ratio was found in MRS medium to be approximately 0·10 for *Str. lactis*, 0·15 for *Str. cremoris*, 0·50 for *Str. diacetilactis* (5·3 for *Str. diacetilactis* PIM 13 and K2 strains) and 0·80 for *Str. thermophilus*; in autoclaved skim-milk medium the corresponding values were 0·08, 0·8, 0·4 (5·2) and 0·3.

As shown in Table 2, *Str. thermophilus* produced diacetyl and acetaldehyde in the approximate ratios of 3·5 to 0·04 in MRS medium and 1·20 to 0·03 in skim-milk medium.

In view of the established significance of this ratio in the flavour of lactic cultures, our data suggest that in the group of mesophilic homofermentative streptococci all single-strain cultures produce these compounds in an unfavourable ratio. Lindsay *et al.* (1965) observed 'green flavour' when the ratio of the 2 compounds was lower than 3·0; on this basis only the *Str. diacetilactis* strains PIM 13 and K2 would be likely to produce a good culture flavour.

Many strains of *Str. thermophilus*, isolated from natural milk cultures, produced in autoclaved skim-milk medium significant quantities of diacetyl and for some strains the ratio with acetaldehyde reached 1·2. The quantities of diacetyl produced in MRS medium by *Str. thermophilus* were found to be from 0·30 to 10·00 ppm. and in skim-milk medium from 0·30 to 3·80 ppm.; the acetaldehyde concentration varied from 2·60 to 6·50 ppm. in MRS medium and from 1·60 to 8·30 ppm. in skim-milk medium. This production of diacetyl in MRS and skim-milk media from strains of *Str. thermophilus* was higher than that of all the other homofermentative lactic streptococci.

Further investigations are now in progress to determine the diacetyl:acetaldehyde ratio in mixed-strain cultures of *Str. thermophilus* and also to determine other carbonyl compounds with particular reference to the neutral monocarbonyl class.

The authors thank Dr C. Corradini for his assistance and helpful suggestions.

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an automated procedure for the determination of diacetyl in beer

By Arthur L. Brandon,
Technical Center,
Anheuser-Busch, Inc.
St. Louis, Mo.

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IN RECENT years the determination of diacetyl in beer has become increasingly important in the brewing industry because of the unpleasant flavor and aroma it imparts. As little as 0.3 ppm can be detected by some tasters (2).

A number of methods for the determination of diacetyl have been proposed. The Voges-Proskauer method (6) was modified by Westerfield (8) for the determination of diacetyl in blood and by Owades, Jakovac, and Vigilante (4) for the determination in beer, but is not satisfactory for samples containing acetoin. The method proposed by White, Krampitz, and Werkman (9) is not sensitive enough for the small amounts of diacetyl found in beer and also produces lower values in the presence of ethyl alcohol. Kato and Nishikawa (3) published a method in which they combined the distillation procedure of West, Lautenbach, and Becker (7) and the colorimetric procedure of Prill and Hammer (5).

A paper on a semiautomated procedure for the determination of diacetyl in beer was presented by the author at the 1964 Technicon Symposium (1). The present paper covers the work completed on a fully automated procedure. Comparative manual data were obtained with the Kato-Nishikawa method (3) with the exception that all-glass distillation equipment was used.

Experimental Details

Apparatus

(1) AutoAnalyzer modules. Large liquid sampler, two proportioning pumps, digestor with 3-rpm motor and 52T and 108T gears (6.2 Helix rpm), heating bath, colorimeter with tubular flow cell, range expander, recorder (10 inches per hour), and two vacuum pumps. The vacuum pumps are equiped with needle valves for better control of vacuum.

(2) 18 by 150-mm. test tubes.

(3) 125-ml. Erlenmeyer flasks.

(4) Ultrasonic unit used for degassing beer samples. The unit used in the author's laboratory is the Son-Blaster Ultrasonic Generator, Series 600, manufactured by the Narda Ultrasonics Corp., Westbury, L.I., N.Y. Put about one inch of water in the tank of the unit.

(5) CO_2 system. (See **Fig. 1**). Consists of a five-pound CO_2 cylinder connected to two 2-liter aspirator bottles as follows: Connect the two aspirator bottles with a piece of rubber or plastic tubing through the side tubes at the bottom of each bottle. Place bottle No. 2 on a level above bottle No. 1, then fill bottle No. 1 with distilled water. Wire a stopper, containing two glass tubes bent at right angle, to bottle No. 1 and lower bottle No. 2. Connect one of the glass tubes to the outlet of the CO_2 cylinder and the other tube to the CO_2 manifold tubes.

Reagents

D-1: *Denatured alcohol*, five per cent. Dilute 1000 ml. of denatured alcohol (Formula 3A) to 20 liters with distilled water.

D-2: *Sulfuric acid*, 10 per cent. Cautiously add 509 ml. of concentrated sulfuric acid (s.g. 1.84) to approximately 8,000 ml. of distilled water in a 2.5-gal. bottle, calibrated at 9,000 ml. Mix well and cool to room temperature. Dilute to 9,000 ml. with distilled water and mix.

D-3: *Hydroxylamine acetate*. (a) Hydroxylamine hydrochloride. Dissolve 394 grams of hydroxylamine hydrochloride in approx. 2,000 ml. of distilled water in a 2.5-gallon bottle, calibrated at 9,000 ml. Dilute to 9,000 ml. with distilled water and mix. (b) Sodium acetate. Dissolve 422 grams of anhydrous sodium acetate or 700 grams of hydrated sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) in approximately 1,500 ml. of distilled water. Dilute to 2,000 ml. with distilled water and mix. (c) Mixed reagent. Mix 1600 ml. of hydroxylamine hydrochloride solution with [illegible] ml. of sodium acetate solution. Filter through No. 1 Whatman filter [illegible] before use.

D-4: *Acetone–potassium phosphate solution*. Dissolve 648 grams of anhydrous dibasic potassium phosphate or 855 grams of hydrated dibasic potassium phosphate ($K_2HPO_4 \cdot 3H_2O$) in approximately 5,000 ml. of distilled water in a 2.5-gallon bottle, calibrated at 9,000 ml. Add 900 ml. of reagent grade acetone and 35 ml. of Brij 35, dilute to 9,000 ml. with distilled water, and mix. Filter through two thicknesses of No. 1 Whatman filter paper before use.

D-5: *Sodium potassium tartrate*, 45 per cent with ammonium hydroxide. Transfer 4050 grams of sodium potassium tartrate (Rochelle salt) to a 2.5-gallon bottle, calibrated at 9,000 ml. Fill the bottle to the 9,000 ml. mark with distilled water and shake until all of the salt has dissolved, or heat in a water bath at 70° C. until the salt has dissolved, then cool to room temperature. Add 1233 ml. of concentrated ammonium hydroxide, stopper, and mix. Filter through two thicknesses of No. 1 Whatman filter paper before use.

D-6: *Ferrous sulfate*, 1.5 per cent in three per cent sulfuric acid. Dissolve 15 grams of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1000 ml. of three per cent sulfuric acid. Store in a glass-stoppered reagent bottle; prepare fresh once a week. Filter through No. 1 Whatman filter paper before use.

D-7: *Sulfuric acid*, three per cent. Cautiously add 152 ml. of concentrated sulfuric acid to approx. 8,000 ml. of distilled water in a 2.5-gallon bottle, calibrated at 9,000 ml. Dilute to 9,000 ml. with distilled water and mix.

Standards

Standards are prepared in finished beer that contains less than 0.10 ppm. diacetyl and is free of any diacetyl precursor. Since it is difficult to obtain pure diacetyl and also to weigh an exact amount, the standards are prepared to contain the approximate level of diacetyl required, then the diacetyl value of each standard is determined by the manual procedure (3). Thus, the diacetyl value of each standard is known and the automated procedure is correlated with the man-

TECHNICON AUTOANALYZER METHOD Diacetyl
CONCENTRATION RANGE 0-1.00 ppm
TYPE SAMPLE Beer
REFERENCE Kato, S. and Nishikawa, N., Bull. Brew. Sci. 6, 23 (1960/1961)

DATE August 23, 1966
SIGNATURE
AFFILIATION Anheuser-Busch, Inc.

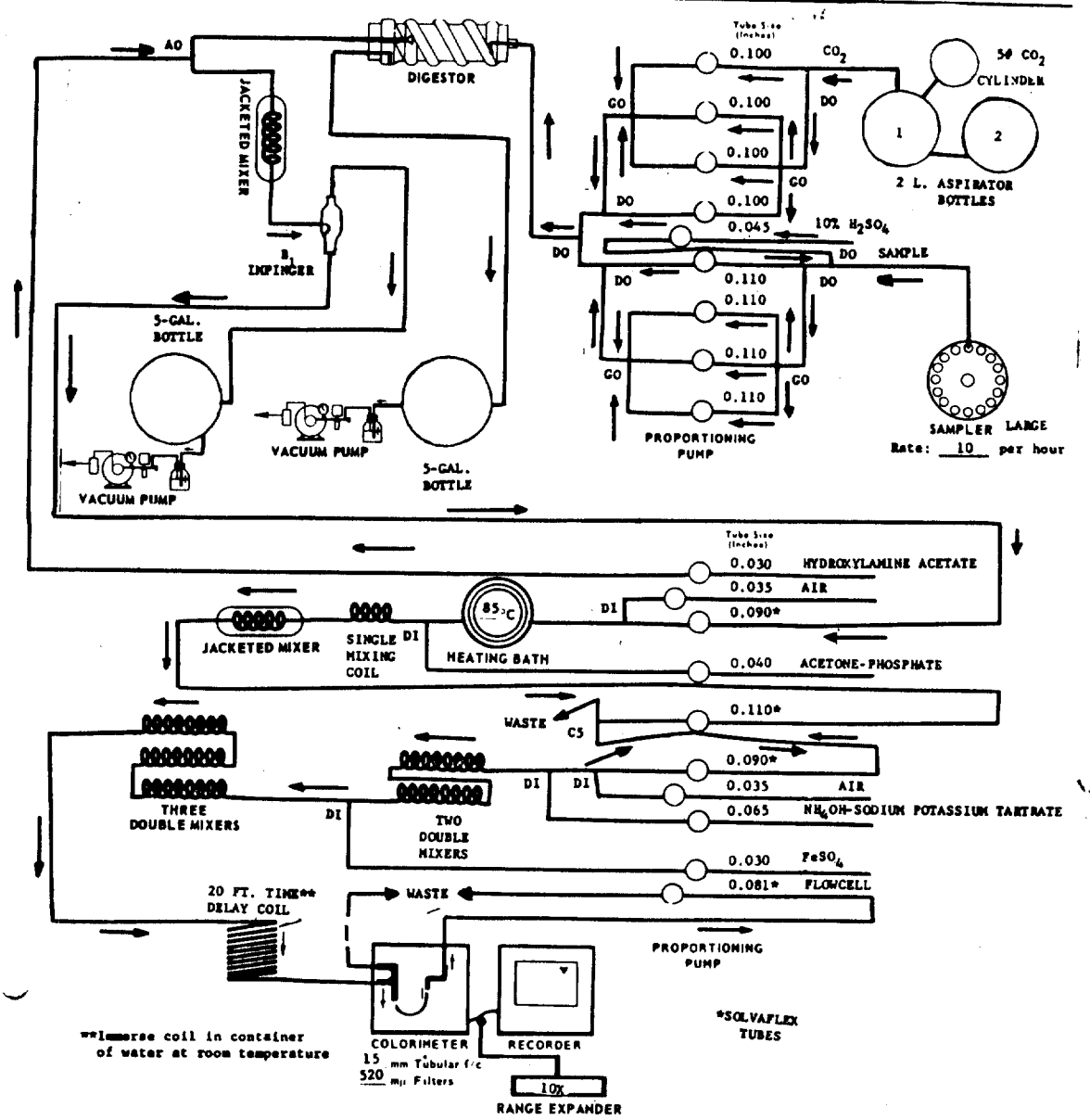

Figure 1: *Flow diagram.*

ual procedure.

(1) *Stock Solution A.* Dissolve 1.0 ml. of diacetyl in degassed beer, dilute to 1000 ml. with degassed beer, and mix. After preparation of stock solution B, as described below, transfer the remaining portion of stock solution A to 12-ounce amber beer bottles, crown, and pasteurize.

(2) *Stock Solution B.* Dilute 100 ml. of stock solution A to 1,000 ml. with degassed beer and mix. Remove 10 ml., as described below, for manual determination, then transfer the remaining portion of stock solution B to 12-ounce amber beer bottles, crown, and pasteurize.

Dilute 2.0 ml. of stock solution B to 1,000 ml. with degassed beer and mix. Determine the diacetyl content of this sample and the beer used for dilution in triplicate, by the manual procedure (3). Calculate the diacetyl content of stock solution B by the formula $D = (A - B)/2$, in which D = mg. of diacetyl per ml. in stock solution B, A = ppm. of diacetyl in diluted sample, and B = ppm. of diacetyl in beer used for dilution.

(3) *Working Standards.* Prepare a series of *six* standards to include 0.05-0.10, 0.15, 0.25, 0.50, 0.75, and 1.00 ppm. of diacetyl.

The 0.05-0.10 ppm. standard is a composite of 20 liters of degassed, finished beer that contains 0.10 ppm. or less of diacetyl and is free of diacetyl precursor. The composite is mixed well and then transferred to 12-ounce amber beer bottles, crowned, and pasteurized.

Calculate the volumes of stock solution B needed to prepare 9,000 ml. of each of the remaining standards by the formula $V = 9000(A - B)/1000C$, in which V = ml. of stock solution B, A = ppm. of diacetyl in the standard, B = ppm. of diacetyl in the beer used for dilution, and C = mg. of diacetyl per ml. of stock solution B.

Add the calculated volume of stock solution B to a 2.5-gallon bottle, calibrated at 9,000 ml., dilute to 9,000 ml. with degassed beer containing less than 0.10 ppm. of diacetyl and free of diacetyl precursor, and mix. Transfer to 12-ounce amber beer bottles, crown, and pasteurize. Determine the

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diacetyl content of each standard, in duplicate, by the manual procedure (3).

Store all the standards at 35–40° F. The above procedure will furnish a three- to six-month supply of all standards.

Preparation of Samples

Transfer approximately 50 ml. of sample to a 125-ml. Erlenmeyer flask. Momentarily immerse the flask in the tank of the ultrasonic unit. Remove, allow the foam to settle, then return the flask to the ultrasonic unit for three or four minutes.

Procedure

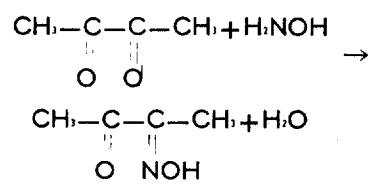
Place an 18 by 150-mm. test tube, filled with five per cent alcohol, in position 1 on the inner row of the large sampler turntable. Two tubes of each standard or sample are then placed on the turntable, each one being separated by two tubes, filled with five per cent alcohol, which acts as a wash between standards or samples. Standards are placed in the sampler in increasing order of concentration. In order to determine recorder drift, two tubes of the 0.05–0.10 ppm. standard are placed in every tenth sample position on the turntable. Three tubes of five per cent alcohol should be placed after the last standard and before the standard used to determine recorder drift if the preceding sample is suspected of having a high diacetyl content. Samples taken during and immediately after active fermentation should be degassed, then kept cold (5–7° C), and placed in the sampler just before the sample probe is ready to pick up the sample. The sampler timer is set at 1.5 minutes, thus allowing a three-min. sample pickup, and three-min. five per cent alcohol pickup.

As shown in the flow diagram of the automated procedure (**Fig. 1**), 10 per cent sulfuric acid (0.8 ml./min.) is pumped into the sample at the entrance end of the manifold (this prevents formation of bubbles in the manifold and excessive foaming in the helix). The acid–sample mixture is then pumped at a rate of 15.6 ml./min., giving a net of 14.8 ml. of sample/min., and is segmented with CO_2, after which it enters the revolving helix (6.2 rpm.). The first-stage heater is set at 3.2 amp. (235° C.), and the second-stage heater at 4.0 amp. (200° C.). As the sample passes through the helix, volatile compounds are evolved and are pulled off continuously by the vacuum pump through the long-stem funnel at the end of the helix.

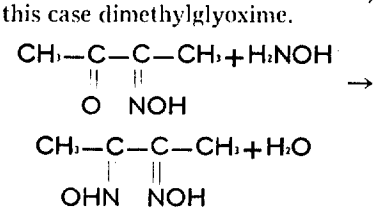
Hydroxylamine acetate (0.3 ml./min.) is added to the distillate (in the vapor phase), after which the mixture is cooled and condensed in a jacketed coil, and then passes into the B_1 impinger. The cooled distillate is removed continuously from the impinger at a rate of 2.4 ml. per minute (Solvaflex tubing), is segmented with air (0.4 ml./min.), and then is passed through the heating bath at 85° C. (double coil). Upon emerging from the heating bath (Acidflex tubing is used for all connections between heating bath and jacketed coil), the hot distillate stream joins with a stream of acetone–phosphate (0.6 ml./min.), passes through a single mixing coil, and then passes through a jacketed coil, where it is cooled.

The distillate is pumped into a C5 debubbler at a rate of 3.4 ml./per minute (Solvaflex tubing) and then is pumped from the debubbler at a rate of 2.4 ml./per minute (Solvaflex tubing) with the air and excess distillate going to waste. Then the distillate stream is segmented with air (0.4 ml./min.) and joined with the ammonium hydroxide–sodium potassium tartrate stream (1.6 ml./min.), after which it is mixed by passing through two double mixing coils. Next the stream is joined with the ferrous sulfate stream (0.3 ml./min.) and passed through three double mixing coils and the time-delay coil (half-length). Next the stream is pulled through the flow cell of the colorimeter at a rate of 2.0 ml./per minute (Solvaflex tubing), and the signal from the colorimeter is expanded by 10 with the range expander.

When hydroxylamine acetate is added to the distillate, diacetyl monoxime is formed:



After passing through the heating bath at 85° C., the dioxime is formed, in this case dimethylglyoxime.

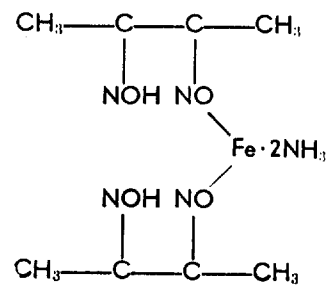


Excess hydroxylamine must be removed from the reaction mixture before color development, since hydroxylamine in alkaline solution oxidizes ferrous iron to ferric iron (5). When acetone is added to the distillate, the excess hydroxylamine is converted to an acetoxime which does not interfere in the subsequent color development. However, unless the pH of the distillate is nearly neutral (pH 6.7), diacetyl dioxime will be converted to acetoxime and diacetyl monoxime. To avoid this, dibasic potassium phosphate is added to give the desired pH.

Ammonium hydroxide is added to supply the alkaline condition necessary to produce the color desired. It is also necessary since it is probable a part of the colored complex is formed on the addition of ferrous iron (5).

Sodium potassium tartrate acts as a sequestering agent and prevents the precipitation of ferrous hydroxide and also prevents the formation of ferric complexes with dimethylglyoxime (5).

When ferrous sulfate is added to the distillate containing ammonium hydroxide and sodium potassium tartrate, an ammono-ferrous dimethylglyoximate complex is formed with the probable formula (5):



Before starting the day's run the system is cleaned by running three per cent sulfuric acid through all the reagent lines for 10 minutes followed by a distilled-water wash for 10 minutes; five per cent alcohol is continuously sampled and distilled, the reagents are started, and the baseline is set at 99 per cent transmission after reagents and alcohol have been pumped for approximately 30 minutes.

Results and Discussion

Finished beers contain four to five per cent alcohol by volume. However, samples containing more or less than five per cent alcohol by volume may be submitted for diacetyl analysis. Samples taken during the early stages of fermentation will contain zero to

Table I

Effect of Alcohol Concentration on Diacetyl Values

Alcohol, %	Diacetyl, A ppm
0	0.19
5	0.18
10	0.18
15	0.19
20	Off chart

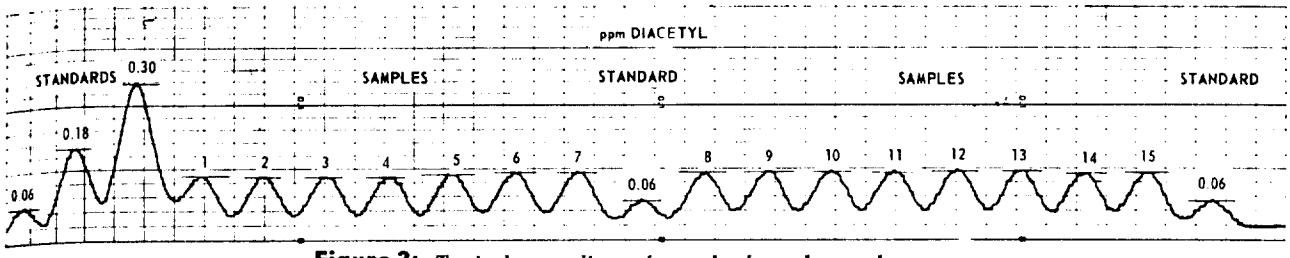


Figure 2: *Typical recording of standards and samples.*

Table II

Effect of Dilution on the Determination of Diacetyl

Sample No.	Dilution*	Diacetyl, ppm. Diluted Sample	Diacetyl, ppm. Original Sample
1	10:100	0.11	1.10
2	25:100	0.28	1.12
3	50:100	0.54	1.08
4	75:100	0.82	1.09
5	None	1.16	1.16

*Samples diluted with five per cent alcohol.

four or five per cent alcohol, while some samples from fermentation studies or samples other than beer may contain more than five per cent alcohol. Therefore, it was necessary to determine the effect of alcohol concentration on diacetyl values. **Table I** shows the diacetyl values obtained with varying concentrations of alcohol. There is no apparent effect on the diacetyl values when the alcohol concentration is between zero and 15 per cent, but there was a noticeable precipitation in the system at the 20 per cent alcohol level, which probably accounts for the recording obtained.

When samples containing over 1.0 ppm. of diacetyl are analyzed, a dilution is necessary to bring the values within the range of the standards. A sample containing 1.2 ppm. of diacetyl (manual value) was prepared and various dilutions were made with five per cent alcohol. The data tabulated in **Table II** indicate that dilution with five per cent alcohol is feasible.

During the development of the procedure, considerable trouble was encountered with recorder drift. We finally came to the conclusion that the drift was caused by a coating of the flow-cell walls with one or more of the reagents, since we could bring the recorder pen back to the baseline when the flow cell was cleaned with three per cent sulfuric acid. To overcome this difficulty, the addition of a wetting agent to one of the reagents was tried. Tween 20 was added to one reagent at a time but without success. In some cases precipitation would occur in the reagent after a short period of time. The addition of Brij 35 to the acetone-phosphate reagent finally proved successful. However, it did not eliminate drift completely. In order to compensate for the small drift still obtained, the standard having the lowest concentration of diacetyl is inserted in every tenth sample position in the sampler. Since the drift is linear, a simple arithmetic calculation is all that is necessary to correct the value of each sample.

A typical strip chart recording of standards and samples is shown in **Fig. 2** and the calculated values are tabulated in **Table III.** A calibration plot of the standards is illustrated in **Fig. 3.** Since the calibration is a

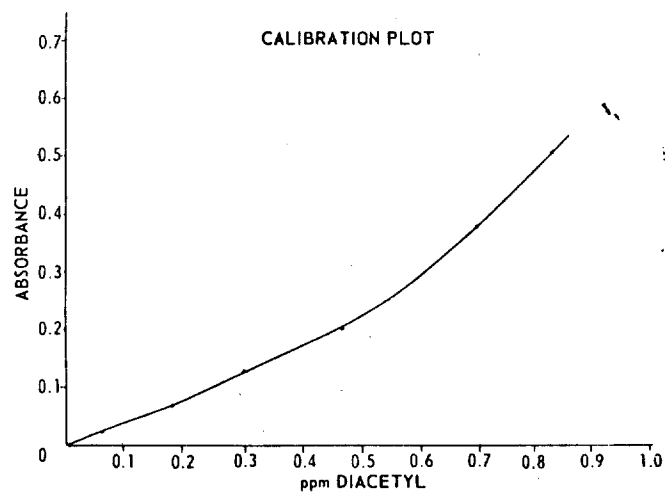


Figure 3: *Calibration plot of standards.*

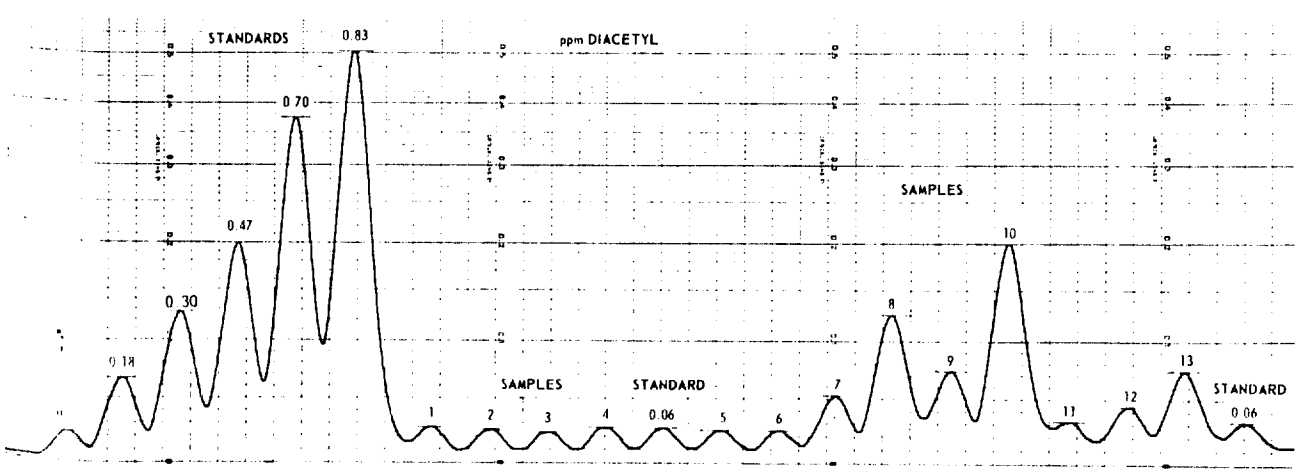


Figure 4: *Recording of standards demonstrating efficiency of washout.*

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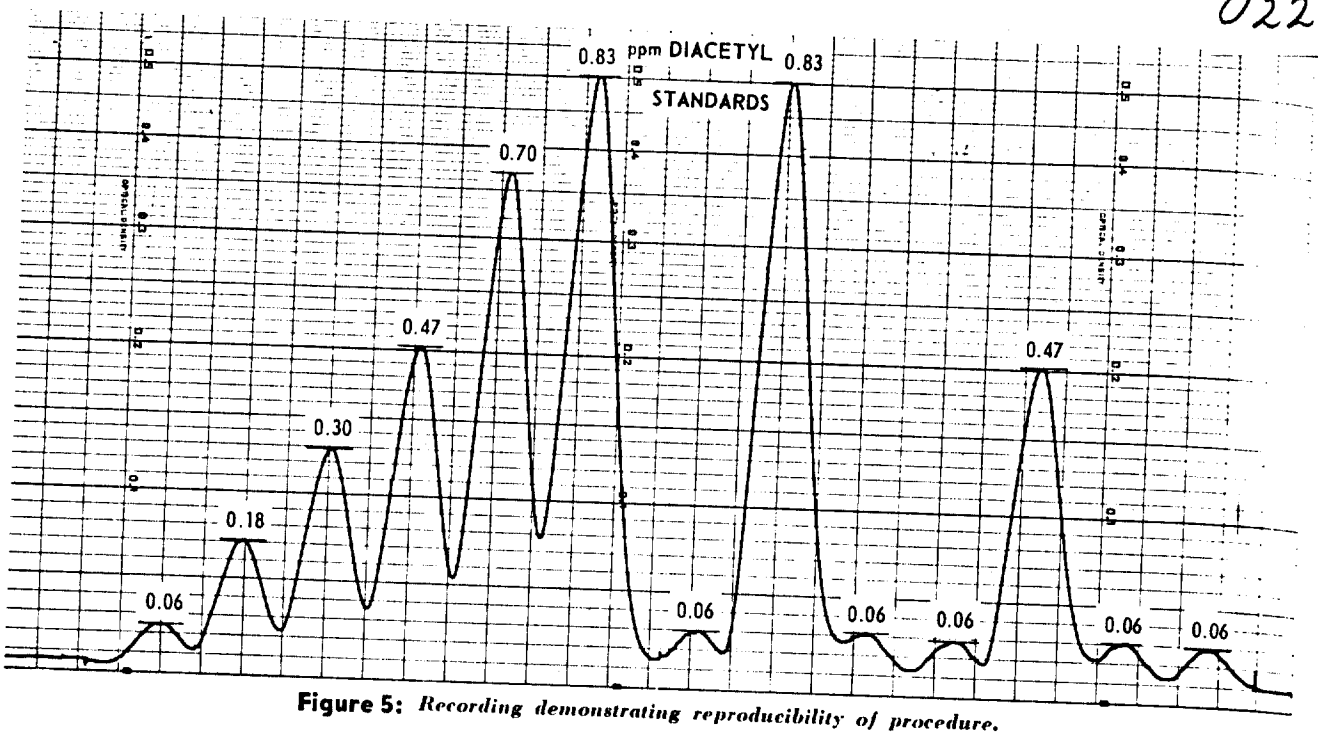

Figure 5: *Recording demonstrating reproducibility of procedure.*

curve, and not a straight line, the chart reader is used for calculating the diacetyl values of the samples. The value of each of the 0.06-ppm. standards is determined. Then 0.06 is subtracted from each of these values, giving the recorder drift correction, which is then subtracted from each of the sample values.

Normally two tubes of five per cent alcohol furnishes sufficient washout between samples. However, if a sample high in diacetyl precedes one that is low, three tubes of five per cent alcohol are required. This is illustrated in the strip chart recording in **Fig. 4,** which was run using standards of known diacetyl content. Three tubes of five per cent alcohol separated the first 0.83-ppm. standard from the following 0.06-ppm. standard, but only two tubes of alcohol were used to separate the second set of these same standards. It will be noted that the second 0.06-ppm. standard had a higher peak than the first one, indicating that two tubes of alcohol did not give sufficient washout. When the standard containing 0.06 ppm. of diacetyl followed one containing 0.47 ppm. separated by two tubes of alcohol, the peak of the 0.06-ppm. standard was slightly higher, but not significantly so. Thus, if a sample containing more than 0.5 ppm. of diacetyl precedes one low in diacetyl, three tubes of alcohol should be used to separate them.

The reproducibility of values by

Table III

Diacetyl Values Calculated from the Chart in Fig. 2

Sample No.	Diacetyl from Chart, ppm.	Recorder Drift Correction	Corrected Diacetyl, ppm.
1	0.07	−0.01	0.06
2	0.06	−0.01	0.05
3	0.06	−0.01	0.05
4	0.07	−0.01	0.06
0.06*	0.07	−0.01	0.06*
5	0.06	−0.01	0.05
6	0.06	−0.01	0.05
7	0.14	−0.01	0.13
8	0.32	−0.01	0.31
9	0.19	−0.02	0.17
10	0.47	−0.02	0.45
11	0.08	−0.02	0.06
12	0.12	−0.02	0.10
13	0.19	−0.02	0.17
0.06*	0.08	−0.02	0.06*

*Standard.

Table IV

Reproducibility of Diacetyl Values from Fig. 5*

Replicate No.	Diacetyl, ppm.
1	0.12
2	0.12
3	0.11
4	0.11
5	0.11
6	0.12
7	0.12
8	0.11
9	0.11
10	0.11
11	0.12
12	0.12
13	0.12
14	0.11
15	0.11

*Standard deviation = ±0.0055. Coefficient of variation = ±4.8%. Reproducibility = ±0.0055.

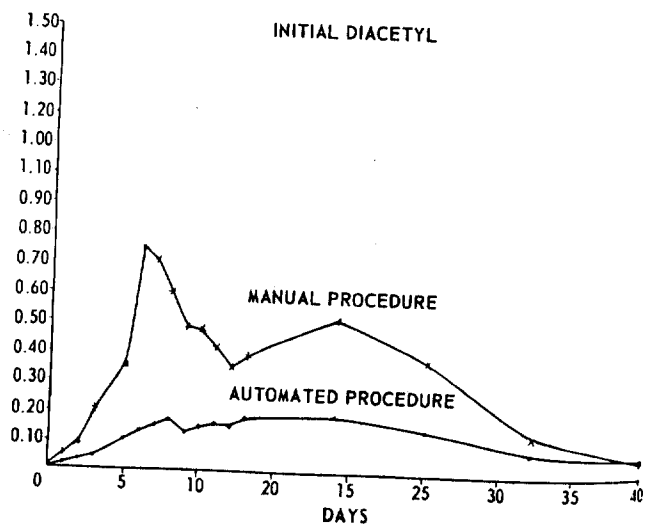

Figure 6: *Tracking study—initial diacetyl—automated vs. manual procedure.*

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the automated procedure are shown in **Fig. 5** and **Table IV.** The values were obtained by sequential sampling of the same sample with an 0.06-ppm. standard inserted to correct for recorder drift. The coefficient of variation was 4.8 per cent and the reproducibility was ±0.0055 at the 0.1-ppm. level.

Diacetyl is determined daily on a finished beer by both the automated and the manual procedure. Representative values of the data obtained are shown in **Table V.**

In the course of our studies comparing manual and automated diacetyl values, it was noted that manual values were considerably higher than those obtained by the automated procedure, especially when the samples were taken during active fermentation. We had also found that when these same samples were aerated and heated, that the manual and automated values compared favorably. In an attempt to elucidate these facts, a tracking study was initiated whereby daily samples were taken from the start of the fermentation to the finished product. The following analyses were then performed on each sample:

I. initial diacetyl by both procedures;

II. diacetyl by both procedures after aeration only; and

III. diacetyl by both procedures after aeration and heating for one hour at 150° F.

The data obtained in this study are shown graphically in **Figures 6, 7, and 8.** Initial and aerated diacetyl values were higher when determined by the manual procedure. Both procedures produce comparable values after the sample is aerated and heated. These results would seem to indicate that the longer heating period used in the manual procedure is responsible for the higher values. These data also indicate the possibility of the presence, during active fermentation, of more than one compound that can be oxidized to diacetyl since the values obtained by both procedures were higher after aeration and heating than the value obtained by the manual procedure after aeration only.

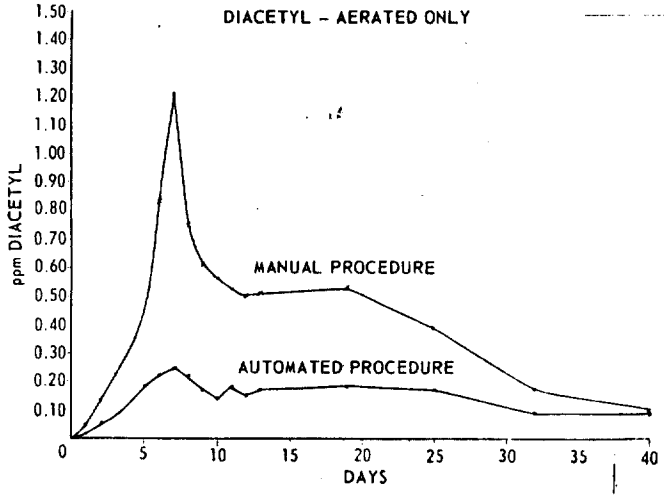

Figure 7: *Tracking study —diacetyl after aeration —automated vs. manual procedure.*

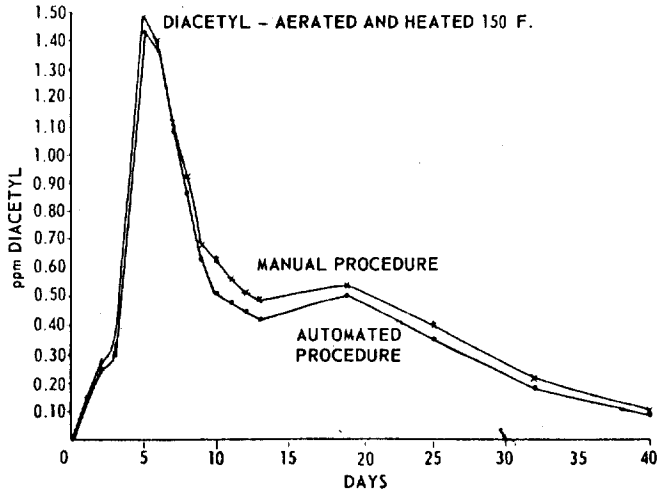

Figure 8: *Tracking study —diacetyl after aeration and heating at 150°F.— automated vs. manual procedure.*

Table V

Comparison of Diacetyl Values, Automated Versus Manual Procedures

Sample No.	Diacetyl, ppm. Automated Procedure	Diacetyl, ppm. Manual Procedure
1	0.05	0.06
2	0.08	0.08
3	0.07	0.08
4	0.08	0.09
5	0.07	0.07
6	0.07	0.06
7	0.14	0.13
8	0.10	0.09
9	0.09	0.09
10	0.09	0.10
11	0.10	0.11
12	0.13	0.13
13	0.13	0.12
14	0.14	0.14
15	0.12	0.13
16	0.09	0.07
17	0.07	0.05
18	0.05	0.05
19	0.07	0.06
20	0.07	0.05

Summary

An automated procedure for the determination of diacetyl has been outlined. Good reproducibility and agreement with the manual procedure can be obtained under the conditions described. Considerable saving of time and personnel can be attained with the automated procedure. The AutoAnalyzer could be used advantageously in studying diacetyl precursors formed during fermentation.

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A PRACTICAL BREWER'S VIEW OF DIACETYL

By M. W. BRENNER

Just about a year ago, American brewers were given the opportunity to read a review article by Lewis[1] on recent research on diacetyl and to listen, at the 1968 MBAA convention, to another paper on the same topic by Latimer.[2] The aim of the present paper is to examine the factors bearing on diacetyl in beer and, without being too theoretical, to offer some simple suggestions which should give the practical brewer control of the situation.

To begin, it should be obvious that there seem to be conflicting reports on causes and cures. The problems of running absolutely identical fermentation experiments are staggering. Consider a simplified list of factors which should be identical — not merely similar — but *identical*, if the fermentation tests are to have higher reliability (see Table I).

M. W. Brenner is President, Schwarz Services International Limited, Mount Vernon, New York.

TABLE I

FACTORS INFLUENCING FERMENTATION

A. *Wort*	
Individual amino acids	pH
Assimilable peptides	Acidity
Sugar spectrum	rH
Oxygen content	Sulfites
Bios factors	Tannins
Mineral nutrients	Trub
Fermentation inhibitors	Clarification
Microbiological purity	
B. *Yeast*	
Microbiological purity	Pitching rate
Strain or type	History and condition
Respiratory competence	Nutrition
Enzymes	
C. *Fermentors and Fermentation Conditions*	
Tanks	Temperature cycle
Shape	Wort depth
Material	Yeast selection
Open or Closed	by skimming or "cropping"
Attemperators	Agitation
Arrangement	Duration of cycle
Temperature of refrigerant	Vatting procedures
	Air pick-up

It would be almost impossible to run a sufficient number of single-factor experiments to obtain a numerical index or guide to the magnitude of the influence of each of these items and the subdivisions of each. Fortunately, however, there are some mileposts on the road, set down by earlier workers. Furthermore, even if we do not know and cannot measure all the factors exactly, we can take some comfort in the fact that uniformity in raw materials, brewing practices, and cellar procedures can avoid trouble.

But rather than dwelling on the problems, let us review the positive, constructive steps which will give the brewer control of diacetyl in his beer. In this paper, which is directed to those who are primarily lager beer brewers, we will be referring to lager yeast, lager beer, and lager beer procedures unless otherwise specified.

Starting with yeast, let us say at once that the standard of yeast purity must be very high. While brewers formerly accepted the use of yeast containing 0.5% bacteria, or even more, a simple procedure for a one step reduction of bacterial count of such yeast to less than 0.01% by use of an acid-persulfate wash[3] should permit acceptance of 0.01% bacteria as an *upper limit*.

This limit cannot be observed if one tries to control yeast purity with a microscope. After all, 0.01% means one bacterium in 10,000 yeast cells. The usual microscopic examination calls for checking twenty fields with 150-200 yeasts per field, or a total of 3,000-4,000 cells. Hence, a microscope may be used only as a rough guide against gross contamination. Reliable quality control data are obtained only by plating on an actidione-containing medium. Hence, some brewers rely more on regular washing than they do on testing.

In addition to bacteria, foreign yeasts or variant strains may cause serious problems. As pointed out by Czarnicki and Van Engel,[4] Lindegren,[5] Kato,[6] Kleyn and Vacano,[7] and others, some yeasts have a greater reducing activity than others. These yeasts, which are capable of reducing some dyestuffs, seem to be able also to reduce diacetyl to low levels. In Figure 1, it will be seen that some colonies are white and are respiratory competent, while others are various shades of green (from brom cresol green) and are respiratory deficient. Pitching yeast built up from a white colony will generally yield beer with lower final diacetyl content than that from a green colony. Thus, a brewer should periodically have his pitching yeast plated on such a medium to see if it is remaining true to type. He should also have it checked for freedom from wild yeasts.

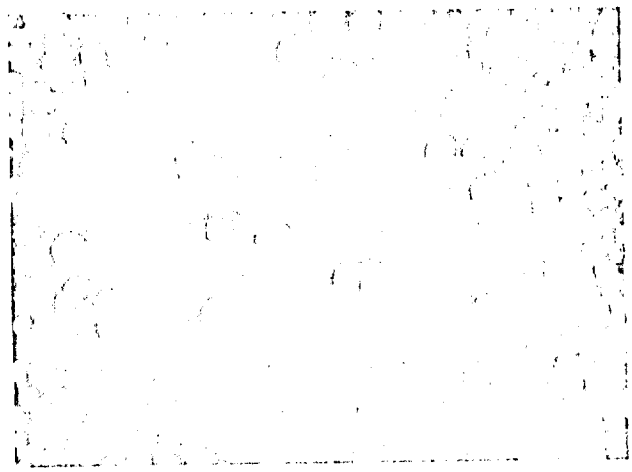

Figure 1. Illustration of mixture of respiratory competent and respiratory-deficient yeast colonies.

On the other hand, there is considerable data to show that the usual brewing yeasts are quite capable of producing finished beers with low final diacetyl content, so long as brewing and cellar procedures are rational and so long as fermentations go to completion in normal time.

General yeast condition plays an important part. As demonstrated by Hayduck[8a] and by Tait and Fletcher,[8b] the fermenting activity of yeast is proportional to its nitrogen content. Their results are transcribed in Figure 2; the data, in both cases on *S. cerevisiae*, were obtained in different countries, with different yeast strains, and different media, and over 40 years apart in time. The parallelism of the results is excellent, which may be surprising, but the relationship between protein content and fermenting power should not be. Fermentation and all the processes associated with it, such as reduction of diacetyl, depends primarily on en-

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zymes. Since the enzymes are all proteins, a yeast deficient in protein content may be deficient in enzymes responsible for fermenting or reducing power.

It is relatively simple to determine the nitrogen or protein content of the yeast, which depends to a considerable extent on the soluble nitrogen content of the wort it is fermenting. Because the yeast may include trub and other impurities, careful preparation of the sample before testing is necessary.

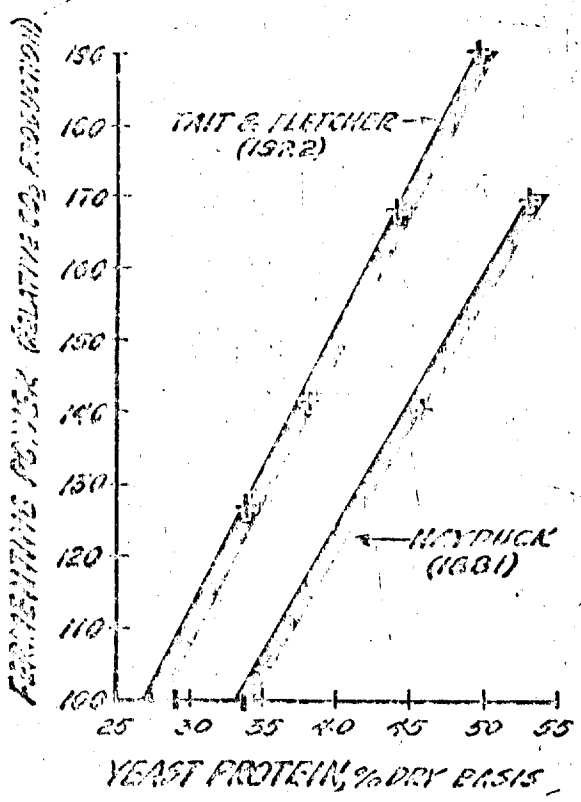

Figure 2. Relationship of yeast protein content to fermenting power.

Before going further, it is appropriate to discuss the formation of diacetyl during fermentations. Figure 3, based on Harrison's work[9] and his GLC method of analysis (three years after first publication), clearly demonstrates that diacetyl and pentanedione are *formed* during the first stage of fermentation and both are *reduced* during the last few days. There are two processes, which may overlap: (a) formation and (b) reduction of diacetyl.

We have reported data to support the belief that yeast alcohol dehydrogenase (YADH) plus reduced nicotinamide adenine dinucleotide (NADH) reduces diacetyl.[10] This is the same system which performs the last step of alcohol production during normal fermentation, namely, the reduction of acetaldehyde to ethyl alcohol. This is why we want a good, active yeast which ferments well and why we can then expect it to effectively reduce diacetyl.

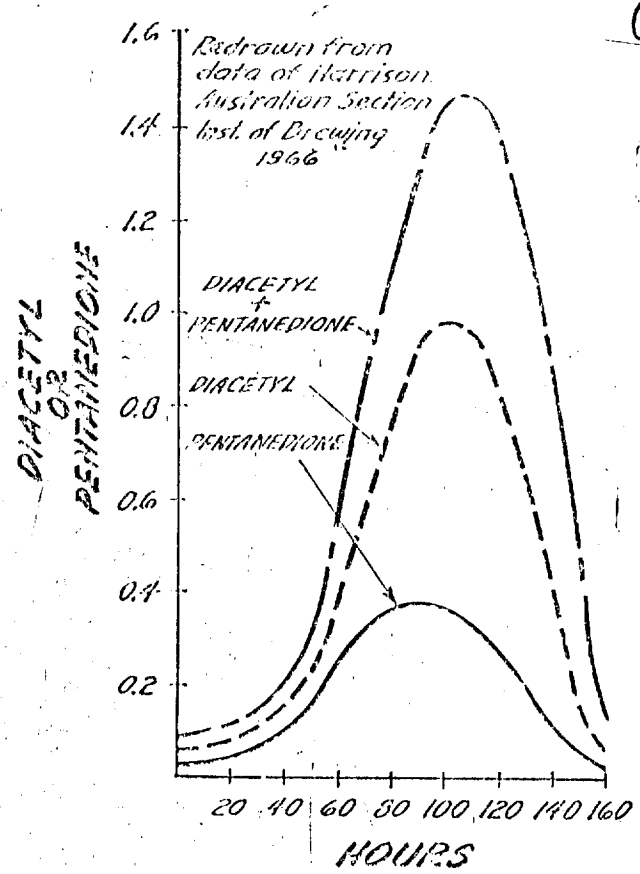

Figure 3. Diacetyl and pentanedione formation during fermentation.

To get these effects, one must not keep the yeast too long and too warm, or permit loss of cell contents by autolysis, as the brewery data in Table II will show. This was observed in a plant with fairly low malt usage, which sometimes permitted the beer to stay in the fermentor for four days "overtime" without cooling below the cellar temperature.

TABLE II

Fermentation Cycle*	Last Previous Cycle	Diacetyl, ppm. 2nd Day	Diacetyl, ppm. 8th Day
8 days	12 days**	1.2	0.7
8 days	8 days	1.2	0.2

*5 days fermenting; 3 days attemperating.
**5 days fermenting; 7 days slow cooling.

Figure 4 illustrates the fact that anything that accelerates the general fermentation process tends to compress the *formation* phase of diacetyl into a relatively short time and generally results in a better opportunity for eventual *reduction* of this type of compound. The diacetyl content of the beer produced will depend to a large extent on the time of contact of beer with the yeast *after* the fermentation of the sugar is substantially complete. Figure 4 shows that a sample of a room tem-

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perature fermentation taken 36-48 hours after the start might contain very little residual sugar, but the diacetyl level could be anywhere between 0.5 and 1.0 ppm. Eventually—particularly if the fermentation is stirred or agitated, which might be necessary because of the beer depth—the diacetyl will be reduced to lower levels, below 0.2 ppm.

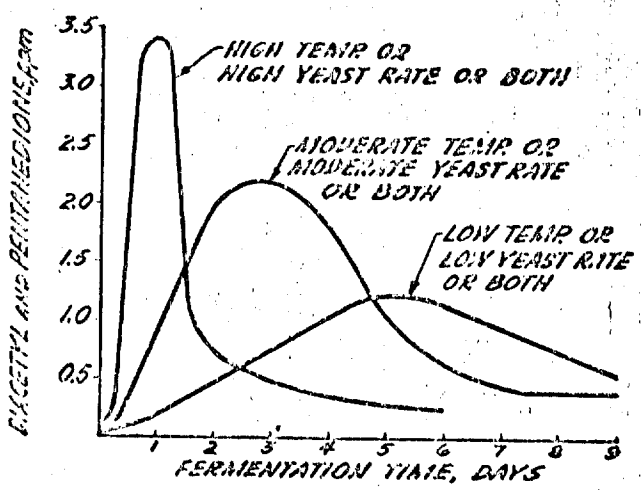

Figure 4. Alteration of diketone production and reduction by changes in temperature and yeast rate.

If not agitated, when there is little CO_2 production because the fermentation is substantially complete, the yeast will settle out of contact with the beer in a laboratory fermentation rather rapidly. After all, there may be a beer depth of only a few inches. If this happens, the diacetyl in the beer cannot be absorbed by the yeast, as it must be reduced to acetoin and 2,3-butanediol by YADH. In a deep fermentor, where settling of the yeast may take several days, the opportunity for removing diacetyl is far better and the same yeast-wort combination may give a beer with lower final diacetyl content. If the yeast is not pure, however, the slow settling of diacetyl-producing bacteria may adversely affect the product.

The practical management of fermentation should take into account the facts mentioned in Table III.

We should explain what is implied by "high" pitching temperature. The concept is illustrated in Figure 5, which is intended to show a possible change from one typical cycle to a new one based on the original or "normal" cycle. It involves starting warmer, but holding to the same maximum as before. This should result in the fermenting beer approaching end gravity appreciably sooner, especially if cooling is deferred.

The relationship of beer gravities resulting from the two different cycles is shown in Figure 6, which emphasizes the extra time available for the reduction of diacetyl. Perhaps mention should be made of the fact that this does not suggest that the fermentation temperature be allowed to fluctuate or to go any higher than the brewery's traditional maximum.

TABLE III

FERMENTATION MANAGEMENT

A. Diacetyl *formation* reaches maximum sooner with:
   (1) "high" pitching temperatures
   (2) "high" pitching rates
   (3) nutritively-fortified yeast
   (4) fresh yeast
   (5) easily fermentable wort.

B. Diacetyl *reduction* is promoted by:
   (1) "high" temperature (to facilitate YADH activity)
   (2) high suspended yeast counts
   (3) yeast with high YADH-NADH contents
   (4) avoidance of cooling while fermentation is active
   (5) avoidance of air pick-up while there is fermentable sugar present

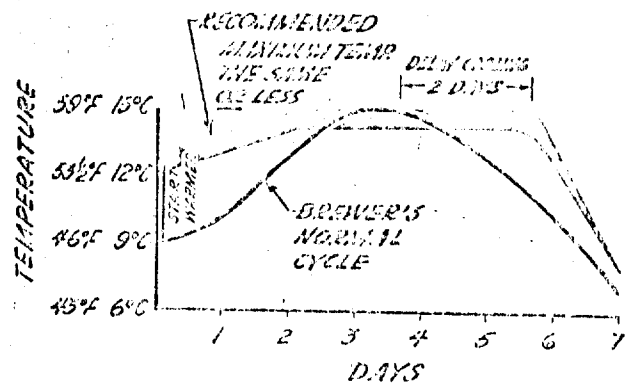

Figure 5. Typical changes in fermenting temperatures to improve diacetyl reduction.

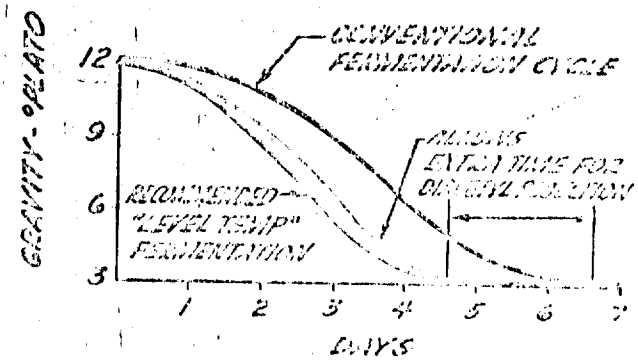

Figure 6. Typical changes in gravity during fermentation accompanying temperature changes of Figure 5.

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Pitching rate changes may or may not be necessary, but experience tends to indicate that very low rates probably favor high final-beer diacetyl, unless the fermentation cycle is protracted. Yeasting with one to two pounds of liquid yeast, equal to about 0.15-0.25 lb. of yeast dry solids, per barrel of wort is certainly within the range of conventional practice, though we would favor the higher amounts in the range given.

We will not attempt to give any involved theory as to why diacetyl is formed during the first part of fermentation. For one thing, there very probably is more than one pathway. Secondly, if one accepts the valine theory of suppression,[11] one must recognize that it cannot be demonstrated unless from 50 to 500 ppm. of valine is added to wort which may normally contain between 70 and 100 ppm.[12] Even if this was the best way to minimize diacetyl formation, it would remain purely theoretical so long as practical and cheap procedures for diacetyl *reduction*, during the later phase of fermentation, exist.

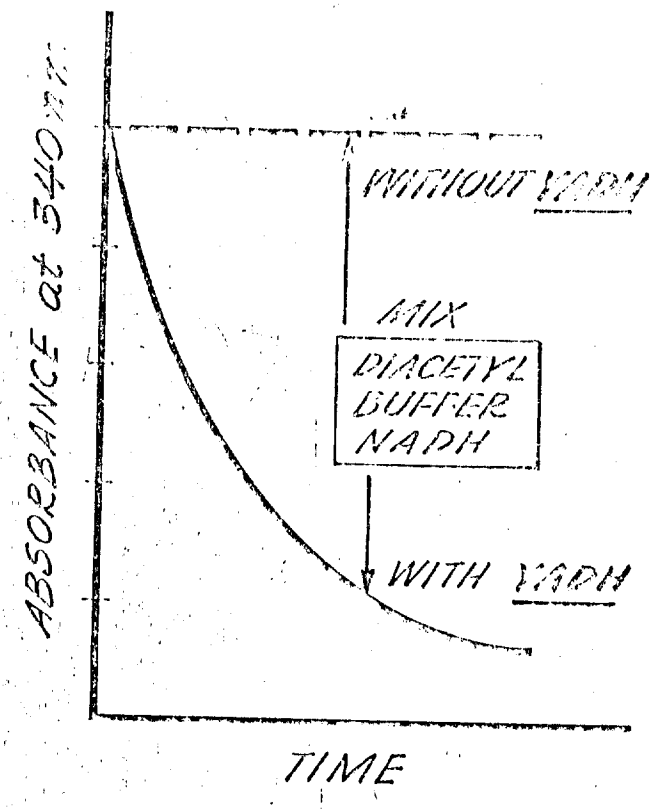
In 1963, we published data on the lowering of beer diacetyl levels by adding nutrilites to the wort.[13] After six years, perhaps we may be allowed to mention that the fortification was accomplished by addition of our product "Yeastex 61" to worts, at the rate of about 75 ppm. (2 lb./100 bbl. of wort). Yeastex-61 is a broad-spectrum nutrient mixture, and does not bring about the results shown in Table IV via its content of valine. As a matter of fact, the treatment would add less than 3 ppm. of valine to the wort, far short of the quantities reported to be effective.

TABLE IV

EFFECTS OF YEASTEX-61 FORTIFICATION OF WORT ON BEER DIACETYL

Wort	Yeastex-61 Added, ppm.	Yeast	Diacetyl In Beer, ppm.
Brewery C	—	Brewery C	1.1
	75	C	0.22
Brewery E	—	Brewery E	0.61
	75	E	0.20

There are two patents[14, 15] which suggest the use of diacetyl reductases from non-yeast sources to achieve low final levels of diacetyl, but these would be very expensive to adopt, since the production and purification of these enzyme systems would be quite costly. We have already shown[10] that normal components of brewers yeast, YADH and NADH, are capable of reducing diacetyl, as shown in Figure 7. Other data indicate that the reduction goes all the way to 2,3-butanediol, which occurs in lager beers to the extent of 80-100 ppm. in German beers[16] and to between 400-1,000 ppm. in French wines.[17]



REACTION: NADH IS OXIDIZED TO NAD, WHILE DIACETYL IS REDUCED ENZYMATICALLY BY YADH

Figure 7. Demonstration of diacetyl reduction by yeast alcohol dehydrogenase.

It is interesting to note that reduction of acetaldehyde to alcohol by YADH proceeds about 7,000 times as fast as the reduction of diacetyl to 2,3-butanediol. We must recognize that diacetyl formation and diacetyl reduction are going on as overlapping processes during normal yeast fermentations. When there is plenty of sugar available to the yeast, the diacetyl content (although we are not sure of the mechanisms of diacetyl formation) increases somewhat, and levels of 2 or 3 ppm. may be reached. When the supply of fermentables is limited towards the end of fermentation, then the YADH-NADH system* continues to act on diacetyl and reduces it, if given enough time, to below threshold levels. Remember that the diacetyl will disappear at 1/7,000th the rate of sugar conversion. These comments on diacetyl apply also to 2,3-pentanedione.

*YADH is an endo-enzyme; that is, it operates within the yeast cell and is far too large a molecule to pass through the living cell wall.

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It is for the foregoing reasons that we advocate the provision of a "warm" holding period at the end of active fermentation. This should be about 24-36 hours after reaching to within about ¼° P. of end-fermentation. After this warm holding period, the attemperators can be turned on full, because both the fermentable sugars and the diacetyl should now be at a very low level.

It should not be necessary to emphasize the need for clean tanks, pure culture yeast, and freedom from bacteria and wild yeasts. A well-run brewery should establish those conditions first, then use the fermentation procedure recommended. If we refer again to Figure 6, we can see the rationale.

We need not worry about starting the fermentation very cold to prevent bacteria from taking command because we should have clean wort and good yeast. If so, we can save time and wort cooler loads by starting the fermentation within about 1° C. of the usual maximum temperature your brewery prefers to employ. Use cellar temperature and modest attemperation to prevent excessive peak temperatures. When the gravity drops and the fermentation slackens, close down or shut off the attemperators to prevent the temperature from dropping; a slight upward kick will do no harm. Allow 24 to 36 hr. warm (at very close to end gravity) to hasten final reduction of fermentable sugars, acetaldehyde, pentanedione, and diacetyl; then turn the attemperation on full.

Since there will be no more heat of fermentation to counteract, the beer temperature will drop more rapidly than when fermentation is still going on, and yeast settling is more rapid after fermentation is complete.

Even though there may not be much fermentable sugar present, care should be taken to prevent air pick-up on the transfer. Oxygen at this point tends to put the yeast or beer back into a diacetyl-forming attitude. Typical results of air pick-up during pumping from fermentor to rub with relation to diacetyl are shown in Figure 8. The same sort of thing, even more exaggerated, occurs when beer is carbonated by kraeusening or by secondary fermentation (lagering). Under the latter circumstances, the first days of the process are very similar to the first days of a cool primary fermentation. For this reason, among others, closed fermentors are preferred to open fermentors. Every possible source of oxygen pick-up should be checked and eliminated. As is apparent, this will also help to get low-air beer in the bottling tanks.

Incidentally, just as oxygen, sugars, and yeast seem to combine to encourage a surge in diacetyl production, they also seem to cause increased generation of hydrogen sulfide. The fermentation gas has a relatively high content of H_2S in the early hours of fermentation, as is also true of the first hours of kraeusening or lagering (secondary fermentation) if oxygen is permitted to be present.

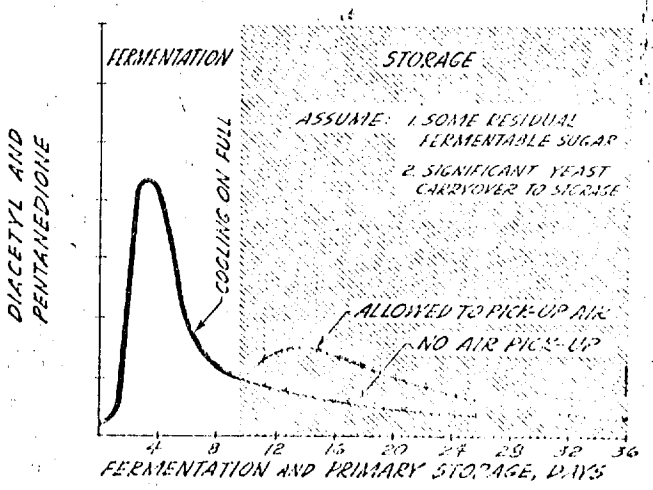

Figure 8. Influence of air absorption at transfer of beer on diacetyl content.

Once the beer is in primary storage with low diacetyl content and protected from access of oxygen for the reason already discussed, the remaining concern of the brewer is only with contamination by foreign organisms, primarily *P. cerevisiae* (sarcina) and lactobacilli which can thrive under anaerobic and microaerobic conditions. As mentioned before, clean yeast and clean plant are the best safeguards and appropriate microbiological testing has diagnostic value. Some brewers tend to ignore the importance of yeast cleanliness and the consequences of pitching bacterially contaminated yeast.

The same brewers who accept 0.1-0.2% bacteria in pitching yeast, which could be adding up to 40,000 bacteria to one milliliter of wort, would probably be quite disturbed if they were to be told that their wort contained 10,000-40,000 bacteria per ml. (see Table V).

TABLE V

Yeast Cells At Pitching, Millions/ml.	Bacteria		
	With 0.1% in Yeast	With 0.01% in Yeast	With 0.001% in Yeast
5	5,000/ml.	500/ml.	50/ml.
10	10,000	1,000	100
15	15,000	1,500	150
20	20,000	2,000	200

Pitching yeast is most often the largest reservoir of bacteria in a brewery; these numbers should make our earlier recommendation of better standards for pitching yeast seem to have been quite conservative. Clean yeast and reasonable standards for cleaning of equipment should erase the worries about bacteria as a source of diacetyl in storage beer.

Not much has been said about the analytical problem, but in our earlier paper,[10] it was suggested that binding of diacetyl to yeast alcohol dehydrogenase could be a part of the difference between electron capture methods used on the vapor above fermenting beer and the earlier methods which employed heating at 60-65° C. or distillation. The heat has been thought by some to *produce* diacetyl, but it may only be liberating it from the enzyme or some other complexing agent. Nor has much been said about mechanisms and theories; again we would like to repeat our earlier statement[10] regarding the different conclusions concerning diacetyl that have appeared in the literature. We said:

"There are many such disparities and all sides may very well be correct. It is extremely difficult under any circumstances to reproduce fermentation results because of the complexities of the substrates employed and the variations in composition which may be important. Furthermore any one yeast strain could scarcely be identical with all the others used. Full elucidation of the whole diacetyl story will require much more investigation and cooperative, or at least complementary, research."

In the meantime, however, an attempt has been made to cover practical procedures to assist the operating brewer in producing beer with low final diacetyl content, below the flavor threshold.

Summary

The most important aspects of the problem may be summarized as follows:

1. The pitching yeast should be free of bacteria, contain less than 5% of respiratory deficient variant yeasts, and be nutritionally satisfied.

2. The wort should be free of bacteria, adequately supplied with yeast nutrients, free of enzyme or yeast inhibitors, and sufficiently oxygenated to promote vigorous yeast growth and fermentation.

3. The fermentation cycle should be designed to encourage rapid conversion of the sugars to alcohol and carbon dioxide, and to have sufficient time after exhaustion of the sugars to permit the YADH to continue its relatively slow reduction of diacetyl, at a temperature which will favor YADH action.

4. The transfer to primary storage should avoid air pick-up, to prevent the fermentive-state yeast from going over to the respiratory phase, since diacetyl formation would then dominate until reversion to anaerobic conditions occurs.

5. In kraeusening or secondary fermentations, diacetyl formation and reduction follow the same pattern as in the main fermentation, so oxygen levels and temperatures can be manipulated similarly to achieve control if the yeast is vigorous.

6. Storage beers should be free of diacetyl-forming bacteria. The main reservoir of such bacteria is usually the brewery's yeast. Good yeast washing and general equipment cleaning are pretty reliable safeguards against this hazard.

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FROZEN FOODS STORAGE EFFECTS

Formation of Alcohol, Acetaldehyde, and Acetoin in Frozen Broccoli Tissue

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P. A. BUCK[1] and M. A. JOSLYN
University of California, Berkeley, Calif.

The carboxylase activity of broccoli tissues was investigated to determine the role of pyruvic carboxylase in the production of volatile aldehydic and ketonic compounds which might serve as precursors of the off-flavors developing in underscalded frozen broccoli. Broccoli carboxylase was found to catalyze synthesis of acetoin and diacetyl chiefly from added pyruvate and to a much smaller extent from acetaldehyde, unlike pea and wheat germ carboxylase. Acetaldehyde inhibited broccoli carboxylase activity. In frozen broccoli, both inhibition by and restricted diffusion of acetaldehyde would favor production of acetoin. The concentration of acetaldehyde, acetoin, or diacetyl was not related to the organoleptically objectionable formation of off-flavors, whereas ethyl alcohol content was related to extent of off-flavor.

INVESTIGATIONS on the accumulation of acetaldehyde, ethyl alcohol, and related products in broccoli during freezing storage (5) were continued, with particular emphasis on pyruvic carboxylase activity. These investigations of the nature of the biochemical reactions which result in abnormal flavors in improperly scalded, frozen vegetables also led to a study of the behavior of pyruvic carboxylase in situ. The immature floral shoot of sprouting broccoli was used as the source of the enzyme system. Additional data on the accumulation of ethyl alcohol and acetoin are reported here and in more detail in the thesis of Buck (2).

[1] Present address, Department of Dairy and Food Industries, University of Wisconsin, Madison, Wis.

Materials and Methods

Commercially grown Italian green sprouting broccoli (whose structure is shown in Figure 1), packed in crates with ice, was obtained from a local wholesale distributor and stored at 1° C. The broccoli was obtained in several lots in order to ensure fresh tissues at the time of utilization. Broccoli shoots were

[illegible] chilled or frozen before homogenization. Sterilization of the surface of broccoli floral shoots with chemical disinfectants required vigorous agitation for at least 30 minutes, followed by several rinsings with sterile, distilled water. This treatment altered enzymatic activity. Control samples agitated with distilled water had increased gas exchange due to the bruising; and samples soaked in a solution of disinfectant without agitation were contaminated sporadically, hence were unreliable. Solutions of 1 to 3000 Roccal (alkyl dimethyl benzyl ammonium chloride compound supplied by Winthrop Chemical Co.), 7% calcium hypochlorite, or 1% mercuric chloride disinfectants reduced the enzyme activity slightly, whereas less concentrated solutions which did not affect the activity were ineffectual. Therefore, only the anaerobic experiments were performed using antiseptic technique. The regular experiments were performed using broccoli which had been thoroughly washed in flowing, cold tap water and rinsed in flowing distilled water.

After the broccoli had been washed thoroughly, it was drained in clean cheesecloth and chilled by storing the intact floral shoot at 1° C. for several hours. Sometimes the tissues were frozen at −17° C. in order to facilitate [illegible] freezing. The floral shoots were then either cut into the desired tissue sections and organs using a stainless steel knife, or ground to a uniform mixture in a Universal food chopper using the fine blade. The food chopper and all equipment for preparation of samples were stored continuously at 1° C. to maintain a uniformly chilled environment throughout the preparation. (Preliminary investigations showed that the broccoli floral shoot must be extracted at low temperature to prevent denaturation of carboxylase.) After the shoot had been sectioned or ground, the material was mixed and aliquots were taken for analysis. The remainder of the material was stored in a glass-stoppered container at 1° C. for not exceeding 3 days, or at −17° C. for 1 day to several months. Control samples which were not macerated were stored simultaneously.

In addition, samples of broccoli were available which had been scalded for varying lengths of time and stored at −17° C. for 7 years. The preparation of these samples has been reported (*6*).

A partially purified fairly stable carboxylase preparation was obtained by macerating cut broccoli in a Waring Blendor with an equal amount of added water at 1° C., and centrifuging the resulting mixture first at 1000 × gravity [illegible] 16,000 × [illegible] to remove more finely suspended plastid and mitochondrial particles. This preparation was used in investigations on acetaldehyde and acetoin formation from added pyr[illegible] Yeast carboxylase (*7, 9*) and whea[illegible] carboxylase (*10*) have been sho[illegible] possess both decarboxylating and acetoin synthesizing activities.

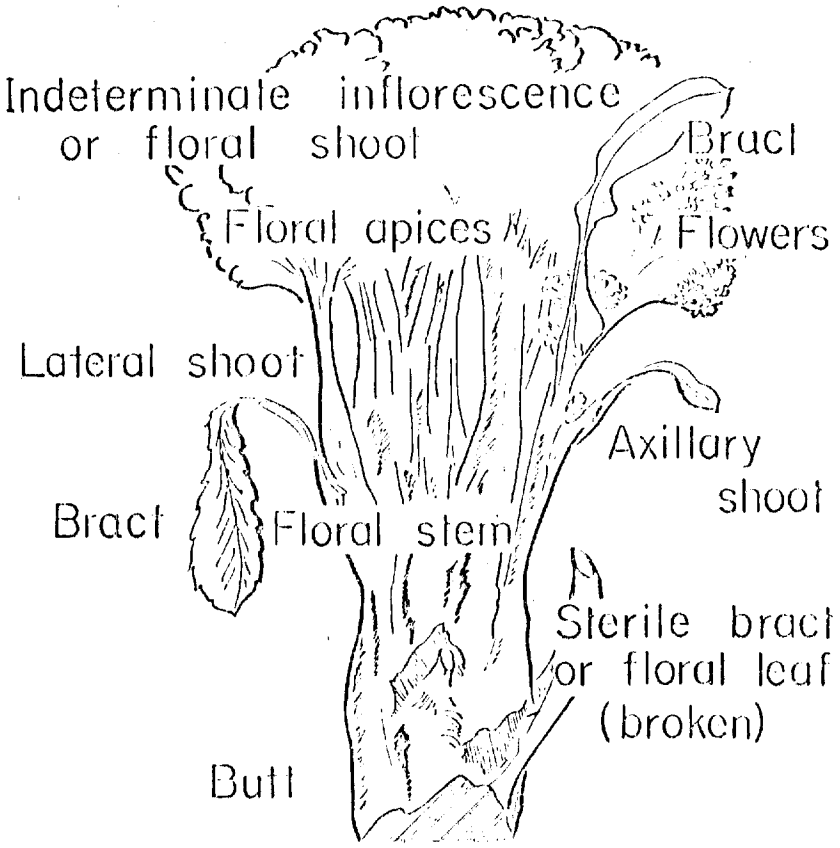

Figure 1. Morphology of the floral shoot of broccoli

Analytical Methods. The volatile aldehyde, ethyl alcohol, total solids, and ascorbic acid content were determined by methods used previously. Total nitrogen was determined by the micro-Kjeldahl distillation method of the Association of Official Agricultural Chemists (*1*). Acetoin was determined qualitatively using the modified Barritt reagent of Vaughn, Wedding, and Tabachnick (*13*), which was mixed just before use. A sensitive test was obtained when the 1-naphthol was resublimed under vacuum (*5*). Acetoin and diacetyl were determined quantitatively on the steam distillate collected for acetaldehyde determination by the colorimetric method of White, Krampitz and Werkman (*15*). Acetoin was oxidized before its determination by the modified Werkman method (*11*), and the amount of acetoin was the difference between the calculated amounts of diacetyl in the oxidized and unoxidized samples. For the micro amounts of acetoin, an efficient fractionating column was employed. Micrograms of diacetyl were read from a calibration curve for pure diacetyl, using a Klett-Summerson photoelectric colorimeter with a 420 mμ filter; 100 γ of of diacetyl gave a colorimetric value of 130.

Results

Acetaldehyde and Acetoin Formation by Broccoli Carboxylase Preparation. During decarboxylation of pyruvic acid, acetaldehyde was the predominant product, but in certain experiments increased amounts of acetoin were formed. The accumulation of acetoin by different enzyme preparations, as well as the rate of evolution of carbon dioxide, varied with the method of extraction. A possible shift in the catalysis of carboxylase from straight decarboxylation of pyruvic acid to decarboxylation and condensation of the product was indicated.

Changes in the amount of acetoin formed often did not parallel changes in the rate of carbon dioxide evolved, despite precautions that were taken to avoid errors of procedure. The cause of this phenomenon was sought by evaluating the modifications in the method of extraction. The addition of thiamine did not alter the ratio of acetoin to carbon dioxide. When added during extraction, thiamine increased not only the

Table I. Effect of Ratio of Thiamine Pyrophosphate to Pyruvic Acid on Synthesis of Acetoin by Broccoli Carboxylase

Pyruvate Molarity	Thiamine Pyrophosphate Molarity × 10^5	Ratio of Cocarboxylase / Pyruvic Acid × 10^3	Q_{CO_2}[a]	Acetoin[b]
0.0015	6.4	4	121	570
	33	22	156	80
0.003	6.4	2	152	609
	33	11	183	166
0.006	6.4	1	...	712
	33	5.5	221	650
0.009	6.4	0.7	168	520
	33	3.6	243	656
0.015	6.4	0.4	182	300
	33	2.2	256	736
0.03	6.4	0.2	...	106
	33	1.1	283	361
0.045	6.4	0.14	222	30
	33	0.7	283	289
0.060	6.4	0.1	234	0
	33	0.55	288	266
0.090	6.4	0.07	234	0
	33	0.36	422	259
0.15	6.4	0.04	...	0
	33	0.22	369	170
0.3	6.4	0.02	...	...
	33	0.11	...	74

[a] Microliters of CO_2 liberated per ml. of tissue extract per hour.
[b] Relative concentration measured as Klett colorimeter reading.

carboxylase activity, but also the synthesis of acetoin.

The partially purified preparation of carboxylase used for the study of its carboligase activity was obtained by a modification of the procedure described initially. Five hundred grams of broccoli were ground in a food chopper at 0° C. and the macerate was squeezed in a Carver press at 5000 to 10,000 pounds per square inch at 0° C. The exudate was collected in 100 ml. of chilled cysteine-thiamine solution containing 0.01*M* of each reagent. After centrifuging at 2000 × gravity to remove coarse particles, the solution was centrifuged at 16,000 × gravity for 20 minutes as before. To this supernate, neutralized ammonium sulfate was added slowly during stirring until the solution was half saturated. The solution was centrifuged and decanted, and the precipitate was dialyzed in distilled water at 1° C. for 6 hours. Further details of this enzyme preparation will be published (*8*).

With carboxylase isolated as described, addition of thiamine decreased carboxylase activity and acetoin formation. The carboxylase activity and synthesis of acetoin were not affected by the addition of magnesium ion to extracts. The atmosphere during analysis did not influence the formation of acetoin. The amount of acetoin formed during decarboxylation under nitrogen, when carbon dioxide accumulated, was similar to the amount formed in the presence of alkali, when no carbon dioxide accumulated. Likewise, the amount of acetoin formed in nitrogen and in air was similar, though the concentration of oxygen differed.

The relative concentration of pyruvic acid and thiamine pyrophosphate in the reaction mixture markedly affected the extent of acetoin formation, as shown in Table I. Increased amounts of pyruvic acid, added to the enzyme preparation containing $6 \times 10^{-5}M$ thiamine pyrophosphate, inhibited the formation of acetoin, while the evolution of carbon dioxide increased to a maximum. Magnesium ion, buffer, and enzyme were added. Although the amount of carbon dioxide evolved from the lowest concentration of pyruvic acid totaled only about 90 μl., the rate within the first 20 minutes was uniform, so that the amount has been expressed in the table in terms of milliliters of Q_{CO_2} for comparison with other values. The amount of acetoin has been expressed as a relative value determined by measurement of absorption of light by the colored solution obtained from the qualitative test for acetoin. The absorption of light was measured in a Klett-Summerson photoelectric colorimeter with a 540-mμ green filter.

Two levels of thiamine pyrophosphate were used with varied levels of pyruvic acid. Maximum evolution of carbon dioxide was dependent upon the concentration of pyruvate and occurred in these experiments at 0.09*M* acid. The greatest synthesis of acetoin, however, occurred at thiamine pyrophosphate-pyruvic acid ratios ranging from 2×10^{-2} to 1×10^{-2}. Aliquots obtained by combining duplicate manometric samples were analyzed quantitatively for acetoin and diacetyl. The sample that gave the maximum amount of acetoin, determined by light absorption, contained 37 γ per ml. This amount of acetoin formed was 1.1% of the added pyruvic acid. Diacetyl was detected only in trace amounts.

Acetaldehyde, Ethyl Alcohol, Acetoin, and Diacetyl Formation in Broccoli Tissues. The formation of compounds originating from the decarboxylation of pyruvic acid by broccoli tissues was investigated. Differential determinations of acetoin and diacetyl were performed on samples of tissues stored anaerobically at room temperature, and on samples stored at −17° C.

Analysis of Anaerobic Samples. Because reaction rates are slow at low temperatures, samples of tissue with added pyruvic acid and acetaldehyde were placed under anaerobic conditions at room temperature for 4 hours in order to investigate the effect of pyruvic acid and acetaldehyde on the formation of acetoin. Control samples were placed under similar anaerobic conditions for varying periods of time.

Broccoli was cleaned, so that the contribution from microbial contamination was insignificant when the broccoli was held at room temperature under nitrogen. The counts obtained by the plate method ranged from 10 to 10^2 microorganisms per gram of fresh tissue. Two hundred grams of broccoli shoots were shaken for 20 minutes with 200 ml. of 1% calcium hypochlorite solution. The sample then was aseptically drained, rinsed three times with 100-ml. portions of sterile, distilled water, cooled by freezing at −17° C., and finally ground in a Universal food chopper at 1° C. Twenty-five grams of the pulp were placed in sterilized, glass-stoppered flasks. Pyruvic acid was added to duplicate preparations of tissues to produce $3.6 \times 10^{-2}M$ and $1.8 \times 10^{-2}M$ concentrations. To similar preparations, acetaldehyde was added in final concentrations of $35 \times 10^{-3}M$ and $7 \times 10^{-3}M$. Acetone containing 1.8×10^{-2} and $7 \times 10^{-3}M$ acetaldehyde was added to fill another preparation. Control samples were prepared simultaneously. All samples were flushed with nitrogen at a rate of 800 ml. per minute for 1 minute. The flasks were hermetically sealed, and the samples were held for 4 hours at room temperature (about 25° C.), and then were frozen prior to analysis.

In another experiment, samples of 100 grams of broccoli shoots were prepared similarly, but the tissues were not ground and no substrates were added. The air was removed from the tissues by evacuating the flasks using a Nelson pump, and nitrogen was introduced into the flasks before they were sealed. These samples were held for varying lengths of time at room temperature before being frozen and ground for analysis.

The flasks were opened and quickly connected to glass steam distillation equipment, and 250 ml. of distillate were collected for analysis. The results of the analyses are given in Table II. Even under anaerobic conditions, diacetyl accumulated in all shoots, whereas acetoin accumulated in the macerated tissue. The addition of pyruvic acid to the pulp caused accumulation of ethyl alcohol, acetoin, and diacetyl. In the tissues to which acetaldehyde was added, 99% of the lower level and 67% of the higher level of acetaldehyde were recovered when the products were included. Ethyl alcohol accumulated in large amounts, but acetoin accumulated only slightly. The acetoin and diacetyl synthesis was suppressed compared to the pyruvate series. In macerated tissue to which both substrates were added, ethyl alcohol and diacetyl accumulated, but no acetoin was detected.

Analysis of Frozen Broccoli Samples. Frozen samples of raw and scalded broccoli shoots which had been stored at −17° C. for 7 years were analyzed to determine if acetaldehyde, ethyl alcohol, acetoin, and diacetyl had accumulated. The tissues initially had been analyzed for acetaldehyde and ethyl alcohol, but not for acetoin or diacetyl (*5*). The tissues which had been subjected to the most severe scalding did not accumulate acetaldehyde or ethyl alcohol and were regarded as controls. Because any initial amount of acetoin might have decreased in these tissues during the scalding process, several samples of fresh broccoli were also analyzed. Duplicate samples were frozen and stored for various periods of time up to 1 year.

The analyses are given in Table III. Acetaldehyde did not accumulate significantly, if at all, during storage at −17° C. for 1 year. The increase in the amount of acetaldehyde in the raw sample stored for 2 months was atypical, and might have occurred before the packaged broccoli became frozen. A sample held aerobically for 1 month at 0° C. accumulated 3.97 mg. of acetaldehyde and only 26.6 mg. of ethyl alcohol per 100 grams of tissue.

During 7 years of storage at −17° C., raw broccoli accumulated acetaldehyde and ethyl alcohol. Samples which had been scalded for less than 3 minutes accumulated diacetyl. Acetoin was not detected in any sample of frozen broccoli shoots, either initially or after storage for 7 years.

Table II. Acetaldehyde, Ethyl Alcohol, Acetoin, and Diacetyl Formation during Anaerobiosis of Broccoli Pulp and Broccoli Shoots

(Pyruvic acid and acetaldehyde added to some tissue preparations)

Sample	Holding Time, Hours	Substrate and Molarity × 10^2	Products, Mg./100 G. Broccoli Tissue: Acetaldehyde	Ethyl alcohol	Acetoin	Diacetyl
Pulp	4	1.8 pyruvate	4.0	123.8	1.9	2.0
		3.6 pyruvate	5.1	131.0	1.7	2.5
		7 acetaldehyde	15.8	288	0.3	0.1
		35 acetaldehyde	29.6	1010	0.3	0.45
		1.8 pyruvate + 7 acetaldehyde	14.5	323	0	2.0
		Control O	3.9	33.4	1.6	0.05
Shoots	0	0	1.4	27.8	0	0.07
	4	0	3.0	30.4	0	0.71
	12	0	3.6	63.8	0	1.07
	23	0	4.7	96.0	0	1.48
	39	0	3.4	159.6	0.7	1.43

Discussion

Accumulation of Acetaldehyde and Ethyl Alcohol. Alcohol dehydrogenase has been reported to occur in many plants (*6, 12*), but the acetaldehyde-ethyl alcohol system in broccoli has not been previously studied. Broccoli tissues have been shown to contain pyruvic carboxylase (*cf. 11*). Alcohol dehydrogenase also was assumed to be present, since ethyl alcohol served as a substrate for an enzymatic reaction which was poisoned by $10^{-4}M$ iodoacetate. When broccoli tissues were stored at 0° C., acetaldehyde accumulated, while ethyl alcohol increased only slightly. When the tissues were stored at −17° C., or were stored anaerobically, ethyl alcohol accumulated. The ratio of ethyl alcohol to acetaldehyde varied as follows:

	Ethyl Alcohol / Acetaldehyde
Tissues stored 1 month at 0° C.	6.7
Tissues stored 1 year at −17° C.	28
Tissues stored 7 years at −17° C.	58
Tissues stored anaerobically up to 24 hours	20
Tissues stored anaerobically up to 40 hours	47
Fresh tissues, average	10

In frozen tissues, acetaldehyde accumulated slightly, but ethyl alcohol accumulated markedly, as it did in anaerobic tissues. The ratios of ethyl alcohol to acetaldehyde in frozen tissues were typical of ratios of anaerobic tissues, and

Table III. Acetaldehyde, Ethyl Alcohol, Acetoin, and Diacetyl Formation during Storage of Frozen Broccoli Shoots

Scalding Processes at 100° C., Minutes	Storage Period at −17° C.	Products, Mg./1000 G. Broccoli Tissue: Acetaldehyde	Ethyl alcohol	Acetoin	Diacetyl
0	0	0.56	31.1	0	0
0	2 months	1.40	30.6	0	0.072
1	2 months	0.87	27.1	0	0
1	2 months	0.67	12.0	0	0
4	2 months	0.45	9.8	0	0
0	6 months	0.97	18.2	0	0
0	1 year	0.93	26.3	0	0
0	7 years	2.30	133	0	0.42
1	7 years	1.43	37.3	0	0.25
2	7 years	0.96	24.2	0	0.251
3	7 years	0.75	15.7	0	0.03
4	7 years	0.77	14.2	0	0

indicate that in frozen tissues pyruvic acid was metabolized anaerobically, and most of the acetaldehyde was converted to ethyl alcohol.

Accumulation of Acetoin and Diacetyl. The formation of acetoin has been reported in the investigations of yeast and wheat germ carboxylase, but diacetyl seldom has been determined separately. David and Joslyn (*1*) analyzed a slurry of peas after 16 hours of storage at room temperature and found that at $2 \times 10^{-2}M$ pyruvate, large amounts of diacetyl and little acetoin were formed. However, the pea slurry was shaken for 16 hours in air, and spontaneous oxidation of acetoin could have occurred. They also found that at $5 \times 10^{-2}M$ acetaldehyde, only half the amount of acetoin and diacetyl were formed in equal amounts. When both $2.5 \times 10^{-2}M$ acetaldehyde and $10^{-2}M$ pyruvate were added, the amount of products at the end of 16 hours was the same as for acetaldehyde, but diacetyl was in greater concentration. Singer and Pensky (*10*), using purified wheat carboxylase, found that addition of pyruvate always increased the rate of acetoin formation — four times that obtained from acetaldehyde alone. When both acetaldehyde and pyruvate were present, maximal activity was reached at approximately $5 \times 10^{-3}M$ pyruvate and $5 \times 10^{-2}M$ acetaldehyde. When acetaldehyde was the substrate, saturation was reached at $2 \times 10^{-2}M$ acetaldehyde, and half saturation at $2 \times 10^{-2}M$. When pyruvate was the substrate at a concentration of $5 \times 10^{-3}M$, Singer and Pensky found that the amount of acetoin synthesized was only 5% of the amount formed in the presence of excess acetaldehyde. They state that when the pyruvate concentration was raised to $5 \times 10^{-2}M$, a significant amount of acetoin formed, although never as much as with acetaldehyde in excess.

In this study, samples of frozen broccoli were found to contain diacetyl, but little acetoin. Oxidation of acetoin to diacetyl might have occurred, even though frozen tissues were sufficiently anaerobic to permit ethyl alcohol to accumulate. In broccoli tissue to which pyruvic acid was added at $1.8 \times 10^{-2}M$, acetoin and diacetyl both accumulated, more diacetyl than acetoin. The addition of acetaldehyde resulted in the formation of only one-tenth as much of the amount of acetoin and diacetyl formed from pyruvic acid. Acetaldehyde added in the same concentration as was added by Singer and Pensky. When acetaldehyde was added with pyruvic acid, the same amount of diacetyl was formed, but acetoin was not detected. The relative amounts of acetoin and diacetyl formed in these three plant tissues are shown in Table IV.

Isolated broccoli carboxylase, in the presence of thiamine pyrophosphate, synthesized acetoin from pyruvate, and in anaerobic tissues pyruvate caused extensive synthesis of acetoin and diacetyl. Carboxylase undoubtedly was involved in their synthesis in both anaerobic and frozen tissues.

Although diacetyl contributes to the flavors of many types of food products, typically, the flavor change in broccoli did not seem to be due to the increase of diacetyl. The amounts of acetaldehyde, acetoin, and diacetyl in the tissues of the floral shoots could not be correlated with the intensity of the undesirable flavor.

Table IV. Relative Amounts of Acetoin and Diacetyl Formed in Plant Tissues from Added Substrates

Preparation	Added Substrate	Relative Ratio: Acetoin	Relative Ratio: Diacetyl	Relative Ratio: Acetoin + diacetyl
Pea slurry	$2 \times 10^{-2}M$ pyruvate	1	940	2
	$5 \times 10^{-2}M$ acetaldehyde	16	27	1
	$1 \times 10^{-2}M$ pyruvate + $2.5 \times 10^{-2}M$ acetaldehyde	5	39	1
Wheat carboxylase	$5 \times 10^{-3}M$ pyruvate	..	...	1
	$5 \times 10^{-2}M$ pyruvate	..	...	20
	$2 \times 10^{-2}M$ acetaldehyde	..	...	20
	$5 \times 10^{-3}M$ pyruvate + $5 \times 10^{-2}M$ acetaldehyde	..	...	40–80
Broccoli pulp	$1.8 \times 10^{-2}M$ pyruvate	19	20	10
	$3.6 \times 10^{-2}M$ pyruvate	17	25	10
	$7 \times 10^{-2}M$ acetaldehyde	3	1	1
	$3.5 \times 10^{-2}M$ acetaldehyde	3	5	2
	$1.8 \times 10^{-2}M$ pyruvate + $7 \times 10^{-2}M$ acetaldehyde	0	20	5

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Diacetyl Studies. III. Further Studies on the Prevention and Removal of Diacetyl in Beer

MARTIN BURGER, PAUL R. GLENISTER, and ALBERT F. LAUTENBACH, *J. E. Siebel Sons' Co., Inc., Chicago, Illinois*

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IN OUR PAPER of last year (2) we presented our findings concerning the origin of diacetyl in beer, as well as some practical suggestions for preventing its formation in beer and for its removal should it develop.

We have continued our studies of diacetyl in beer because a number of brewers have told us that they continue to meet with occasional difficulties in their efforts to rid their beers of the undesirable diacetyl flavor, even though they have followed our recommendations closely.

In this present paper we wish to report our findings as to factors which can influence the action of yeast in removing diacetyl from beer or in preventing its formation.

Diacetyl at Various Stages of Fermentation

We ran a test in our pilot brewery to measure the amounts of diacetyl present during and shortly after fermentation. The brew, on an extract basis, contained 65% malt and 35% common corn grits; it had an original extract of 12% Plato and a volume of 2 bbl. The yeast used was clean, healthy, and vigorous. The wort was at 8.5°R. when pitched; the highest temperature reached during fermentation was 11.5°R. Fermentation was practically complete after 120 hours. The diacetyl findings are shown in Table I; they indicate that the diacetyl-reducing power of yeast is greatest during the period of intense yeast propagation, and that subsequent to this period a concentration of about 0.2 ppm. is the smallest concentration of diacetyl which is likely to be obtained in the presence of living but not actively propagating yeast. These findings corroborate those reported earlier (2), which showed that an active yeast fermentation reduces abnormal diacetyl contents to the normal 0.2 ppm. range.

TABLE I
Diacetyl during and after Fermentation

Hours after Pitching	Diacetyl
	ppm
24	< 0.05
48	0.05
72	0.17
96	0.15
120	0.10

Yeast Flocculence and Diacetyl

The differences in flocculence which different batches or races of brewing yeast possess have been shown to be the result of both the innate properties of the yeast (7,9) as well as of the nature of the yeast environment (4,5), the wort and beer. When we learned that some brewers had met with difficulty in attempting to use their own yeast for treating beer for the removal of diacetyl, we came to realize that the yeasts they employed differed in their flocculence.

A simple experiment which we ran to test our hypothesis concerning the relationship between the flocculence of a yeast and its ability to remove diacetyl from beer was as follows: Commercial bottled beer of clean normal flavor and low diacetyl content was used as the test substrate. The bottles (12-oz. size) were at 32°F. when treated and were recrowned immediately afterwards. The treatment consisted of the addition to each bottle of 0.5 g. of compressed yeast and 2 ml. of a stock solution of diacetyl strong enough to add approximately 0.7 ppm. of diacetyl to the beer. This fairly large amount of diacetyl was used so as to give an opportunity for comparing the diacetyl-removing powers of the different yeast treatments. Two distinctly different types of

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yeast were used: one was a commercial baker's yeast; it behaved as a powdery yeast in beer; the other was a brewers' yeast having good to excellent settling properties. The brewers' yeast was washed and compressed just prior to its use in the experiment. To further exaggerate the effects of yeast flocculence in some of the tests, the desired weight of yeast was added in one lump to the beer and no attempt was made to disperse it uniformly throughout the beer. The bottles of treated beer were then stored at 32–34°F. for 5 days and the beer was then assayed for diacetyl according to the method of West *et al.* (10). It was noticed, during the storage period, that the bakers' yeast tended to remain well suspended in the beer and the brewers' yeast settled out rapidly.

The data of this experiment are given in Table II; they indicate that the ability of a yeast to remove diacetyl from beer is directly related to the amount of yeast which remained in suspension in the beer during the period of treatment. These findings help to explain why it is that a brewer will sometimes find a variation in the diacetyl content of his beer during storage. If a yeast flocculates rapidly, a variation of a day or more in the time of transfer from the fermenter to ruh-storage could result in considerable variation in the amount of yeast carried over and therefore to considerable variation in the diacetyl content of the beer.

TABLE II

Yeast and the Removal of Diacetyl from Beer

Test No.	Diacetyl Stock Solution Added per Bottle	Yeast Treatment	Diacetyl Found after 5 Days	Diacetyl Removed by Yeast
			ppm	*ppm*
1	none	none	0.22	(control)
2	2 ml.	none	0.87	0.0
3	2 ml.	bakers' yeast well dispersed	0.22	0.65
4	2 ml.	baker's yeast poorly dispersed	0.43	0.44
5	2 ml.	brewers' yeast, well dispersed	0.58	0.29
6	2 ml.	brewers' yeast, poorly dispersed	0.72	0.15

Practical observations in a number of breweries have shown that a carry-over of ½ to 1 lb. of yeast per bbl. of beer has led to satisfactory removal of diacetyl and, at the same time, has not given any undesirable yeasty flavor to the beer.

The practice of *kräusening*, which is still used as such or in a modified form by some brewers, is really a kind of yeast treatment, and we have noticed that brewers who *kräusen* their beer are seldom troubled with the diacetyl flavor.

Fermentable Matter and Diacetyl Removal

In our previous paper (2) we described a procedure in which a diacetyl-contaminated beer could be freed of its diacetyl flavor by a refermentation with one-half its volume of fresh finished wort and with yeast pitched at the regular rate on total volume. We reported also that the mere addition of fresh pitching yeast to a diacetyl-contaminated beer without any refermentation at all is sufficient to remove moderate amounts of diacetyl. We were surprised, therefore, to learn from a brewer who had tried the yeast-treatment technique for the removal of a diacetyl flavor from his beer and had had only indifferent success, that, in his opinion, the presence of fermentable matter in the beer during the yeast treatment actually tends to increase the diacetyl content of the beer. We conducted a simple experiment to test this hypothesis.

A number of 12-oz. bottles of a commercial beer of clean normal flavor and low diacetyl content were each treated with diacetyl, with 0.5 g. compressed yeast, and with 1 g. of dextrose. The bottles were at 32°F. when treated and were recrowned immediately afterwards. The yeast was dispersed thoroughly in the beer. The beer

TABLE III

Fermentable Matter and Diacetyl Removal

Test No.	Diacetyl Stock Solution Added per Bottle	Yeast Treatment per Bottle	Dextrose Added per Bottle	Diacetyl Found After 5 Days	Flavor Remarks After 5 Days
				ppm	
1					[illegible] normal
2	none	none	none	0.21	[illegible] diacetyl
3	1 ml.	none	none	0.58	[illegible] normal
4	1 ml.	none	none	0.18	[illegible] normal
5	1 ml.	0.5 g. bakers' yeast	none	0.28	[illegible] normal
6	1 ml.	0.5 g. brewers' yeast	1 g.	0.19	[illegible] normal
7	1 ml.	0.5 g. bakers' yeast	1 g.	0.25	[illegible] normal

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was stored at 32°–36°F. for 5 days after the treatment and was then analyzed for diacetyl. Our findings are presented in Table III; they indicate that the presence of fermentable matter in beer does not diminish the efficacy of yeast treatment for the removal of diacetyl.

A Large-Scale Test of the Yeast-Treatment Technique

Some time ago, a brewery submitted a sample of beer for a routine analysis; the beer had a strong diacetyl character. A chemical analysis indicated a diacetyl content of 0.60 ppm. We treated a number of the bottles of this beer with compressed yeast at the rate of 0.75 lb. liquid yeast per bbl. The bottles were stored at 32°F. Five days after treatment, the flavor of the beer was found to be clean and the laboratory assay revealed a diacetyl content of only 0.20 ppm. We told the brewer about this experiment and recommended the use of a yeast treatment should any more of his beer develop the diacetyl flavor.

Not long after that, this brewer found that the beer in a number of his open fermenters had a diacetyl flavor. This beer was all blended together and transferred to some closed storage tanks. It was dusted with CO_2 during transfer. Half of these tanks of beer were charged with fresh pitching yeast at the rate of 1 lb. per bbl.; the remainder were not given a yeast treatment. The beer treated with yeast lost the diacetyl flavor after 3 days; the untreated beer continued to have a pronounced diacetyl flavor. The yeast used was of a somewhat powdery type.

Attempts at Enzyme Extraction

Previous work (2) has indicated that the action of yeast in removing diacetyl from beer is an enzymatic process. If so, it might be feasible to remove diacetyl from beer by merely adding a small amount of enzyme material to the beer rather than the amounts of yeast we have discussed.

We prepared crude cell-free extracts of yeast by means of plasmolysis and subsequent autolysis, using such chemical agents as ethyl acetate, or toluene, or secondary ammonium phosphate, as outlined by a number of authors (3) for the separation of various enzymes from yeast. We also extracted cell constituents from yeast by a process of alternately freezing and thawing a thin slurry of yeast and then preparing a cell-free extract by centrifugation.

Various amounts of these different crude yeast extracts were added to a number of diacetyl-contaminated beers to discover their action on the diacetyl. None of these extracts removed diacetyl from beer.

These findings indicate that the enzyme material responsible for diacetyl destruction by yeast is rather tightly bound to the substance of the yeast cell, perhaps, as Nossal (8) has indicated for some respiratory enzymes, to the mitochondria.

Interactions of Bacteria and Yeast in the Formation of Diacetyl in Beer

In our previous paper (2) we mentioned a long, slender, Gram-positive rod bacterium which was able to develop rapidly in beer at room temperature and give the beer a pronounced diacetyl character. This microorganism, as well as a coccus form which we encountered in a beer in the course of this work, was isolated by Mrs. Bessie May, our staff bacteriologist. The media used for the isolation were Difco tomato juice agar in an atmosphere of 10% CO_2, and Difco W-L differential medium and the medium of Kissel and Dakin (6), both in an atmosphere of 100% CO_2. These three media were used both with and without added phenethyl alcohol (at about 2,500 ppm.) for the suppression of some Gram-negative bacteria which

Fig. 1. A Gram-stained smear preparation of the pure culture of *Lactobacillus* used in the tests described in the text under the heading "Interactions of bacteria and yeast . . ." Some of the rods shown here are Gram-negative. Continued propagation in artificial media led to a Gram-variable condition in the culture. (Approx. × 1300)

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Fig. 2. A Gram-stained smear preparation of the pure culture of *Pediococcus* used in the test described in the text under the heading "Interactions of bacteria and yeast." (Approx. × 1300)

were present. The organisms, when isolated, were found to be a species of *Lactobacillus* and a species of *Pediococcus* (1). They are shown in Figs. 1 and 2. Preliminary tests with beer indicated that, of the two forms isolated, the *Lactobacillus* form was a more active producer of diacetyl than the *Pediococcus*. Stock cultures of the bacteria were maintained on Difco thioglycollate medium at 2°–3°C.

We set up an experiment in which we pitted these bacteria against beer yeast in an unfinished beer with the intention of finding out just what would be the effects of different contents of yeast, air, and fermentable matter on the formation of diacetyl in the beer. The beer we used was a 2-bbl. pilot brew; it was filled into 12-oz. bottles with a generous amount of yeast (about 1 lb. per bbl.) still suspended in it. The bottles were crowned and allowed to stand at 30°–32°F. for 48 hours. It was our plan to have some of the bottles contain only a scanty amount of yeast; this was accomplished by taking those bottles and decanting the beer carefully from the well-settled yeast sediment into clean bottles. The remaining bottles of beer were left as-is, with the ample supply of yeast in them. Some of the bottles were filled only half-full, to allow the beer to have plenty of contact with air. As another of the variables in the experiment, we added 1 g. of dextrose to each of a number of the bottles of the beer; this was intended to provide an opportunity to measure the influence of fermentable matter on the formation of diacetyl. The bacteria were added as 2-ml. portions of a richly grown stock culture to each 12-oz. bottle. The half-filled

TABLE IV

Diacetyl in Beer: Bacteria versus Yeast

Test No.	Conditions of Test	Test Temperature	Flavor After 2 Weeks	Flavor After 10 Weeks	Diacetyl	Microscopic Findings, Liquid and Sediment
		°F			ppm	
1	Bottles full, yeast ample	30–32	clean	clean	0.21	yeast normal, occasional rods (see Fig. 3)
2	Bottles full, yeast scanty	30–32	clean	clean	0.26	some second crop yeast, occasional rods
3	Bottles full, yeast ample	70–72	clean, slightly yeasty	clean, slightly yeasty	0.18	yeast poor, a few rods and cocci (see Fig. 3)
4	Bottles full, yeast scanty	70–72	diacetyl, stale	oxidized, acidic	0.18	yeast poor, a few rods and cocci
5	Bottles half full, yeast scanty	70–72	winy, aromatic, estery	aromatic, estery	0.66	some elongated yeast cells, many rods and cocci
6	Bottles full, yeast ample, rods added	70–72	fermn	oxidized	0.28	yeast fair, many rods and cocci
7	Bottles full, yeast scanty, rods added	70–72	aromatic, spoiled	oxidized, syrupy	0.51	yeast poor, some rods and cocci
8	Bottles full, yeast scanty, cocci added	70–72	sl. diacetyl winy	oxidized	0.35	yeast poor, many tetrads
9	Bottles full, yeast ample, cocci added	70–72	clean, sl. yeasty	clean, sl. yeasty	0.30	yeast poor, cocci and rods
10	Bottles half full, yeast ample, rods added	70–72	diacetyl, earthy	strong, acetaldehyde	0.40	yeast poor, some rods, few cocci
11	Bottles half full, yeast scanty, rods added	70–72	strong, diacetyl	cardboard, acetic	0.70	yeast poor, many rods
12	Bottles half full, yeast ample, cocci added	70–72	diacetyl	yeasty, estery	0.49	yeast fair, many rods, few cocci
13	Bottles full, yeast ample, rods added, dextrose added	70–72	clean, sl. yeasty	sl. acidic, acceptable	0.24	yeast fair, many rods, some cocci (see Fig. 3)
14	Bottles full, yeast scanty, rods added, dextrose added	70–72	clean, sl. yeasty	sl. acidic, acceptable	0.24	yeast fair, new crop; many rods, some cocci
15	Bottles full, yeast ample, dextrose added	70–72	clean, sl. yeasty	sl. acidic, acceptable	0.19	yeast fair, many rods, some cocci

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bottles received only 1 ml. of bacteria culture. After treatment, some of the bottles were stored at 30°-32°F. and some were stored at 70°-72°F. The over-all plan and the data of the experiment are presented in Table IV. The diacetyl assays were made 2 weeks after the treatments.

Examining the data, it is evident that at low temperatures (tests 1 and 2) the beer by itself developed only the low amount of diacetyl usually associated with a normal product (10) (see Fig. 3, top). The beer held at room temperature kept a clean flavor when given an ample yeast supply, but it developed considerable diacetyl when given only a scanty yeast supply. The presence of air tended to augment the formation of diacetyl in this instance (tests 3, 4, and 5) (see Fig. 3, middle).

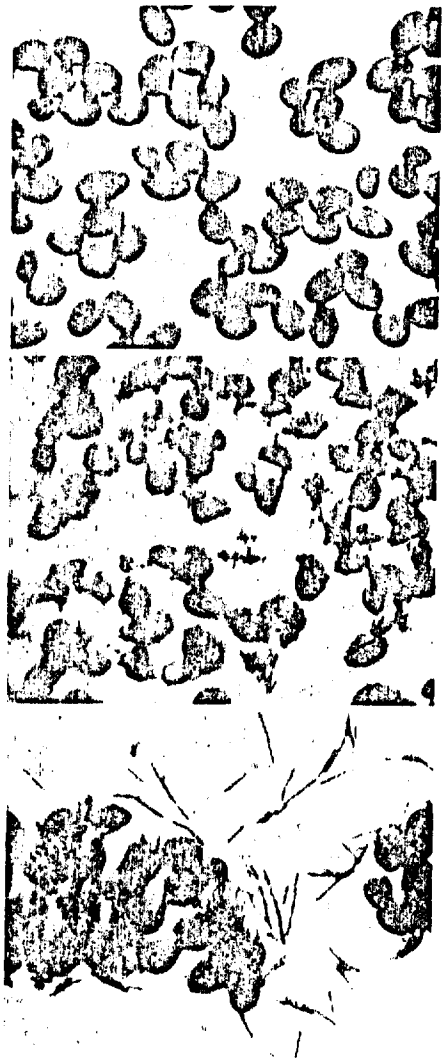

Fig. 3. Gram-stained smear preparations of the yeast sediments of the beer of tests 1, 3, and 13, Table IV. Top, test 1; middle, test 3; bottom, test 13. (Approx. × 1300)

The beer with added cultures of *Lactobacillus* or *Pediococcus* tended to develop the largest amounts of diacetyl when the yeast was in scanty supply and when considerable air was present in the beer; ample supplies of yeast and low air content clearly tended to limit the development of diacetyl (tests 6 to 12).

The beer itself, it should be pointed out, contained bacteria of its own before it was ever used in this experiment and, when put into contact with a great deal of air and given only a scanty yeast supply, it developed an appreciable amount of diacetyl all by itself (tests 4 and 5).

The presence of fermentable matter, as added dextrose, tends to help the yeast suppress the development of the diacetyl flavor even when the initial yeast supply is scanty and lactobacilli are present in fairly large numbers (tests 13, 14, and 15) (see Fig. 3, bottom).

The data indicate also that it may not be desirable to strive for a very low sugar content in the finished beer, as has been the trend in some breweries, for sugar can be useful, in conjunction with yeast, in preventing the development of the diacetyl flavor.

In tests 5 and 7, the diacetyl content of the beers was high, but no diacetyl flavor was noticed; probably because it was masked by other, stronger, flavors.

It is interesting to note that diacetyl was not detected in a number of the tests 10 weeks after treatment, although it had been detected earlier and appreciable amounts of it had been found in the laboratory analyses. In tests 4, 8, and 11, the loss of the diacetyl flavor can be attributed to the oxidative changes taking place in those beers, for the diacetyl character is replaced by a stale, oxidized flavor. In beers of tests 10 and 12 the oxidation reactions gave rise to estery and aldehydic flavors.

Yeast in Long Contact with Beer

Yeast autolysis is often cited as a cause of an undesirable flavor in beer (2). Our

experience has indicated to us that this danger is remote if the yeast is clean and if the beer is kept under CO_2 and at a temperature of about 32°F.

Aside from the fact that such treatment does not damage the beer, we have evidence which indicates that the yeast itself does not suffer unduly during such storage. Figure 4 is a photomicrograph of a Gram-stained preparation of a yeast which had been kept under fermented beer for 5 weeks at about 32°F. The yeast was in good condition, as can be seen from the size and contour of the cells and from their excellent staining properties. The beer in this instance had a clean normal flavor and a diacetyl content of only 0.21 ppm.

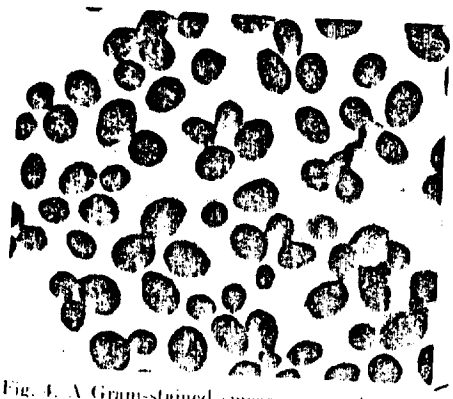

Fig. 4. A Gram-stained smear preparation of a yeast which had been kept 5 weeks under beer. See text under heading "Yeast in long contact with beer." (Approx. × 1300)

This same yeast was collected and used to pitch a later brew: the fermentation was a little slow in starting, but the beer which resulted was clean and normal in all respects.

Summary

The occasional difficulties which some brewers have encountered when they attempted to use their own yeast for treating beer for the removal of diacetyl appear to be caused by the yeast flocculating too soon, with the result that it does not remain in suspension long enough to accomplish the desired action.

Fermentable matter present in beer does not interfere with the action of yeast in removing the diacetyl flavor.

Yeast is best able to counteract the action of diacetyl-producing bacteria if it is present in ample supply in the beer, and if the air content of the beer is as low as possible.

Yeast does not suffer from long storage under beer, and such storage does not lead to the development of the undesirable yeast autolysis flavor in the beer, provided that the beer is under carbonation and kept at a temperature of about 32°F.

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A STUDY OF THE DIACETYL IN CHEESE
II. THE CHANGES IN DIACETYL CONTENT OF CHEDDAR CHEESE DURING MANUFACTURING AND CURING[1]

HAROLD E. CALBERT AND WALTER V. PRICE
Department of Dairy Industry, University of Wisconsin

A previous study has shown that diacetyl was present in all lots of Cheddar cheese analysed (3). In many of the lots the amount of diacetyl present in the cheese was much greater than could be accounted for by the diacetyl content of the original milk and starter culture from which the cheese had been made. Since the diacetyl content of Cheddar cheese has been related to the flavor of the cheese and appears to be an element of cheese flavor, a further study of the diacetyl in Cheddar cheese seems advisable.

The purpose of this investigation was to measure the development of diacetyl during the manufacturing and early stages of curing of both pasteurized and raw-milk Cheddar cheese.

METHODS

Manufacturing procedures. Six lots of Cheddar cheese were made on six different days. Three lots were manufactured from milk pasteurized at 144° F. for 30 minutes. The other three lots were made from raw milk which had a reduction time of 4 hr. on the standard methylene blue test (1). The method of Price (11) was followed for all making operations.

Two lots of identical milk were made into Cheddar cheese, the milk being pasteurized for one lot and raw for the other. These lots were used to study the diacetyl content of cheese during the early stages of curing. The curd was placed in square hoops, pressed, cut into 2-lb. blocks, then pressed again overnight. The blocks of cheese were removed from the press the following morning and placed on shelves in the curing room which was maintained at 60 to 62° F. and at a relative humidity of 80 to 85 per cent. After 2 days, when the surfaces were sufficiently dry, each block of cheese was dipped into paraffin and packed in a wooden box. Each wooden box contained 10 of the 2-lb. blocks of cheese. The boxes were covered to minimize drying of the cheese and were stored at an average temperature of 45° F.

Sampling procedure. In the six lots used to study the development of diacetyl during the making process, the diacetyl content of the starter and of the milk was measured. Samples were removed for diacetyl determinations at setting, cutting, dipping (curd and whey) and milling (curd and whey).

Blocks of the two lots of cheese used to study the diacetyl content during the early stages of curing were ground and duplicate aliquot samples were prepared from each block.

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The diacetyl content of all samples was determined by the modified colorimetric procedure (3).

EXPERIMENTAL RESULTS

Development of diacetyl during the manufacturing procedure. The diacetyl measurements at the various steps during the making of the 6 lots of Cheddar cheese are shown in table 1. The diacetyl contents of the three lots

TABLE 1
Diacetyl measurements during the making of six lots of Cheddar cheese

Material	Diacetyl					
	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
			(mg./100 g.)			
Milk	0.002	0.002	0.002	0.003	0.002	0.002
Starter	0.037	0.085	0.115	0.040	0.037	0.070
Milk at setting	0.009	0.012	0.015	0.005	0.006	0.007
Curd before cutting	0.010	0.013	0.017	0.005	0.007	0.008
Dipping						
Curd	0.034	0.042	0.021	0.024	0.037	0.031
Whey	0.007	0.015	0.015	0.007	0.008	0.010
Milling						
Curd	0.035	0.047	.....	0.014	0.030	0.058
Whey	.....	0.023	0.022	0.024	.....	0.030

of raw milk at setting were lower than those of the three lots of pasteurized milk. This also was true of the measurements made of the diacetyl in the curd just prior to cutting.

Lots 2 and 4 were used as typical lots to calculate the quantity of diacetyl produced during the making of pasteurized-milk and raw-milk Cheddar cheese. The amount of diacetyl present at each step during the making procedure was calculated as the total milligrams of diacetyl present in either the weight of milk in the vat or the combined weights of curd and whey.

The quantity of diacetyl produced during the making of lot 2, a pasteurized-milk type of Cheddar cheese, was as follows: from the start of the making and up to the time of setting, 19.44 mg.; from setting to cutting, only 2.15 mg.; from cutting to dipping, 12.50 mg.; and from dipping until milling, 11.90 mg. The total increase of diacetyl was 45.99 mg.

A total of 39.16 mg. of diacetyl was produced during the making of lot 4, a raw-milk type of Cheddar cheese. Of this total, 1.42 mg. was produced prior to setting; none from setting to cutting; 9.50 mg. from cutting to dipping; and 28.74 mg. from dipping to milling.

Diacetyl content during the early stages of curing. The dia[illegible]nt of a sample from a block of cheese representing each vat lot wa[illegible]ed at pressing and at 3, 18, 28, 34 and 45 days after pressing.

The gradual decrease in the diacetyl content of each type of Cheddar cheese during the early stages of curing [illegible]

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DISCUSSION

It is very likely that the diacetyl which is in cheese originates in a manner comparable to its formation in starter cultures. In starter cultures diacetyl is formed by bacteria acting upon the lactose and citrates. Van Beynum and Pette (13) have described the formation of the diacetyl from the citrates.

Many investigators have shown that diacetyl is a constituent of the desirable flavor and aroma in butter cultures (8). The organisms responsible for the production of this desirable flavor and aroma are *Streptococcus lactis*, *Leuconostoc dextranicum* (*Streptococcus paracitrovorus*) and *Leuconostoc citrovorum* (*Streptococcus citrovorus*) (7). Wilster and Price (15) have pointed out that these same organisms always are present in a good cheese starter as used in this country. It is primarily through their action that the lactose and citrates of the milk might be converted into fermentation products such as diacetyl in the cheese.

TABLE 2

Diacetyl content of Cheddar cheese during early stages of curing

Age	Diacetyl content	
	Pasteurized-milk type	Raw-milk type
(*d.*)	(*mg./100 g.*)	(*mg./100 g.*)
0	0.046	0.035
3	0.042	0.032
18	0.033	0.017
28	0.025	0.021
34	0.025	0.016
45	0.027	0.017

Many workers have shown that aeration and increased acidities favor the production of diacetyl in starter cultures. Prill and Hammer (12) noted that aeration of ripening starter cultures by shaking produced significant increases in the diacetyl, while the lack of aeration caused decreases in the diacetyl. They also noted that lowering the pH of the starter culture tended to increase the diacetyl content. Hedrick and Hammer (9) pointed out that an increased diacetyl content could be obtained in ripening cream by development of higher acidities and use of agitation. Increases up to several hundred per cent in the diacetyl content of starter cultures were noted by Brewer *et al.* (2) when air was bubbled through the starter culture. Cox (4) studied the effect of acidity on the production of diacetyl by betacocci in milk. His investigations were in the pH ranges of 5.5 to 4.4. He found that the rate of growth of the organisms was slower progressively with decreasing pH, but at least as much diacetyl was formed eventually at the low pH values as at high pH values. The results of Cox's investigations showed that there was an alteration in the [illegible] per unit cell increasing with decreasing pH. According to van Beynum and Pette (13), the diacetyl was produced from citric acid only when the medium was acid and the conditions aerobic.

The same factors as discussed above also influence the rate of diacetyl production during the making of Cheddar cheese. The experimental data of this study with Cheddar cheese show that while the milk and starter are being stirred, prior to the time of setting, there is an increase in diacetyl. This stirring or agitation provides a means of aeration that would be expected to favor diacetyl production. Little diacetyl is produced between the time of setting and the time of cutting. During this period the material in the vat is quiescent, and the aeration that occurs at other times during the making procedure is lacking. Also, the pH at this time is much higher than it is later in the making procedure. This combination of factors does not encourage diacetyl production. From the time of cutting until the time of dipping, the material in the vat is stirred continuously. This aeration favors the production of diacetyl. From the time of dipping until milling diacetyl production is favored by the rapidly decreasing pH in the curd and whey.

In the six lots of cheese used in this investigation, the rate of diacetyl production during the early steps of the making procedure appears to be slower in the raw-milk than in the pasteurized-milk Cheddar cheese. The nature of the experimental procedure does not establish this as an actual fact. The trend is most interesting and might be investigated further because it suggests the action or influence of: (a) either substances or microorganisms in the raw milk that slow down or retard the production of diacetyl by the "aroma-formers" until later in the making procedure, (b) lack of oxygen or a lower oxidation-reduction potential in the raw milk that prevents or slows down the formation of diacetyl.

The microorganisms responsible for the formation of diacetyl in starter cultures probably are present during the making of cheese. Factors that influence the formation of diacetyl in starter cultures undoubtedly also influence its formation in cheese. Therefore, the diacetyl formed during the making of Cheddar cheese probably comes from the same source and is produced in the same manner as the diacetyl produced in starter cultures.

A gradual decline in the diacetyl content of both the pasteurized-milk and raw-milk Cheddar cheese was noted during the early stages of curing. This parallels observations which have been made by several investigators on the decline in the diacetyl content of butter during storage. According to Langton (10), the gradual disappearance of diacetyl in butter is caused by volatilization. On the other hand, many investigators, including Elliker (6), have indicated that some of the diacetyl in butter is reduced to acetyl[illegible] nol and 2,3-butylene glycol by the action of microorganisms. Virta[illegible] on-[illegible] (14) added known amounts of diacetyl to samples of milk; [illegible] was inoculated with cultures of microorganisms that had been [illegible] butter. A decline in the diacetyl content of the milk samples [illegible] by

These factors responsible for the decline in the diacetyl content of butter during storage also may explain the decline of diacetyl in cheese. Carbon dioxide and moisture are lost in large amounts by volatilization from the surfaces of the cheese in the early stages of curing. This action would be expected to remove volatile substances like diacetyl from the cheese. The changes in the oxidation-reduction potential of the cheese during curing (5) may cause the diacetyl to undergo chemical changes. Undoubtedly, the microorganisms present in the cheese during this period are responsible in part for the decline in diacetyl. The decline of the diacetyl content in the cheese probably can be attributed to a combination of these physical, chemical and biological influences.

SUMMARY

Diacetyl is produced throughout the manufacturing process of Cheddar cheese except during the interval between setting and cutting. Agitation and increased acidity were factors associated with the increase in diacetyl content.

The diacetyl in Cheddar cheese probably is produced by the action of microorganisms upon the citrates in a manner similar to its production in starter cultures.

A gradual decrease in the amount of diacetyl in both raw-milk and pasteurized-milk Cheddar cheese was noted during the early stages of curing. This decrease probably is caused by volatilization of the diacetyl and by its reduction to acetylmethylcarbinol and 2,3-butylene glycol as a result of chemical reaction or the action of microorganisms.

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A STUDY OF THE DIACETYL IN CHEESE. I. DIACETYL CONTENT AND FLAVOR OF CHEDDAR CHEESE[1]

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HAROLD E. CALBERT AND WALTER V. PRICE
Department of Dairy Industry, University of Wisconsin

Diacetyl is a prominent element in the flavor of many food products. This is true particularly of food products that are produced by a fermentation process.

Prill *et al.* (9) have discussed the various properties of diacetyl as well as its higher homologues. In their work the general chemical structure of the *alpha*-dicarbonyl compounds was presented with particular reference to the contribution of diacetyl and its homologues to butter flavor and aroma. While the influence of the diacetyl content on the flavor and aroma of butter and starter cultures has been investigated to a considerable extent, little work has been done to determine the diacetyl content of cheese. Both Crocker (2) and Davies *et al.* (6) have indicated that diacetyl could be a possible constituent of cheese aroma. In listing the various flavors of cheese, Davis (7) referred to the buttery aroma and suggested that the substance responsible was diacetyl. Davies *et al.* (6) attempted to determine the effect of adding diacetyl to the curd in making cheese. They reported that when diacetyl was added in the concentration of 1.7 p.p.m. to the curd, a cheese was produced that had a stinging effect on the tongue.

In 1941, Csiszár and Bakos (3) and Csiszár *et al.* (4, 5) conducted studies in Hungary on the diacetyl content of such types as Emmenthal, Ovári, Trappist, Edam, Roquefort, Camembert and Romadour. These investigators reported that the aroma and quality in cheese were better when the cheese was made with a culture which was highly active in acetylmethylcarbinol-diacetyl formation. The diacetyl content of the various types of cheese as reported by Csiszár *et al.* (4) ranged from none to as much as 1 mg. per 100 g. of cheese. This study was undertaken with these objectives: (a) to determine whether diacetyl is a component of cheese; (b) if diacetyl is present, to determine to what extent; and (c) to determine whether there is a correlation between cheese flavor and the amount of diacetyl present in the cheese.

METHODS

The colorimetric method of Prill and Hammer (10) seemed to offer the best means of determining the relatively small diacetyl content of cheese. This method was very sensitive and could be adapted easily to routine analysis. To adapt this method to the determination of diacetyl in cheese, alterations which involved the preparation of the sample for analysis, a slight modification of the distillation apparatus and measurement of the concentration of ammono-ferrous dimethylglyoximate by means of a Beckman spectrophotometer were made.

Preparation of the cheese sample. Cheese, unlike butter cultures, cultured milks and butter, does not lend itself readily to steam distillation. Upon heating,

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the cheese becomes a plastic mass with a tendency to retain any volatile components ordinarily removed by steam distillation of a liquid. Even after grinding, all of the diacetyl that is present cannot be removed from the cheese unless the sample is converted into a liquid form.

Consistent results can be obtained if the sample of cheese is treated in the following manner: A given weight of ground cheese (usually 200 g.) plus an equal weight of distilled water are placed in a Waring blendor. The blendor is run at low speed for 2 min., followed by mixing at high speed for 3 min. Such treatment produces, for all practical purposes, a homogeneous suspension, as the data presented in table 1 indicate. The differences between the theoretical solids

TABLE 1

Solids measurements of cheese-water mixtures as a criterion of homogeneity

Sample no.	Cheese	Solids/100 g. cheese-water mixture[a]		Difference	Cheese equivalent to the difference
		Measured	Calculated		
	(*g.*)	(*g.*)	(*g.*)	(*g.*)	(*g.*)
1	65.05	32.90	32.53	+ 0.37	0.57
2	65.50	32.60	32.75	− 0.15	0.29
3	64.95	32.80	32.47	+ 0.33	0.51
4	65.40	33.00	32.70	+ 0.30	0.46
5	64.60	32.80	32.35	− 0.45	0.68

[a] Solids content determined by *Official Methods* (1).

content of the cheese-water mixture, and the measured solids content were converted by calculation to their equivalent weights of cheese. The standard deviation of these differences was calculated and found to be equivalent to ± 0.38 g. of cheese. The maximum difference rarely should exceed three times the standard deviation or approximately ± 1.14 g. of cheese when a 200 g. aliquot of the cheese-water mixture is used to represent 100 g. of cheese.

Subsequent analyses indicated that only rarely would the diacetyl content of cheese exceed 0.1 mg. per 100 g. Therefore, the error in the amount of diacetyl measured by using this procedure would not exceed ± 0.001 mg. This amount of diacetyl is so small that it would not affect materially the final results of the determination.

Modification of distillation apparatus. The distillation apparatus used for the determination of diacetyl in cheese was essentially the same as described by Prill and Hammer (10). However, instead of using glass discs in the reflux-fractionating column, the columns were packed with small pieces of 2 mm. glass tubing, about 5 to 10 mm. in length.

A simple steam generator was made to insure a constant source of uncontaminated steam. This generator was constructed in the following manner: Eleven ft. of nichrome wire with an approximate total resistance of 24 ohms was wound in the form of a coil and placed in the bottom of a 3-l. round-bottomed flask. Each end of the wire was connected to a copper lead, sealed in glass tubing that passed through the rubber stopper. The other end of the copper lead was con-

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nected to a rheostat connected to the 110-volt, A.C. outlet to control the flow of steam. A glass funnel with a section of rubber tubing and a pinchcock in the stem was passed through the rubber stopper. This provided a means of introducing distilled water into the flask. A suitable outlet for the steam and a glass steam trap to collect the condensate also were provided. A steam generator of this design furnished an ample supply of steam for all distillations.

Dimethylglyoxime was used as the diacetyl standard as originally proposed by Prill and Hammer (10).

Spectrophotometric measurement of color. By measuring the intensity of the rose-red color complex with the Beckman spectrophotometer only one or two standards were needed for each group of determinations, rather than the range of standards mentioned in the original procedure.

The absorption spectrum was established by measuring the transmittance for a particular concentration of the diacetyl standard at various wave lengths. The results were plotted in the form of a curve relating optical density to wave length. For these determinations a Beckman spectrophotometer (model DU) and 1.000 cm. Corex cells were used. A nominal band width of 1 to 2 mμ. was used throughout the absorption spectrum determinations. The optical density was read directly from the spectrophotometer.

Solutions containing the equivalent of 0.02, 0.05 and 0.10 mg. of diacetyl per ml. were made from the diacetyl standard. The colored ammono-ferrous dimethylglyoximate derivative was prepared from each of these solutions according to the procedure of Prill and Hammer (10). A blank determination on all reagents was prepared at the same time. The optical density of these solutions was measured at various wave lengths ranging from 500 to 560 mμ. Maximum absorption was obtained at 530 mμ. Within the range of concentrations tested, Beer's law is applicable for this determination.

The accuracy of this modification of the Prill and Hammer method for the determination of diacetyl in Cheddar cheese was determined by means of recovery tests. To samples of each cheese, which had been analyzed previously for diacetyl by the modified method, known quantities of purified diacetyl were added. The total diacetyl content of these cheese-diacetyl mixtures then was determined by the modified method. The results of these recovery tests are shown in table 2. The percentages of recovery ranged from 95 to 102.3 per cent. The method appeared to be of sufficient sensitivity to measure the diacetyl content of cheese.

This modified colorimetric procedure was used for the diacetyl determinations of all lots of cheese. Duplicate samples of each cheese were analyzed. Samples were obtained by grinding either the whole cheese or a wedge of the cheese as described in *Official Methods* (1).

EXPERIMENTAL RESULTS

Twenty-eight lots of Cheddar cheese and eight lots of other varieties of cheese were selected for this investigation. The majority of the lots of Cheddar cheese had been manufactured at the Dairy Department of the University of Wisconsin The rest of the lots were manufactured at various plants within the state of Wis

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consin. Typical cheese was selected so that it would resemble the average cheese found on the commercial market. Usually little was known concerning the making process of any particular lot of cheese. Fourteen lots of the Cheddar cheese had been manufactured from pasteurized milk, the remainder from raw milk. The cheese ranged in age from 1 to 48 months. Each lot of Cheddar cheese was examined by a judging panel composed of from one to three competent cheese judges. Comments upon the flavor of each lot of cheese were made by each judge. The flavor criticisms used were the standard comments usually employed in evaluating the quality of a cheese on the basis of flavor (8).

TABLE 2

Recovery tests

Test no.	Diacetyl in cheese	Diacetyl added	Diacetyl recovered	Recovery
	(*mg.*)	(*mg.*)	(*mg.*)	(%)
1	0.026	0.009	0.034	97.25
2	0.026	0.018	0.045	102.30
3	0.026	0.027	0.052	98.20
4	0.026	0.045	0.069	97.20
5	0.026	0.090	0.111	95.70
6	0.035	0.017	0.050	96.30
7	0.035	0.034	0.068	98.75
8	0.035	0.051	0.084	97.80
9	0.035	0.068	0.100	97.23
10	0.035	0.085	0.114	95.00

Other varieties of cheese included in this investigation with the diacetyl content of each lot (expressed as mg. per 100 g.) were as follows: Swiss (0.036), Brick (0.017), Limburger (0.021), Camembert (0.008), Blue Mold (0.030), Edam (0.043), Port Salut (0.074) and Cottage (0.029).

To illustrate the variations in the diacetyl content of the different lots of Cheddar cheese, the 28 lots were classified according to the diacetyl content. The results of this grouping are shown in table 3. The majority of all lots contained less than 0.05 mg. per 100 g. of cheese.

TABLE 3

Diacetyl in cheddar cheese[a]

Diacetyl content	No. of lots	% of lots
(*mg./g. of cheese*)		
0.000–0.049	16	57.2
0.050–0.099	5	17.8
0.100–0.149	2	7.2
0.150 and over	5	17.8

[a] The diacetyl content of the Cheddar cheese ranged from 0.016 to 0.335 mg./100 g.

Flavor characteristics were used to divide the 28 lots of Cheddar cheese into two groups. One group of "Excellent Flavor" consisted of lots which had no adverse flavor criticisms. The other group contained all lots that had adverse flavor criticisms. These adverse flavor criticisms included anything from a very

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slight off-flavor to a definitely objectionable flavor. This latter group was called the group with "All Other Flavors." Each of these two flavor groups then was divided into two sub-groups based on the diacetyl content of the lot. One sub-group contained all lots with less than 0.05 mg. of diacetyl per 100 g. of cheese. The other sub-group consisted of all lots with more than this amount of diacetyl.

The number of lots in each sub-group then was expressed as the per cent of the total lots in that flavor group. Seventy-nine per cent of the lots in the group with "Excellent Flavor" had a diacetyl content of less than 0.05 mg. per 100 mg. In the group with "All Other Flavors" 63 per cent of the lots had more than this amount of diacetyl.

DISCUSSION

The experimental data have shown that diacetyl was present in cheese both when it had an excellent flavor and when it was criticized adversely for flavor. The majority of the lots of cheese with an excellent flavor had a diacetyl content of less than 0.05 mg. per 100 g. of cheese. On the other hand, most of the lots of cheese with adverse flavor criticisms had a larger diacetyl content than this. The production of diacetyl probably was associated with other biological activities that produced substances that detract from the flavor of a cheese. The presence of these substances, and not the diacetyl content, probably was responsible for the adverse criticisms of the cheese flavor. The data show that it was possible to have more than 0.05 mg. of diacetyl per 100 g. of cheese without the production of the "flavor detracting" substances. Probably there is a maximum diacetyl content which, if exceeded, will cause adverse flavor criticisms. Frequently cheese judges identify the flavor as "acidy" when the cheese is normal in hydrogen ion concentration and has no acid defects in body. Crocker (2) described the flavor of diacetyl as "pseudo-sour." It is highly probable that this "pseudo-sour" flavor of diacetyl gives the impression of "acidy" flavor in a cheese that shows no other signs of high acidity.

Diacetyl seems to be an essential part of the flavor complex of Cheddar cheese. In amounts smaller than 0.05 mg. per 100 g. of cheese it is associated with excellent flavor qualities, but amounts in excess of this appear to be associated with adverse criticisms.

SUMMARY

Diacetyl is a prominent element in the flavor of many food products. To determine its presence in cheese and to make quantitative measurements of it, the colorimetric method described by Prill and Hammer (10) was modified. The color of the ammono-ferrous dimethylglyoximate formed in this procedure was measured spectrophotometrically.

Diacetyl was found in all lots and kinds of cheese examined. The majority of the lots contained less than 0.05 mg. of diacetyl per 100 g. of cheese. The diacetyl content of Cheddar cheese ranged from 0.016 mg. to 0.335 mg. per 100 g. of cheese.

A small quantity of diacetyl probably contributes to the typical flavor of Cheddar cheese. The majority of the lots of Cheddar cheese with an excellent

flavor had a diacetyl content of less than 0.05 mg. per 100 g. Larger amounts of diacetyl than this frequently appear to be associated with flavor defects.

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CHR. HANSEN'S LABORATORY, INC.

9015 WEST MAPLE STREET

MILWAUKEE, WISCONSIN 53214

STARTER DISTILLATE

General Description, Ingredients, Origin:

Starter distillate is the mixture of flavor compounds distilled from specially cultured reconstituted skim milk. It contains the flavor compounds which are volatile with water at 212°F (100°C).
Chr. Hansen's Laboratory has developed a standard manufacturing procedure so that a duplication from batch to batch is possible.
The main component is diacetyl but starter distillate, as a whole, gives a flavor much more pleasant than synthetic diacetyl because of the other flavor substances produced by specific strains of bacteria used in culturing the milk.

Applications:

Butter, margarine, cottage cheese, sour cream, non-dairy type products, candies, etc.

Strength:

Standardized on diacetyl.

ısen's Standard Starter Distillate contains 1 mg diacetyl / ml.

Hansen's 15X Starter Distillate contains 15 mg diacetyl / ml.

Apart from the difference in strength, the total flavor obtained from regular starter distillate and 15X is not quite the same so it is suggested that both be tried for each specific use.

How To Use:

Starter distillate is a clear liquid which is completely water soluble It is, therefore, easy to add to any water-containing product. The flavor components of starter distillate have a certain solubility in oil also. If a product has two phases (oil/fat and water) there will be a distribution between the two phases. At high temperatures the flavors tend to volatilize and disappear but some products may retain the flavor better than expected, so it is very important to make trials similar to actual conditions.

How Much To Use:

1 mg diacetyl / ml = 1,000 p.p.m.
15 mg diacetyl / ml = 15,000 p.p.m.

For products like cottage cheese and margarine it is desirable to ave about 1 - 5 p.p.m. in the final product so this can serve as a ıide. As mentioned before, evaluation of each application is ıecessary.

STARTER DISTILLATE

Availability:

Gallon plastic bottles packed one or four per case. Five-gallon Poly-Pak containers.

Storage, Shelf Life:

Keep containers closed! Store as cool as possible but not below 32°F. Starter distillate is very stable for a flavor product. It is good practice to limit inventories to a three-month supply.

Approval:

Butter starter distillate is covered by 21CFR § 121.1164 of the food additive regulations on flavoring substances and adjuvants.

0322

Christensen, H.E. (Ed.). 1974
Toxic Substances List
FDA, U.S. Department of Health,
Education, and Welfare

0323

Factors Affecting Diacetyl Production by Lactic Acid Bacteria[1]

M. D. Christensen and C. S. Pederson

New York State Agricultural Experiment Station, Cornell University, Geneva, New York

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Recently it has been observed that the lactic acid bacteria are primarily responsible for the production of diacetyl during concentration of orange juice. The amount of diacetyl present is used as an indicator of the degree of contamination, even though it is not a major end product of fermentation. Its presence may be detected even though little change in total acidity or pH has been noted. A better understanding of factors influencing diacetyl production by lactic acid bacteria would be desirable.

The relationship of the lactic acid bacteria to diacetyl production evolved from an early date. Hammer (1920) concluded that the organisms in butter cultures other than *Streptococcus lactis* were responsible for the volatile acidity, aroma, and flavor of the fermented dairy product. Van Niel in 1929 as stated by Hammer and Babel (1943) recognized that diacetyl was either responsible for the aroma of butter or was the principal component of the aromatic material. Michaelian *et al.* (1933) noted that butter cultures with satisfactory character contained considerable quantities of acetylmethylcarbinol and diacetyl. This fact is generally accepted.

Hays (1951) and Hays and Riester (1952) identified spoilage organisms from orange juice as species of the genera *Leuconostoc* and *Lactobacillus*. Murdock *et al.* (1952) and Murdock and DuBois (1955) noted production of acetylmethylcarbinol and diacetyl by lactic acid bacteria grown in orange juice. Brokaw (1952) also employed plate counts and microscopic examination to follow the contamination of orange juice. Hill and Wenzel (1954b) developed a colorimetric method which has been used successfully as a quality control device.

Michaelian *et al.* (1938) and Glenn and Prouty (1955) studied the effects of citric acid on the growth of *Streptococcus lactis*, *Streptococcus citrovorum*, *Streptococcus cremoris*, and *Leuconostoc mesenteroides*.

While most reports recognize the role of the lactics in diacetyl production, conflicting reports do appear. Temisson and Hall (1947) concluded that bacteria are probably not a major factor in influencing quality of orange juice since only one-third of their isolates were bacteria.

Wolford and Berry (1948) and Purko *et al.* (1956) noted the influence of coliform bacteria in orange juice concentrate. They isolated strains of the genera *Aerobacter*, *Escherichia*, and *Serratia*. Berry *et al.* (1954) felt that orange juice was high enough in acidity to prevent growth of most types of bacteria. They isolated strains of the genera *Leuconostoc* and *Lactobacillus*. Faville *et al.* (1951) found that yeasts do not appear to be the predominating organism and found that 40 per cent of their isolates were strains of the genus *Leuconostoc*. Murdock *et al.* (1952) and Rushing *et al.* (1956) isolated lactic acid bacteria.

Additional information on the ability of each of the lactic acid bacteria to produce acetylmethylcarbinol and diacetyl under a variety of conditions would augment the knowledge already available concerning specific characteristics of the lactic acid bacteria. This study involves species of the four genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus*.

Materials and Methods

The organisms used in this study were *Lactobacillus plantarum* strain B246, *Lactobacillus lactis* strain L33, *Lactobacillus bulgaricus* strain 7994, *Lactobacillus helveticus* strains L31, L85, and L43, *Lactobacillus brevis* strains B155 and 2810, and the dextran producing variant L155, *Pediococcus cerevisiae* strain E66, *Leuconostoc mesenteroides* strain C29, and *Streptococcus faecalis* strain E166.

The basic medium A contained 1.5 per cent glucose, 0.5 per cent tryptone, and 0.25 per cent yeast extract. Medium B contained, in addition, 0.137 per cent citrate; medium C contained 0.1 per cent glucose and 0.137 per cent citrate. Medium AC contained only 0.1 per cent glucose, and medium CC contained 0.1 per cent glucose and 0.15 per cent citric acid. Media D through H were commercial orange juices adjusted to pH readings of 4.0, 5.5, 4.0, 5.5, and unadjusted, respectively. The orange juice media were sterilized in flowing steam for 30 min. Media A through E were incubated at 32 C whereas media F, G, and H were incubated at 21 C.

Diacetyl was determined by the method of Hill *et al.* (1954a). This determination measures total diacetyl, acetylmethylcarbinol, and 2,3-butylene glycol as diacetyl. Three hundred ml of media were inoculated with the representative culture from a 24 hr stab, incubated 96 hr, and then distilled. From each sample,

[1] Journal Paper No. 1109 New York State Agricultural Experiment Station, Cornell University, Geneva, New York, February 4, 1958.

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25 ml of distillate were collected and from that a 10 ml aliquot was removed from below the surface to avoid small traces of peel oil which might interfere with colorimeter readings. This 10 ml aliquot was treated with 5 ml of 5 per cent α-naphthol in ethyl alcohol and 2 ml of 40 per cent potassium hydroxide (containing 0.3 per cent creatine), shaken vigorously for 15 sec, allowed to stand 10 min, and shaken again for 15 sec. The concentration of diacetyl was determined by measuring the color intensity in a Bausch and Lomb Spectronic 20 Model[2] colorimeter at 550 mμ using the reagent blank to zero the instrument. Standard calibration curves were prepared using solutions of 1 to 140 ppm diacetyl in distilled water, diluted until a suitable light transmission could be obtained with the instrument. The standard curve for samples requiring dilution of 1:20 is presented in figure 1. In the same way, after the color in the fractions was developed, those samples containing high concentrations of diacetyl were diluted in order to obtain suitable colorimetric readings.

Citric, lactic, and acetic acids were determined after separation by the column chromatographic technique of Bulen *et al.* (1952) and carbon dioxide was determined after titration of $Ba(OH)_2$ in a series of fermentations using Eldredge tubes (Christensen *et al.* 1958).

Results and Discussion

The production of diacetyl by the various lactic acid bacteria was found to vary, depending upon the medium, the pH, and the incubation temperature. The homofermentative species produced diacetyl more readily and in larger amounts than did the heterofermentative species, table 1. *Lactobacillus plantarum* strain 246, *Lactobacillus bulgaricus* strain 7994, *Lactobacillus helveticus* strain L31, *Pediococcus cerevisiae* strain E66, and *Streptococcus faecalis* strain E166 produced diacetyl even in the absence of citric acid. However, more diacetyl was produced when citric acid was added, medium B. The latter two species produced greater quantities when the sugar was limited to 0.1 per cent, medium C.

The heterofermenter, *Leuconostoc mesenteroides* strain C29, produced a small amount of diacetyl in the low

[2] Bausch and Lomb, Rochester, New York.

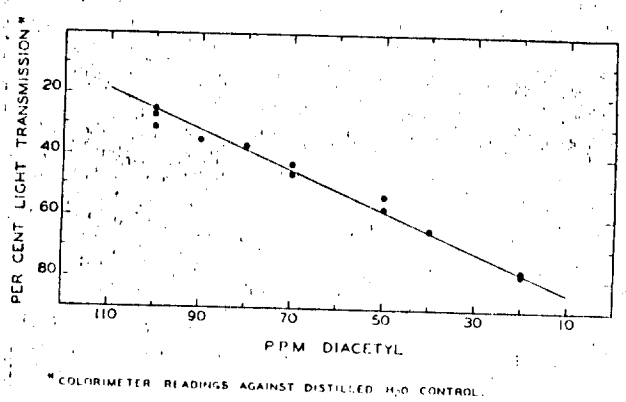

Figure 1. Diacetyl standard curve for sample diluted 1:20

TABLE 1

Production of diacetyl in various media by typical lactic acid bacteria

Organism	Diacetyl as ppm in Medium:*							
	A	B	C	D	E	F	G	H
Lactobacillus plantarum B246	2.18	9.27	6.72	4.55	0.73	5.45	6.18	11.27
Pediococcus cerevisiae E66	0.45	2.00	4.00	0.37	0.73	0.41	0.51	0.31
Streptococcus faecalis E166	0.27	0.27	0.47	3.09	0.73	6.63	4.15	0.29
Leuconostoc mesenteroides C29	0.00	0.00	0.61	6.45	3.18	6.09	0.51	0.29
Lactobacillus brevis B155	0.00	0.00	0.00	0.13	0.23	0.09	6.73	0.31
Lactobacillus brevis L155	0.00	0.00	0.00	0.38	0.73	0.15	9.09	0.35
Control	0.09	0.00	0.00	0.35	0.36	0.41	0.51	0.31
Lactobacillus helveticus L31	2.64	4.91	8.36					
Lactobacillus bulgaricus 7994	12.00	11.00	11.27					
Control	0.09	0.17	0.00					

* Medium A contained 1.5 per cent glucose, 0.5 per cent tryptone, 0.25 per cent yeast extract; medium B the same with 0.137 per cent citric acid; and medium C contained only 0.1 per cent glucose with 0.137 per cent citric acid. Media D to H were orange juices adjusted to: D and F, pH 4.0; E and G, pH 5.5; and H, unadjusted. Media F, G, and H were incubated at 21 C, all others at 32 C.

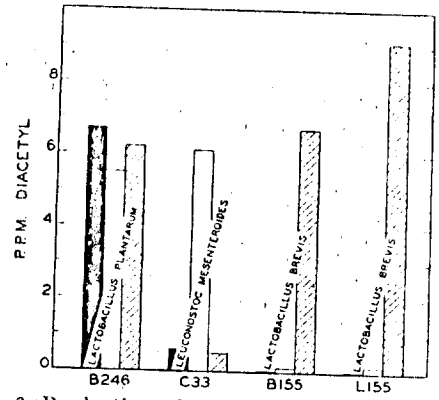

Figure 2. Production of diacetyl by strains of lactic acid bacteria grown in low sugar citrate medium and in orange juice. ■ = Medium C, contained 0.1 per cent glucose, 0.5 per cent tryptone, 0.25 per cent yeast extract, 0.137 per cent citrate, incubated at 32 C, pH unadjusted. □ = Medium F, contained commerically canned orange juice incubated at 21 C, pH adjusted to 4.0. ▨ = Medium G, contained commerically canned orange juice incubated at 21 C, pH adjusted to 5.5.

sugar, citric acid medium. However, the other heterofermenters *Lactobacillus brevis* strain B155 and the dextran producing variant L155 produced diacetyl only in orange juice media. The greatest amount was formed in orange juice adjusted to pH 5.5 and incubated at 21 C, conditions under which the least diacetyl was produced by *Leuconostoc mesenteroides* C33 (figure 2).

Since citric acid is necessary for production of diacetyl in some instances, it seemed desirable to determine how citric acid may affect other fermentation byproducts. Instead of carrying on the fermentation in Erlenmeyer flasks, it was carried out in Eldredge tubes so that the carbon dioxide could be absorbed with barium hydroxide. From the same medium, larger quantities of diacetyl were produced in the Eldredge tubes and still larger amounts in the presence of citric acid (table 2, figure 3). However, neither of the strains of *Lactobacillus brevis* produced diacetyl regardless of whether or not citric acid was added or the sugar was reduced to 0.1 per cent. *Leuconostoc mesenteroides* produced diacetyl only in the low sugar citric acid medium CC.

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TABLE 2

Diacetyl, acid and carbon dioxide production in Eldredge tubes by some typical lactic acid bacteria

Species and Strain No.	Media*	Diacetyl ppm	Carbon Dioxide	Acetic Acid	Lactic Acid	Citric Acid	Acetic/Lactic Acid
			Mg per cent carbon				
Lactobacillus plantarum, 246	A	31.5	9	14	612	—	0.02
	B	68.9	19	11	592	0	0.07
	AC	10.0	10	21	31	—	0.70
	CC	11.0	19	57	42	0	1.35
Lactobacillus bulgaricus, 7994	A	66.0	10	4	304	—	0.01
	B	75.5	15	9	505	—	0.02
Lactobacillus helveticus, L85	A	72.5	5	—	207	—	—
	B	61.0	11	13	620	30	0.02
Lactobacillus helveticus, L31	A	30.2	5	8	616	—	0.01
	B	37.0	7	15	572	33	0.03
Pediococcus cerevisiae, E66	A	12.5	7	20	362	—	0.05
	B	15.5	8	17	382	40	0.04
	AC	9.0	10	12	30	—	0.40
	CC	7.5	12	19	42	30	0.45
Streptococcus faecalis, E166	A	2.2	4	2	163	—	0.01
	B	2.2	3	3	212	39	0.01
	AC	0.9	5	9	44	—	0.20
	CC	0.9	5	9	50	39	0.18
Leuconostoc mesenteroides, C29	A	0	51	6	219	—	0.03
	B	0	57	52	266	0	0.20
	AC	0	2	13	28	—	0.47
	CC	19.0	35	47	39	0	1.20
Lactobacillus brevis, B155	A	0	71	39	294	—	0.13
	B	0	75	72	310	0	0.23
	AC	0	19	31	8	—	3.87
	CC	0	29	47	26	0	1.80
Lactobacillus brevis, L155	A	0	66	36	412	—	0.09
	B	0	66	63	428	0	0.14

* Medium A contained 1.5 per cent glucose, 0.5 per cent tryptone, and 0.25 per cent yeast extract and medium B contained the same plus 0.137 per cent citric acid. Medium AC contained only 0.1 per cent glucose; and medium CC contained 0.1 per cent glucose and 0.15 per cent citric acid.

It may be further noted that the citric acid was utilized, at least partially, by all cultures and that ordinarily carbon dioxide and acetic acid were produced in greater amounts when citric acid was added. In addition, the amount of lactic acid was usually somewhat greater showing partial conversion of citric acid to lactic acid. This occurred in both the high-sugar and low-sugar media.

The data indicate that diacetyl is produced in lesser quantities under optimum conditions for growth. *Leuconostoc mesenteroides* produced lesser amounts in media adjusted to pH 5.5 than pH 4.0 and little, if any, in laboratory media in contrast to the more acid orange juice. *Streptococcus faecalis* also produced less at pH 5.5 than at pH 4.0 and, when incubated at 21 C; *Lactobacillus brevis* produced diacetyl only in orange juice. Even *Lactobacillus plantarum* produced the least diacetyl in the orange juice adjusted to pH 5.5 and incubated at 32 C. In addition, it may be pointed out that citric acid is too poor a carbon source to allow initiation of growth of many of the lactics but will stimulate diacetyl production.

There is little doubt that differences would be found

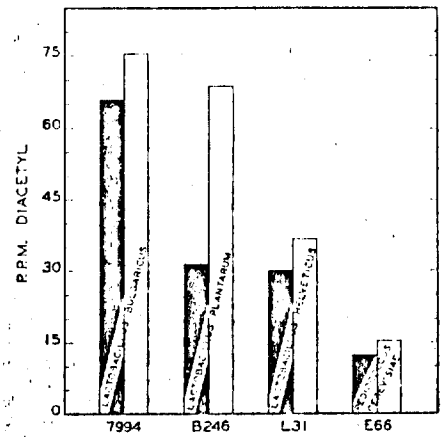

Figure 3. Production of diacetyl by strains of homofermentative lactic acid bacteria in Eldredge tubes using media with and without citric acid. ▩ = Medium A, contained 1.5 per cent glucose, 0.5 per cent tryptone, 0.25 per cent yeast extract, incubated at 32 C, pH unadjusted. ▢ = Medium B, contained 1.5 per cent glucose, 0.5 per cent tryptone, 0.25 per cent yeast extract, 0.137 per cent citrate, incubated at 32 C, pH unadjusted.

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between strains within each species as indicated by Berry *et al.* (1954). However, no marked difference was obtained between the nondextran producing strain of *Lactobacillus brevis* and the dextran producing strain.

SUMMARY

The production of diacetyl by various species of lactic acid bacteria is dependent upon several factors. The homofermentative species produce diacetyl more readily than do the heterofermentative species. Citric acid is essential for diacetyl production by some strains. In all species studied, diacetyl production in low-sugar medium is enhanced by the presence of citric acid. In addition, the greater quantities of diacetyl are produced under the less optimal conditions. From these results, one may conclude that, since certain strains of lactic acid bacteria, particularly the heterofermentative species, may develop without producing diacetyl, diacetyl production is not necessarily an indication of growth of such organisms.

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Biosynthesis of Diacetyl in Bacteria and Yeast

LINDA F. CHUANG AND E. B. COLLINS
Department of Food Science and Technology, University of California, Davis, California 95616

Received for publication 1 April 1968

Both diacetyl and acetoin were produced by cell-free extracts and cultures of *Pseudomonas fluorescens*, *Aerobacter aerogenes*, *Lactobacillus brevis*, and *Saccharomyces cerevisiae* 299, whereas only acetoin was produced by cell-free extracts and cultures of *Streptococcus lactis*, *Serratia marcescens*, *Escherichia coli*, and *S. cerevisiae* strains 513 and 522. Cell-free extracts that produced diacetyl did not produce it from acetoin; they produced it from pyruvate, but only if acetyl-coenzyme A was added to the reaction mixtures. Production of diacetyl by *S. cerevisiae* 299 was prevented by valine, inhibited by sodium arsenite, and stimulated by pantothenic acid. Valine did not prevent the production of acetoin. *E. coli* and the three strains of *S. cerevisiae* did not decarboxylate α-acetolactate but did use acetaldehyde in the production of acetoin from pyruvate. The other organisms produced acetoin from pyruvate via α-acetolactate.

Juni (6, 7) elucidated certain differences in the mechanisms by which bacteria and yeast and mammalian tissues form acetoin (3-hydroxy-2-butanone) from pyruvate. The bacteria he studied formed acetoin by decarboxylation of α-acetolactate, which was formed by enzymatic condensation of two molecules of pyruvate, probably by formation of an acetaldehyde-thiamine pyrophosphate (TPP) complex from pyruvate and condensation of the complex with another molecule of pyruvate. Free acetaldehyde is not involved in this mechanism (6, 8, 11, 18). The yeast and mammalian tissues formed acetoin by a different mechanism, probably by condensation of the acetaldehyde-TPP complex with free acetaldehyde formed from pyruvate (7).

It has been assumed that diacetyl (2,3-butanedione) is formed by oxidation of acetoin in bacteria (17) and in yeast (10, 16, 21). Speckman and Collins (18), however, recently reported that *Streptococcus diacetilactis* and *Leuconostoc citrovorum* do not form diacetyl from acetoin; instead, they form diacetyl by condensation of the acetaldehyde-TPP complex with acetyl coenzyme A (CoA).

This study was done to determine whether the mechanism for diacetyl biosynthesis found by Speckman and Collins (19) is used by other diacetyl-producing microorganisms and to verify the mechanisms that have been described for acetoin production. All bacteria and yeast in the study formed acetoin by the expected mechanisms. Those that formed diacetyl were found to form it by the new mechanism, not from acetoin.

MATERIALS AND METHODS

Organisms and culturing procedures. The following organisms which produced CO_2 and acetoin plus diacetyl from pyruvate were used in this study: *Pseudomonas fluorescens*, *Aerobacter aerogenes*, *Serratia marcescens*, *Escherichia coli*, *Streptococcus lactis* C_2, *Lactobacillus brevis*, *Saccharomyces cerevisiae* var. *ellipsoideus* (UCD Enology strain no. 513, Distillers', and no. 522, Montrachet), and *S. cerevisiae* (UCD Food Science and Technology strain no. 299). *S. cerevisiae* strain NCYC 1200, obtained late in the study and not included, produced an amount of diacetyl similar to that produced by strain 299.

S. lactis was propagated routinely in sterile skim milk fortified with 0.75% nonfat milk solids. For obtaining cells, it was grown at 26 C for 15 hr in lactose-citrate broth (3), *p*H 6.8. *L. brevis* was propagated in tomato-enriched medium (6% Difco-Rogosa SL broth dissolved in filtered tomato juice) and, for obtaining cells, was grown at 21 C for 40 hr in orange-lactose broth (commercial orange juice with 0.5% lactose added, steam-sterilized, with the *p*H not adjusted). The media used for propagating and obtaining cells of the other bacteria were, respectively, Difco Nutrient Agar and Nutrient Broth, *p*H 7.0. To obtained cells of these species, *P. fluorescens* was grown at 21 C for 72 hr, *A. aerogenes* at 32 C for 35 hr, *S. marcescens* at 32 C for 30 hr, and *E. coli* at 37 C for 36 hr. *S. cerevisiae* was grown for 2.5 days at 21 C in YED medium (yeast extract, 2%; glucose, 2%; KH_2PO_4, 0.5%; *p*H 5.95) or (for certain indicated experiments) in ASD medium (ammonium sulfate, 0.1%; KH_2PO_4, 0.5%; glucose, 2%; *p*H 5.85). The glucose content of the YED medium was increased to 20% for certain indicated experiments.

Cell free extracts. Each species was grown in 4-liter Erlenmeyer flasks. Each flask contained 2 liters of

broth and was shaken every 3 to 5 hr. The content of acetoin plus diacetyl was followed by analysis with the Westerfeld test (22). The cells were harvested by centrifugation when the content of acetoin plus diacetyl stopped increasing. They were washed twice with cold 0.2 M phosphate-cysteine buffer at the *p*H of the original growth medium (0.001 M with respect to L-cysteine) and were resuspended in the same buffer. The cells were disrupted by ballistic disintegration with a Mickel tissue disintegrator operated at maximal amplitude for 1 hr (1.5 hr for yeast) in a room maintained at 4 C, and crude extract was separated from the disrupted cells by centrifugation at 2 C for 30 to 40 min in a refrigerated centrifuge at 23,000 × *g*. To purify the crude extract, 1 μmole of ethylenediaminetetraacetate (EDTA) was added per mg of protein in the cell-free extract to remove metal ions; then 15 ml of the extract (containing about 10 mg of protein per ml) was dialyzed for 12 hr against 100 volumes of 0.2 M phosphate cysteine buffer at the *p*H of the original growth medium. CoA was removed from dialyzed extracts by treatment with an anion-exchange resin (acid-washed Dowex 1-X2). One volume of the extract was stirred slowly for 5 min with one-quarter volume of the resin. The *p*H was checked periodically and was maintained between 7 and 7.3 (5.8 to 6.0 for yeast) by dropwise addition of 0.2 M phosphate-cysteine buffer. The resin was sedimented by centrifugation for 3 min at 5,000 × *g*, and the supernatant fluid was removed with a Pasteur pipette.

For experiments with *S. cerevisiae* 522, extracts were prepared by the method of Juni (7) modified as follows. Freshly harvested cells were carefully washed and lyophilized. A 10 g amount of the lyophilized cells was crushed with a mortar and pestle, and the resulting paste was extracted with 40 ml of 0.13 M phosphate buffer, *p*H 5.5. The crude extract (43.5 ml) was cooled to −1 C in an ice-salt bath, and 9 ml of cold (−18 C) 95% ethyl alcohol was added, dropwise, with stirring. A precipitate which formed for 1 hr at −1 C was removed by centrifugation. Then 34.8 ml of cold 95% alcohol was added to the supernatant fluid, and the mixture was allowed to precipitate at −18 C for 12 hr. The precipitate was removed by centrifugation and resuspended in 10 ml of distilled water. Insoluble material, removed from the suspension by centrifugation, was discarded. Finally, the suspension was dialyzed as indicated above, and CoA was removed.

Analytical procedures. The diacetyl and acetoin produced by all organisms except *S. cerevisiae* 299 were separated by salting-out chromatography with a column (1.9 × 27 cm) packed with Dowex 1-X8 anion-exchange resin slurried in 3.0 M $(NH_4)_2SO_4$, as described by Speckman and Collins (19), and were determined quantitatively by the Westerfeld method (22). With *S. cerevisiae* 299, except in the experiment involving radioactive pyruvate or acetyl-CoA, diacetyl and acetoin were determined either by the Westerfeld method after separation with the 1.9 × 27 cm column (Fig. 2) or by the gas chromatographic method of Jennings and Nursten (5; results in Table 1), except that the column was packed with 10% silicone oil SF-9560 (Varian Aerograph, Walnut Creek, Calif.) and operated at 65 C. For the experiment involving radioactive pyruvate or acetyl-CoA (Fig. 3), acetoin and diacetyl were separated with a (3.5 × 54 cm) column packed with Dowex 1-X8 anion-exchange resin slurried in saturated Na_2SO_4, as described by Speckman and Collins (19), and were determined by radioactivity or the Westerfeld test (22). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer with 0.5% 2,5-diphenyloxazole (PPO), 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 5% naphthalene in a total volume of 20 ml of liquid containing 38% toluene, 38% dioxane, and 24% ethyl alcohol. A fraction collector (LKB-Rak, Stockholm, Sweden) was used to collect and distribute effluent during chromatographic separations.

A Beckman spectrophotometer, model DB, was used for all spectrophotometric determinations. *p*H was measured with a Radiometer (Copenhagen, Denmark) *p*H meter, model 22. A Klett-Summerson colorimeter, model 800-3, was used with filter no. 54 for Westerfeld analysis, and with filter no. 29 or 66 for measuring the turbidity of cells during growth. Protein was determined by the biuret method (2), and citrate by the method of Marier and Boulet (12). Decarboxylation was measured by the conventional Warburg manometric technique. Diacetyl reductase activity was measured indirectly by measuring oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) spectrophotometrically at 340 mμ.

Sodium pyruvate, calcium d-pantothenate, CoA, TPP, NAD, $NADH_2$, sodium pyruvate-2-^{14}C (specific activity, 10 mc per mmole), and sodium pyruvate-3-^{14}C (specific activity, 4.6 × 10^4 counts per min per μmole) were obtained from Calbiochem (Los Angeles, Calif.). Acetyl-CoA 1-^{14}C (specific activity, 70 mc per mmole) was obtained from New England Nuclear Corp. (Boston, Mass.). Isotopically labeled acetoin (specific activity, 5.9 × 10^4 counts per min per μmole) was prepared by the action of cell-free extracts of *S. diacetilactis* 18-16 on sodium pyruvate-3-^{14}C (18). Acetyl CoA was synthesized from acetic anhydride and CoA (20). Acetaldehyde, acetoin, and diacetyl were obtained from Eastman Organic Chemicals Co. (Rochester, N.Y.). Acetoin, obtained as the crystalline dimer, was washed with ether until free from diacetyl, as shown by both column and gas chromatography. α-Acetolactate was prepared from its ester (obtained from K & K Laboratories, Jamaica, N.Y.), as described by Speckman and Collins (18), by saponifying with two equivalents of cold 0.025 N NaOH, added slowly to the system held in a water bath at 5 C and continuously purged with a stream of nitrogen.

RESULTS

Production of acetoin or diacetyl and acetoin by growing cultures. Six species of bacteria and three yeast strains reported to produce diacetyl or acetoin were tested for production of the compounds. Each produced acetoin, but five (*S. lactis*, *S. marcescens*, *E. coli*, and *S. cerevisiae* 522 and 513) did not produce detectable diacetyl.

even though one of them (*S. marcescens*) produced large amounts of acetoin. Figure 1 gives results obtained with growing cultures of four of these five. Results with *S. cerevisiae* 513 were similar to those obtained with *S. cerevisiae* 522.

Streptococcus lactis produced acetoin rapidly during the logarithmic growth phase, and converted the acetoin in the growth medium to 2,3-butylene glycol (2,3-butanediol). (A cell-free extract of *S. lactis* was found able to reduce acetoin in the presence of $NADH_2$.) *Serratia marcescens* produced acetoin during and after the logarithmic growth phase, but *E. coli* produced detectable quantities only after the maximal stationary growth phase had been achieved. Apparently these two organisms did not reduce appreciable quantities of acetoin.

Saccharomyces cerevisiae 522 produced more acetoin when the glucose content of YED medium was increased to 20%, and almost 10 times as much in the ASD medium (the medium without yeast extract), though growth in ASD was retarded considerably, and the final cell population was much lower than it was in the more complete medium. Figure 1 includes a comparison of results for *S. cerevisiae* in YED medium (without additional glucose) and ASD medium. The larger amounts of acetoin with additional glucose and in ASD medium possibly reflected, at least in part, differences in the amounts of pyruvate available for acetoin synthesis (4). Detectable diacetyl production by *S. cerevisiae* 522 or 513 was not induced by replacing glucose with maltose, increasing the concentration of glucose up to 40%, or decreasing the *p*H of the medium to 4.65.

P. fluorescens, *A. aerogenes*, *L. brevis* (in orange-lactose broth), and *S. cerevisiae* 299 formed both diacetyl and acetoin. *L. brevis* did not produce detectable quantities of diacetyl or acetoin in tomato-enriched medium, but cell-free extracts of cells harvested from this medium produced both compounds. The uninoculated medium used for comparison in the determinations of acetoin and diacetyl gave discolored controls, and it is possible that small amounts of acetoin and diacetyl were undetected. This medium contained only 0.3% citrate, compared with 1% in the orange-lactose broth. Thus, it seems probable that *L. brevis*, like *S. diacetilactis* (3, 4), does not produce enough excess pyruvate from carbohydrate to result in the production of appreciable acetoin and diacetyl. *P. fluorescens*, *A. aerogenes*, *S. marcescens*, *E. coli*, and *S. cerevisiae*, however, produced acetoin or acetoin and diacetyl in broths that contained no citrate.

S. cerevisiae 299 began producing diacetyl only when the *p*H of the growth medium dropped below 4.9 (Fig. 2). The organism produced both diacetyl and acetoin at 10 and 20 C in YED medium containing 20% glucose (Fig. 2A and Aa) and at 20 C in ASD medium (Fig. 2Ba), but pro-

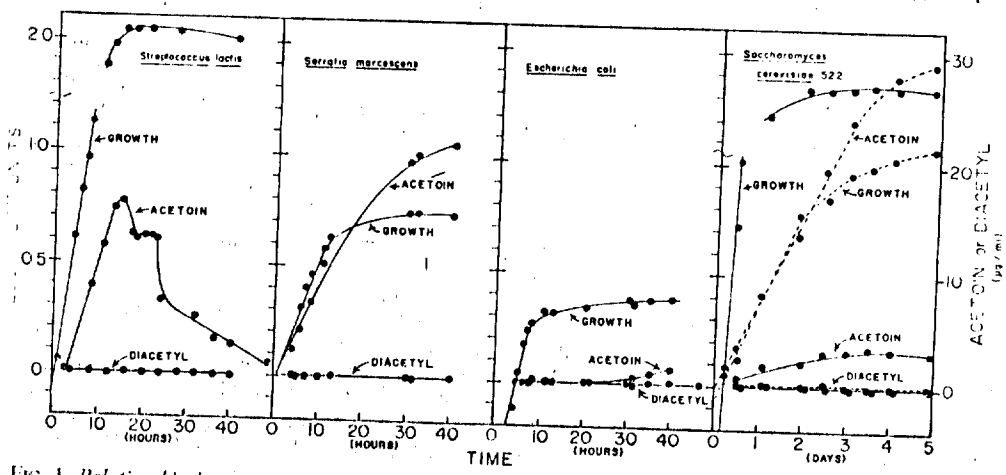

FIG. 1. *Relationship between growth and acetoin production by Streptococcus lactis C_2, Serratia marcescens, Escherichia coli, and Saccharomyces cerevisiae 522. S. cerevisiae was tested in ASD medium (dotted lines) and in YED medium (solid lines). See Materials and Methods for composition of the media. Klett-Summerson filter no. 66 was used for determining the growth of S. cerevisiae, and filter no. 29 was used for the other organisms. Klett units corresponding to 1 mg (dry weight) per ml for S. lactis, S. marcescens, E. coli, and S. cerevisiae were 395, 235, 230, and 210, respectively. For determining these relationships, cells that were growing logarithmically were harvested by centrifugation, washed, and suspended in distilled water; curves relating cell mass (determined after drying to constant weight at 105 C) to Klett units were constructed.*

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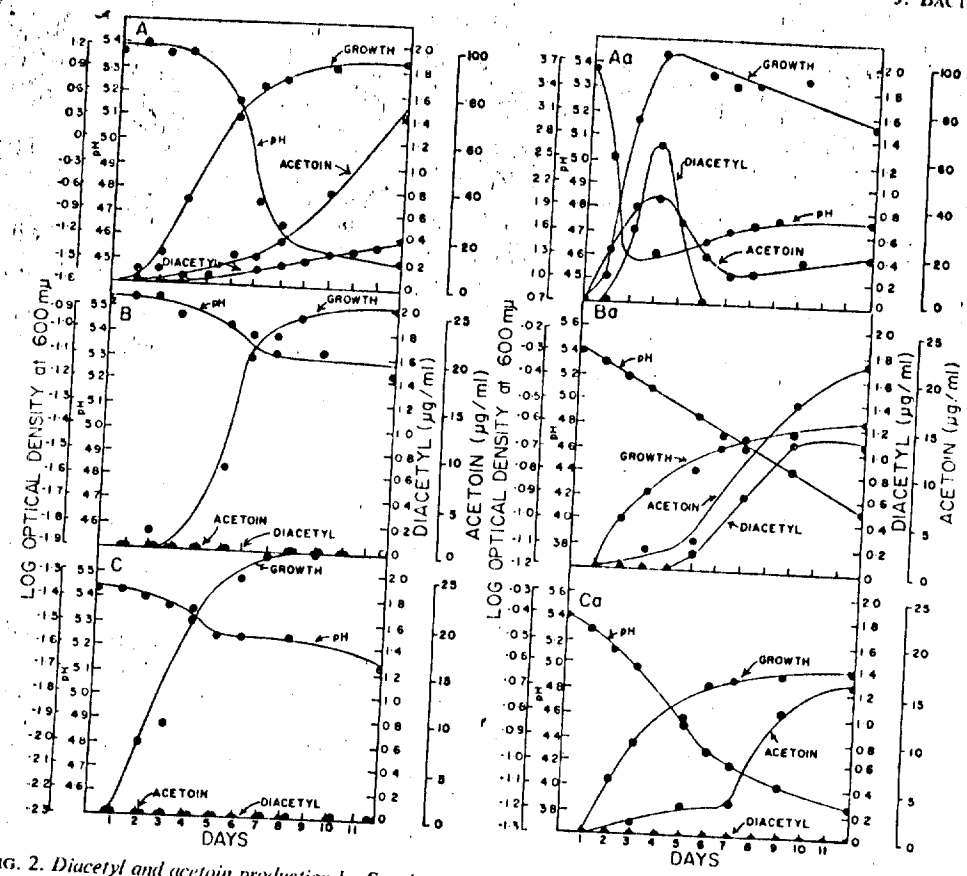

FIG. 2. *Diacetyl and acetoin production by Saccharomyces cerevisiae 299. (A and Aa) YED medium containing 20% glucose at 10 and 20 C, respectively. (B and Ba) ASD medium at 10 and 20 C, respectively. (C and Ca) ASD medium plus 0.1% DL-valine at 10 and 20 C, respectively. One milligram (dry weight) per milliliter of S. cerevisiae 299 corresponded to OD_{600} equals 4.70.*

duced neither compound at 10 C in ASD medium (Fig. 2B). This lack of production probably occurred because the *p*H remained above 4.9 or was related to differences in growth and fermentation that permitted only minimal amounts of excess pyruvate, or resulted from a combination of these conditions. The amounts of diacetyl formed in the media were related to cell population, as previously reported for yeast (15), but the amounts of diacetyl produced per unit of cells were increased by raising the glucose content of the YED medium to 20%, and they were greater in the ASD medium than in the YED medium containing 20% glucose (Fig. 2Aa and Ba).

Influence of DL-*valine, calcium pantothenate, or sodium arsenite on diacetyl formation by S. cerevisiae 299.* The addition of 0.1% DL-valine to ASD medium prevented the formation of diacetyl by *S. cerevisiae* 299, but did not prevent the formation of acetoin (Fig. 2Ba and Ca). Subsequently, we found that pantothenate stimulated the production of diacetyl and arsenite inhibited it. Addition of 0.02% calcium d-pantothenate to YED medium containing 20% glucose resulted in a 62% increase in the diacetyl content at the population maximum of *S. cerevisiae* 299, with a 33% increase in this maximum. Addition of sodium arsenite to give a concentration of 5 mM resulted in a 77% decrease in the diacetyl content, with a corresponding 43% decrease in the population maximum.

Production of acetoin or acetoin and diacetyl by cell-free extracts. The production of acetoin, or acetoin and diacetyl, by cell-free extracts of the organisms confirmed the results that had been obtained with growing cultures. Table 1 gives selected data for four organisms. Each of the

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TABLE 1. *Production of acetoin or diacetyl and acetoin by cell-free extracts of bacteria and yeast*[a, b]

Organism	Substrate					Products	
	Pyruvate	α-Acetolactate	Acetyl-CoA	Acetaldehyde	Acetoin	Acetoin	Diacetyl
	μmoles	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*
Lactobacillus brevis	25.0	—	—	—	—	3.82	0
	—	—	—	—	1.9	1.82	0
	25.0	—	10.0	—	—	1.02	1.78
Escherichia coli	25.0	—	—	—	—	0.44	0
	—	—	—	—	11.4	8.62	0
	—	10.0	—	—	—	0.02	0
	25.0	—	12.5	—	—	0.91	0
	12.5	—	12.5	12.7	—	4.96	0
Saccharomyces cerevisiae 513	50.0	—	—	—	—	2.35	0
	—	10.0	—	—	—	0	0
	100.0	—	25.0	—	—	5.69	0
	50.0	—	—	50.0	—	5.45	
S. cerevisiae 299	27.2	—	—	—	—	Trace	0
	—	—	—	—	11.4	5.43	0
	—	10.0	—	—	—	0	0
	27.2	—	—	22.7	—	Trace	0
	27.2	—	25.0	—	—	Trace	0.14
	27.2[c]	—	—	—	—	1.01	0
	—[c]	10.0	—	—	—	0	0

[a] Reaction conditions and cofactors were as follows. *L. brevis*: Mg^{++}, 22.5 μmoles; TPP, 0.105 μmole; extract protein, 3.3 mg; 0.2 M phosphate buffer; *p*H 5.3; temperature, 30 C; total volume, 5.0 ml; reaction time, 16 hr. *E. coli*: Mg^{++}, 22.5 μmoles; TPP, 0.105 μmole; extract protein, 9 mg; 0.2 M phosphate buffer, *p*H 6.7; temperature, 37 C; total volume, 5.0 ml; reaction time, 17 hr. *S. cerevisiae* 513: Mg^{++}, 22.5 μmoles; TPP, 0.105 μmole; extract protein, 3.5 mg; 0.066 M acetate buffer; *p*H 5.5; temperature, 21 C; total volume, 6.0 ml; reaction time, 16 hr. *S. cerevisiae* 299: extracts prepared from cells grown on YED medium containing 20% glucose; Mg^{++}, 45 μmoles; TPP, 0.21 μmole; extract protein, 5.4 mg; 0.1 M succinate buffer; *p*H 4.65; temperature, 27 C; total volume, 7.0 ml; reaction time, 7 hr.

[b] Reaction times for maximal production of acetoin or diacetyl and acetoin were determined experimentally. Reaction mixtures containing boiled extract were used as controls.

[c] Phosphate buffer (0.05 M, *p*H 5.8) replaced succinate buffer.

extracts required TPP and Mg^{++} for the production of acetoin from pyruvate. The bacteria, except *E. coli*, produced acetoin from pyruvate or α-acetolactate; *E. coli* and yeast did not decarboxylate α-acetolactate, and acetaldehyde stimulated their production of acetoin from pyruvate. Cell-free extracts of those organisms that had been found to produce diacetyl as growing cultures (*P. fluorescens*, *A. aerogenes*, *L. brevis*, *S. cerevisiae* 299) were found to produce diacetyl. However, they did not produce diacetyl from acetoin or α-acetolactate, with or without the addition of NAD. They produced diacetyl from pyruvate, but only if acetyl-CoA was included in the reaction mixtures. Extracts of the five organisms that had been found unable to produce diacetyl as growing cultures did not produce diacetyl, with or without the inclusion of acetyl-CoA in the reaction mixtures.

The synthesis of diacetyl by a cell-free extract of *S. cerevisiae* 299 was studied with radioactive pyruvate and radioactive acetyl-CoA. The results in Fig. 3 show that the extract formed acetoin from pyruvate. However, it did not incorporate acetyl-CoA into acetoin or convert acetoin to diacetyl. Acetyl-CoA was incorporated into diacetyl, and the extract formed diacetyl from pyruvate only if acetyl-CoA was included in the reaction mixture.

DISCUSSION

The primary purpose of this investigation was to determine whether the mechanism described by Speckman and Collins for diacetyl formation by *S. diacetilactis* and *L. citrovorum* (18) is used by other microorganisms. Yeast seemed particularly important. Simultaneously, the study gave information verifying the formation of acetoin

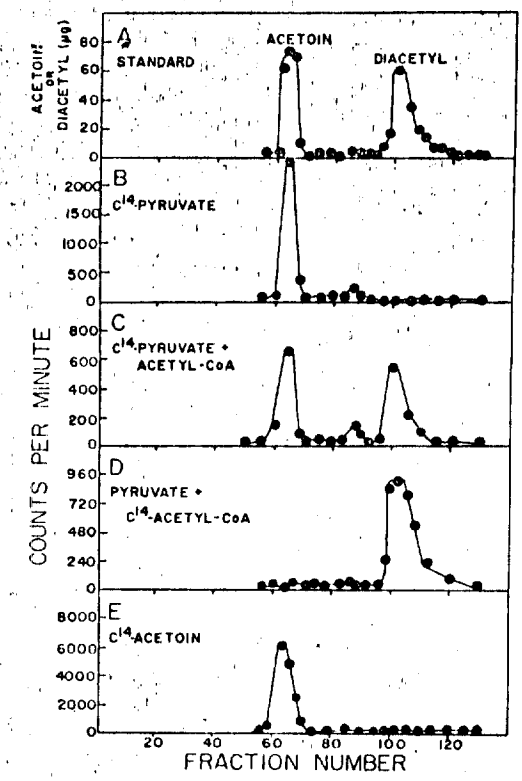

FIG. 3. *Incorporation of labeled pyruvate or labeled acetyl-CoA into diacetyl by dialyzed, resin-treated cell-free extracts of Saccharomyces cerevisiae 299. The standard elution graph (A) was determined with a mixture containing 100 µg each of acetoin and diacetyl. Reaction mixtures contained TPP, 0.21 µmole; Mg^{++}, 45 µmoles; extract protein, 8 mg; succinate buffer, pH 4.6; plus: (B) sodium pyruvate-2-^{14}C, 27.3 µmoles (8.05×10^4 counts per min per µmole); (C) sodium pyruvate-2-^{14}C, 27.3 µmoles (8.05×10^4 counts per min per µmole), and acetyl-CoA, 10 µmoles; (D) sodium pyruvate, 27.3 µmoles, and acetyl-CoA-1-^{14}C, 10.0 µmoles (21.0×10^4 counts per min per µmole); (E) acetoin-^{14}C, 11.5 µmoles (3.5×10^4 counts per min per µmole), and NAD, 10.0 µmoles. Total volume, 6.0 ml; temperature, 27 C; reaction time, 7 hr.*

by previously described mechanisms (6, 7, 18). Yeast did not convert α-acetolactate to acetoin by the mechanism normally used in bacteria (6). The production of acetoin by cell-free extracts of the yeast was stimulated by the addition of acetaldehyde, as would be expected from the work of Juni (7), which indicates that yeast and mammalian tissues form acetoin by condensation of free acetaldehyde with the acetaldehyde-TPP complex formed from pyruvate. The results with bacterial extracts, except that of *E. coli*, were consistent with the mechanism reported for bacteria by Juni (6), i.e., the formation of acetoin by decarboxylation of α-acetolactate formed from pyruvate. *Escherichia coli*, however, appears to use the mechanism normally used by yeast and mammalian tissues. Acetaldehyde stimulated the production of acetoin by cell-free extracts of this organism, and the extracts were unable to decarboxylate α-acetolactate. The minute amounts of acetoin that were formed in two of the experiments to test decarboxylation of α-acetolactate by *E. coli* are attributed to nonenzymatic decarboxylation during the 17-hr reaction (15). Additionally, cell-free extracts of each of the organisms that produced diacetyl were found to oxidize $NADH_2$, indicating the presence of acetyl reductase.

Dialyzed, resin-treated cell-free extracts of each of the four organisms that produced diacetyl from pyruvate produced it only if acetyl-CoA was included in the reaction mixtures, suggesting that all diacetyl-producing microorganisms probably use the mechanism found by Speckman and Collins (18). The involvement of acetyl-CoA in the formation of diacetyl by *S. cerevisiae* 299 was substantiated in experiments with radioactive pyruvate and radioactive acetyl CoA.

The new mechanism for diacetyl synthesis, and the failure of both growing cultures and cell-free extracts of several acetoin-producing organisms to produce diacetyl, place in question the results of a large number of published papers in which the sum of acetoin and diacetyl has been considered to indicate the production of diacetyl. The new mechanism is contrary to the previous assumption that bacteria (9, 17) and yeast (10, 16, 21) form diacetyl by oxidation of acetoin. In yeast, the inhibition of diacetyl by valine incorporated into the growth medium (13, 15) has been considered to substantiate α-acetolactate and acetoin as intermediates in the biosynthesis of diacetyl, since α-acetolactate is an intermediate in the biosynthesis of valine and, in some microorganisms, acetoin. *A. aerogenes* has been shown to have two enzymes that catalyze the formation of α-acetolactate, and valine has been found to inhibit one of the enzymes, leaving enough activity to produce sufficient α-acetolactate to meet the demands for growth without wasteful formation of valine (23).

At least some of those organisms that are able to use α-acetolactate for forming pantothenic acid or valine, or both, are provided with a means of forming acetoin that does not involve α-acetolactate. Failure of valine to prevent the formation of acetoin by *S. cerevisiae* 299 is consistent with results showing that in this organism α-acetolactate does not serve as an intermediate in the

synthesis of acetoin. The inhibition of diacetyl formation by sodium arsenite, and the stimulation by pantothenic acid, substantiate the role of acetyl-CoA in the synthesis of diacetyl. Also, they indicate that involvement of α-acetolactate in the synthesis of diacetyl is determined by whether the organism in question is able to synthesize pantothenic acid.

The mechanism by which valine prevents the formation of diacetyl is not clear. One could suppose that inhibition of valine synthesis by feedback inhibition would suppress the formation of α-ketoisovaleric acid. Consequently, since α-ketoisovaleric acid also is an intermediate in the formation of pantothenic acid, formation of the latter would be suppressed. This, in turn, would suppress the formation of acetyl-CoA, and subsequently the formation of diacetyl, since pantothenate is a constituent of CoA. However, transamination of amino acids by yeast (1) is common, and one would expect *S. cerevisiae* to make sufficient α-ketoisovaleric acid by transamination of the valine added to the medium. *S. cerevisiae* 299 apparently has a partial requirement for pantothenic acid, since it was stimulated by calcium d-pantothenate. Possibly the amount of pantothenic acid formed from valine by the organism at low *p*H is small, the added valine causes enhancement of some of the organism's needs for CoA, and there is little excess CoA that can be directed to diacetyl synthesis.

ACKNOWLEDGMENT

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STARTER DISTILLATE

The original experimental work on the product was carried out the University of Illinois Dairy School by Dr. H. A. Ruehe in 1934.

The object of this experiment was to develop a method whereby the manufacturer could develop and maintain an uniform starter flavor in butter, improve the keeping quality by doing away with live bacteria in the starter and eliminate the possibility of disease organisms being carried from the starter to the butter, as has been the case in both butter and cheese.

The results of this experiment were reported to the American Butter Institute and published in the annual report of the research committee, November, 1937, as follows:

The average scores of the butter stored at 40°F., for two months were as follows:

Lot		Fresh	After 2 months at 40°F.
1.	Control (2.5% starter)	90.12	89.87
2.	Distilled starter	90.25	89.62
3.	Distilled starter	90.37	89.56
4.	Distilled starter	90.12	89.50

The average scores for the same butter stored at 10°F.

Lot		Fresh	After 2 mo.	After 4 mo.	After 6 mo.	After 8 mo
1.	Control	90.12	90.00	89.00	89.67	89.15
2.	Distilled Start	90.25	90.62	90.00	89.75	89.35
3.	Dist. starter	90.37	90.25	89.83	89.67	89.85
4.	Dist. starter	90.12	89.87	89.83	89.67	89.75

In this experiment the butter to which distilled starter had been added, scored approximately the same as the control when fresh, but had a better keeping quality after storage.

Further work in the development of butter starter cultures for distilled starter was carried on at Iowa State College and reported by Hoecker and Hammer in the Iowa State College research bulletins, numbers 205 and 290, 1941. Their conclusions were that a better quality in butter, cheese and buttermilk could be maintained when several strain of lactic acid andbacteria were used in starters, other than S. Lactis which is in general use by plants carrying their own starter.

The technical control needed for the propagation and culturing of these various strains requires quite an expensive outlay of equipment and necessitates the employment of a dairy bacteriologist. The expense of maintaining such a laboratory is beyond the means of most dairy plants, thus making it very hard for the smaller plants to compete on a quality basis with the big dairy companies.

The use of starter distillate affords all operators, regardless of size, the advantage of using the best of starters at a cost suitable to their operations.

The proceedure for making starter distillate is a follows:

A vat of milk is pasteurized at 180°F. for 30 minutes and cooled to 73°F. One strain of lactic acid starter culture is added. After 24 hours, two other strains of lactic acid cultures are added and held for 48 hours. This cultured milk is then pumped into a glass-lined enclosed vat and heated by direct steam. As the temperature reached 210°F., the curd settles to the bottom. The volatile acids and part of the water from the milk are carried by steam distillation through a water-cooled condenser. The resultant product is in a liquid state. 0327

The distillate is next checked for volatile acid and diacetyl content then standardized by the addition of sterile water. It is packed in sterile containers.

As for the composition of ~~butter starter distillate~~, reference is made to the ingredients of the original cultured milk. An excellent authority of information on cultured milk is Dr. Hugo H. Sommer's book MARKET MILK AND RELATED PRODUCTS. Dr. Sommer at the time for publication, was a Professor of Dairy Industry, University of Wisconsin, Madison, Wisconsin.

"Starter Culture. The starter culture which is to be used in making cultured buttermilk and cultured cream should be selected for its ability to produce the desired flavor, and the desired viscosity. A starter should be propagated under aseptic conditions in sterile milk. As a result the commercial starter cultures are frequently called pure culture starters. However, this is not meant in its strict bacteriological sense, which would mean that the culture contains only one species, type or strain of micro-organisms. This is not the desired condition in starter cultures.

"Good commercial starter cultures contain S. lactis and either one or both, S. citrovorous and S. paracitrovorous. Any one of these organisms grown alone in a pure culture will not produce as high a flavor and aroma as is produced by the mixed fermentation which the commercial starter represents. In this mixed fermentation one organism ferments the by-products of the others with the result that the associated growth produces results which the organisms individually are incapable of producing. It is known that citrates and lactates are fermented in starter cultures.

"Lactic acid is the main fermentation product; it has a sour taste, but not a sour odor because lactic acid is not volatile. The characteristic aroma of a good starter must, therefore, be due to other products. Such fermentation products as the following are known to be formed, some in traces, others in appreciable amounts: acetic acid, propionic acid, formic acid, butyric acid (mere trace), ethyl alcohol, acetaldehyde, acetone, diacetyl and methyl acetyl carbinol. The latter two products are especially important in butter flavors. All of these products are volatile and contribute to the aroma. The first four products are acids, and since they constitute the greater part of the volatile products, the starters are frequently analyzed for their volatile acidity to obtain an indication of their flavor and aroma producing qualities."[1]

The ingredients of starter distillate would be the same as the cultured milk. However, there is one exception as the result of the distillation process. Only a small portion of the lactic acid is c and the methyl acetyl carbinol is reduced to diacetyl. 0327

The starter distillate which can be concentrated to any desired degree is added to butter and other products in amounts which equal the flavoring strength of the original cultured milk starter.

At this time, approximately 90 percent of the butter and margarine manufacturers use this method for flavoring their products.

MARKET MILK AND RELATED PRODUCTS, Hugo H. Sommer, Olsen Publishing Co., Milwaukee, Wisconsin, 1938. Page536.

Information obtained through Cino Chemical Company from Chumlea's Laboratories, Lebanon, Indiana.

VOLATILE FLAVOURING CONSTITUENTS OF THE PINEAPPLE

I. SOME ESTERS, ALCOHOLS, AND CARBONYL COMPOUNDS

By D. W. Connell*

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Summary

Investigation of the volatile constituents of pineapples received at a Brisbane cannery in the summer of 1961–62 has resulted in the identification of methanol, ethanol, n-propanol, isobutanol, n-pentanol, ethyl acetate, ethyl n-butyrate, methyl isovalerate, methyl n-caproate, ethyl n-caproate, methyl n-caprylate, n-amyl n-caproate, ethyl lactate, methyl β-methylthiolpropionate, ethyl β-methylthiolpropionate, and diacetyl. The presence of at least 12 other constituents was indicated by gas chromatography.

I. Introduction

Since a basic knowledge of the chemistry of pineapple flavour is considered necessary for an insight into problems connected with the harvesting and processing of Queensland pineapples, an investigation of the volatile constituents of the fruit has been undertaken.

The earliest investigation of the volatile constituents of pineapples was carried out by Haagen-Smit *et al.*[1] in California. Juice expressed from trimmed Hawaiian fruit was distilled at 20 mm and subsequent treatment of the distillate yielded a volatile oil. This oil was fractionated at atmospheric pressure into a number of fractions from which derivatives were prepared and the identifications shown in Table 1 were made. A thioether ester, viz. methyl β-methylthiolpropionate, was isolated in later work[2] and positively identified by oxidation to the crystalline sulphone which was identical with a synthetic sample.

Using Hawaiian fruit Mori[3] prepared an anhydrous volatile essence which was examined by gas chromatography. Comparison of retention times on two semipolar liquid phases enabled the identifications shown in Table 1 to be made.

Gawler[4] examined the volatile acid and carbonyl content of canned Malayan juice prepared from skinned and cored whole fruit. Derivatives were prepared from a distillate of the juice and subjected to paper chromatography, subsequent elution of spots and spectral examination revealed the identifications shown in Table 1.

* Food Preservation Research Laboratory, Queensland Department of Agriculture and Stock, Brisbane.

[1] Haagen-Smit, A. J., Kirchner, J. C., Prater, A. N., and Deasy, C. L., *J. Amer. Chem. Soc.*, 1946, **67**, 1646.

[2] Haagen-Smit, A. J., Kirchner, J. G., Prater, A. N., and Deasy, C. L., *J. Amer. Chem. Soc.*, 1946, **67** 1651.

[3] Mori, R., 1963, personal communication.

[4] Gawler, J. H., *J. Sci. Fd. Agric.*, 1962, **13**, 57.

This paper contains an account of work carried out in this laboratory on the 1961–62 summer crop which has led to the identification of the 16 constituents shown in Table 1.

Table 1

IDENTIFIED PINEAPPLE FLAVOURING CONSTITUENTS

Constituents	Haagen-Smit *et al.*[1,2]	Mori[3]	Gawler[4]	Queensland Fruit
Esters				
Methyl acetate		+†	‡	
Ethyl acetate	++++ W & S*	+		++++ S
Ethyl acrylate	+ S			
Methyl n-butyrate		+		
Ethyl n-butyrate		+		+ S
Methyl n-valerate	+ W			
Methyl isovalerate	+ W			+ S
Ethyl lactate				+ S
Ethyl isovalerate	+ S			
Methyl n-caproate		+		++ S
Methyl isocaproate	++ W			
Ethyl n-caproate	+ S	+		+ S
Methyl n-caprylate	+ W	+		+ S
Ethyl n-caprylate		+		
n-Amyl n-caproate				++ S
Methyl β-methylthiolpropionate	++ S			+++ S
Ethyl β-methylthiolpropionate				+ S
Acids				
Acetic	+ S	‡	+	
Alcohols				
Methanol				+ S
Ethanol	++++ S			++++ S
n-Propanol				+ S
Isobutanol				+ S
n-Pentanol				++ S
Carbonyl compounds				
Acetone			+	
Formaldehyde			+	
Acetaldehyde	+ W & S		+	
Diacetyl				+ S
Furfural			+	
Methyl-n-propylketone	+ S			
5-Hydroxy-2-methylfurfural			+	

* W, winter fruit; S, summer fruit.

† The number of crosses after each compound gives an approximation to its contribution to the total mixture. Ethyl acetate and acetaldehyde had approximately the same percentage composition in summer and winter.

‡ Quantitative results were not given by Mori[3] and Gawler.[4]

II. Methods and Results

(a) *Preparation of Volatile Pineapple Constituents*

During the summer of 1961–62, 475 gallons of juice extracted from the skins, cores, and trimmings of fresh fruit at a Brisbane cannery was heated

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TABLE 2

TENTATIVE GAS CHROMATOGRAPHIC IDENTIFICATIONS

Differences in peak areas are due to incorporation and overlapping of peaks

Liquid Phase	Di-2-ethylhexyl Sebacate*			Polypropyleneglycol			Di isodecylphthalate		
Tentative Identification	Peak Number	Retention Time	% Peak Area of Total	Peak Number	Retention Time	% Peak Area of Total	Peak Number	Retention Time	% Peak Area of Total
Methanol	2	1·40	0·6	2	0·60	1·5	2	0·70	0·4
Ethanol	3	1·60	2·7	2	0·70	33·7	3	0·80	1·6
n Propanol	4	2·60	0·9	3	1·20	0·9	5	1·30	1·2
Isobutanol	5	3·40	1·5	4	1·30	0·7	6	1·60	1·2
Methyl isovalerate	7	4·80	0·2	6	1·70	0·4	9	2·40	0·1
Ethyl n butyrate	8	5·30	1·2	7	1·85	0·8	11	2·90	0·4
n Pentanol	9	5·70	5·2	9	2·35	7·8	10	2·60	0·9
Ethyl lactate	11	8·10	0·4	11	3·10	0·5	12	3·70	0·4
Methyl n-caproate	13	10·65	4·2	12	3·40	6·1	13	4·80	5·0
Ethyl n caproate	15	13·90	0·9	14	4·50	1·4	15	6·70	3·5
Methyl β-methylthiodipropionate	18	21·00	34·5	17	7·65	32·0	18	10·80	36·3
Ethyl β methylthiodipropionate	21	29·70	6·2	19	10·20	7·0	21	14·90	7·5
Methyl n-caprylate	22	32·80	1·3	18	8·50	2·5	20	13·50	1·5
n Amyl n caproate	25	46·00	14·0	22	14·50	11·0	26	23·60	15·6

* See Figure 1.

passed through a cyclone under a vacuum. The distillate from this was fractionated at atmospheric pressure and this second distillate extracted with diethyl ether. Vacuum stripping of the juice was considered necessary to minimize the production of heat generated compounds from proteins and sugars, but it was expected that fractionation of the distillate at atmospheric pressure would not result in any considerable chemical changes.

Fractionation of the ethereal extract concentrated the volatiles to a workable amount but the high ethanol and ethyl acetate content necessitated further fractionation at reduced pressure. This final step gave a yield of 1·4 g of high boiling point material, hereafter referred to as "pineapple oil".

(b) *Examination of Volatile Pineapple Constituents*

The pineapple oil was gas chromatographed on three liquid phases at 170°. Recognition of particular compounds on chromatograms from different liquid phases was made by consideration of retention times and identity of peak areas. Comparison of retention times with standard retention times enabled peaks to be tentatively identified (Table 2). Standard retention times were obtained by injecting pure substances under standard gas-chromatographic conditions while use was also made of logarithm retention time *versus* number of carbon atoms plots with an homologous series to obtain retention times of unavailable substances.

Similarly, gas chromatography at 50° permitted the tentative identification of methanol, ethanol, diacetyl, and ethyl acetate in the lower boiling point fractions obtained during the preparation of the pineapple oil. The presence of diacetyl was confirmed by the preparation of a dinitrophenylhydrazone while ethyl acetate was confirmed by the transesterification procedure described below.

(c) *Identification of Esters*

Transesterification was used to indicate which peaks were esters and of those which were methyl, ethyl, or other esters. Transesterifications were carried out with sodium alkoxide in the appropriate alcohol at 100°. The crude products were then injected into the gas chromatograph and the resultant chromatograms carefully compared with the chromatograms of the original material (Table 3). Peaks due to methyl esters were shown in control experiments to be unaffected by transmethylation and largely shifted by transethylation to the position anticipated for the appropriate ethyl ester.

Similarly ethyl esters were recognized by their converse behaviour (Table 3). One higher ester, viz. n-amyl n-caproate, was recognized by its conversion to methyl or ethyl n-caproate by the appropriate alcohol. The areas of the methyl and ethyl n-caproate peaks thus produced were in good agreement with the areas expected.

(d) *Identification of Thioether Esters*

Preparation of methyl β-methylthiodipropionate by a procedure similar described by Haagen-Smit *et al.*[2] yielded the expected methyl ester from which ester was obtained by transesterification. Peaks appeared in chromatogra

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pineapple oil at the appropriate positions for these thioether esters. The transesterification experiments described below revealed appropriate methyl or ethyl groupings. Ready oxidation of the two thioether esters was observed in control experiments with hydrogen peroxide in acetic acid while a similar oxidation carried out on the whole pineapple oil resulted in the removal of the two peaks under consideration (Table 4). Further evidence for the presence of sulphur compound was given by a qualitative test which showed that sulphur was present in the oxidation products.

TABLE 3
TRANSESTERIFICATIONS

Peak Number*	Result: Methanol	Result: Ethanol	Conclusion
2	Large increase	Unaffected	Methanol
3	Unaffected	Large increase	Ethanol
6	Removed	Removed	Ester of higher alcohol
7	Unaffected		Methyl ester
8	Removed	Unaffected	Ethyl ester
10		Unaffected	Ethyl ester
11		Obscured	Ester of alcohol higher than methanol
13	Unaffected	Removed	Methyl ester
15	Removed	Unaffected	Ethyl ester
17	Removed		Ethyl ester
18	Unaffected		Methyl ester
19	Removed	Obscured	Ester of an alcohol higher than methanol
21	Removed	Large increase	Ethyl ester with methyl ester present
22	Unaffected	Removed	Methyl ester
25	Removed	Removed	Ester of a higher alcohol

* See Figure 1.

Calculation from peak areas of the two thioether esters showed these would contribute 6·6% sulphur to the total sulphur in the pineapple oil. This is in fair agreement with an analysis revealing 8·6% sulphur, as other unrecognized sulphur containing compounds may be present in the oil.

(e) *Detection of Carbonyl Compounds*

The pineapple oil was reacted separately with dinitrophenylhydrazine and Girard's P reagent, carbonyl compounds being detected by careful comparison of gas chromatograms of the original oil and the oil after reaction (Table 5). A number of carbonyl compounds were detected but none was identified except diacetyl.

With peaks 12 and 14 (see Fig. 1), the exit gas was tested with Schiff's reagent which revealed a strong positive reaction with peak 12 and a weak reaction with peak 14.

(f) *Identification of Alcohols*

Tentative gas chromatographic identifications were made of a number of alcohols; the hydroxylic nature of these was confirmed using partition between carbon tetra-

TABLE 4
OXIDATION

Peak Number*	Result	Conclusion
1	Increased	Due to release of O_2 from H_2O_2
2	Removed	Probably dissolved in CH_3COOH
3	Removed	
6	Obscured	—
7		—
8		—
12	Removed	Contains readily oxidized group
16		
18		Contains thioether group
21		

* See Figure 1.

TABLE 5
EFFECT OF CARBONYL REAGENTS

Peak Number*	Result: Girard's P	Result: Dinitrophenylhydrazine	Conclusion
2†	Obscured	Reduced	Not a carbonyl
3			
4	Unaffected	Removed	
5		Reduced	
9			
12	Reduced	Reduced	Carbonyl
14	Slightly reduced		
16	Unaffected	Removed	Not a carbonyl
17	Reduced		Carbonyl

* See Figure 1.

† In the case of the dinitrophenylhydrazine treatment, the substances yielding peaks 2, 3, 4, 5, 9, and 16 were apparently dissolved out by the 2N HCl solution.

chloride and propylene glycol[5] (Table 6). This technique while indicating monohydric and polyhydric alcohols will also respond to alcohols of complex function, e.g. ethyl lactate.

[5] Suffis, R., and Dean, D. E., *Analyt. Chem.*, 1962, 34, 480.

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The occurrence of methanol in the pineapple oil after removal of the low boiling point fractions can be attributed to hydrolysis prior to examination.

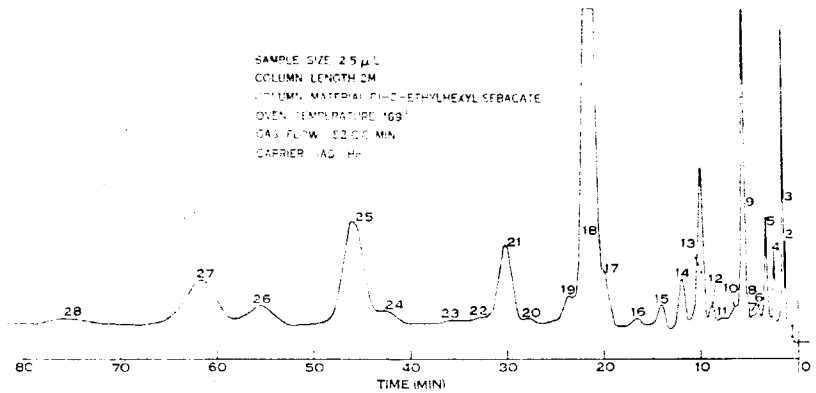

Fig. 1.—Chromatogram of "pineapple oil".

III. Conclusions and Discussion

The full set of results were correlated and compared revealing either a positive identification or useful information as to the groupings present in unidentified compounds (Table 7). A comparison of the identifications thus made with those of previous workers is shown in Table 1.

Table 6

PARTITION BETWEEN CARBON TETRACHLORIDE AND PROPYLENEGLYCOL

Peak Number*	Result		Conclusion
2	Reduced	70% (approx.)	Alcohol
3		80% (approx.)	
4		60% (approx.)	
5	Obscured		
6			
7			
9	Reduced 80% (approx.)		Alcohol
10	Reduced 50% (approx.)		Contains hydroxyl group
11	Reduced		
12	Reduced 80% (approx.)		Alcohol
17	Reduced 50% (approx.)		Contains hydroxyl group
20	Reduced		

* See Figure 1.

With those substances present in largest amounts, viz. ethanol, ethyl acetate, and methyl β-methylthiolpropionate, there is substantial agreement with the work of Haagen-Smit *et al.*[1,2] but with the other constituents there is no obvious pattern of

correlation. The appearance of esters of higher alcohols and free higher alcohols is of interest as these have not been previously detected. The verification of the presence of methyl β-methylthiolpropionate and identification of the corresponding ethyl ester in substantial amounts indicates the importance of these relatively unattractive odorous compounds to pineapple flavour.

Haagen-Smit *et al.*[1] observed a seasonal distribution of esters as being mainly ethyl esters in the summer and methyl esters in the winter but in this case such a distribution was not evident. This could be attributed to climatic and cultural differences.

The presence of acetic acid and furan derivatives in fresh juice as demonstrated by Gawler[4] is doubtful as these may have been produced during processing by the hydrolysis of esters and cyclization of sugars.

IV. Experimental

(*a*) *Preparation of Pineapple Essence.*—The juice used in this study was freshly expressed, at a Brisbane cannery, from the skins, cores, and trimmings of fruit grown in the Yeppoon, North Coast, and Brisbane areas. At the Food Preservation Research Laboratory, Brisbane, it was centrifuged and the volatile flavours were removed from the juice by passing it through the essence stripping section of a fruit juice concentration plant.[6] This consisted of a steam-heated (30 lb in^{-2}) tubular flash heater which discharged into a cyclone which was evacuated via the condenser to 25 mm pressure. The distillate was condensed at 49°F followed by a series of cold traps. It was then fed at 1 l/hr into a continuous fractionation unit (stripping section 1¼ by 23 in. packed with Berl's saddles, rectifying section ¾ by 30 in. vacuum-jacketed and packed with Fenske's [illegible] helices) at atmospheric pressure using a reflux ratio of between 5 and 10. In this manner 1778 l. of fresh juice at 31 p.p.m. gave 347 l. at 82 p.p.m. which resulted in a final concentration of 11·7 l. at 2040 p.p.m. Concentrations are p.p.m. of esters as ethyl acetate estimated using the method developed by Thompson.[7] The concentrate was saturated with ammonium sulphate and extracted with 70% by volume of diethyl ether which was then dried with anhydrous Na_2SO_4. Fractionation, at atmospheric pressure, was carried out in a Bower Cooke type unit, fractions boiling 68–71°, 71–77·5°, and 77·5–77·9° were collected leaving a residue of 8·4 g which was further fractionated in a semimicro still leaving a residue of 1·4 g of pineapple oil boiling above 27°/25 mm.

(*b*) *Gas Chromatography.*—A Perkin-Elmer model 154D Vapor Fractometer fitted with thermistor bead detector and 2 m by ¼ in. stainless-steel columns packed with polypropyleneglycol, di-2-ethylhexyl sebacate, and di-isodecyl phthalate on kieselguhr was used. Work was carried out at 170° column temperature with helium carrier gas, e.g. Figure 1. Peak areas were obtained by cutting the peaks from the recorder chart and weighing.

(*c*) *Reference Compounds.*—Various esters were prepared by the usual synthetic procedures followed by fractional distillation.

Methyl β-methylthiolpropionate was prepared as follows. A solution of Na (5·7 g) in absolute methanol was used to absorb methyl mercaptan (11·9 g) and then was treated with ethyl bromopropionate (41·4 g) as described by Haagen-Smit *et al.*[2] This was worked up to yield methyl β-methylthiolpropionate: b.p. 84–20 mm (Found: C, 44·8; H, 7·5%. Calc. for $C_5H_{10}O_2S$: C, 44·8; H, 7·6%).

Ethyl β-methylthiolpropionate was obtained, by transesterification of the methyl ester with sodium ethoxide in ethanol, in a substantially pure form as shown by gas chromatography, and showed a retention time relative to methyl β-methylthiolpropionate of 1·41.

[6] Leverington, R. E., *Fd. Tech. Aust.*, 1962, **14**, 594.

[7] Thompson, A. R., *Aust. J. Sci. Res.* A, 1950, **3**, 128

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TABLE 7

RESULTS OF TESTS AND CONCLUSIONS DRAWN THEREFROM

Peak Number*	Gas-Chromatographic Identification	Transesterification	Carbonyl Reactions	Oxidation	Partition	Conclusions
1	Air plus other gases	—	—	—	—	Air plus other gases
2	Methanol	Methanol	—	Methanol	Alcohol	Methanol
3	Ethanol	Ethanol	—	Ethanol		Ethanol
4	n-Propanol	Not an ester		Not readily oxidizable		n-Propanol
5	Isobutanol					Isobutanol
6	—	Ester of a higher alcohol		—	—	Ester of a higher alcohol
7	Methyl isovalerate	Methyl ester	Not a carbonyl	—	—	Methyl isovalerate
8	Ethyl n-butyrate	Ethyl ester		—	Not an alcohol	Ethyl n-butyrate
9	n-Pentanol	Not an ester		Not readily oxidizable	Alcohol	n-Pentanol
10	—	Ethyl ester			Contains hydroxyl group	Ethyl ester of an hydroxy acid
11	Ethyl lactate	Ester of an alcohol higher than MeOH				Ethyl lactate
12	—	Not an ester	Contains aldehydic carbonyl	Contains readily oxidized group	Alcohol	Polyfunctional compound
13	Methyl n-caproate	Methyl ester	Not a carbonyl	Not readily oxidizable	Not an alcohol	Methyl n-caproate
14	—	Not an ester	Possibly aldehydic carbonyl			Aldehyde
15	Ethyl n-caproate	Ethyl ester	Not a carbonyl	Contains readily oxidized group		Ethyl n-caproate
16	—	Not an ester				—
17	—	Ethyl ester	Possibly carbonyl	Not readily oxidiazble	Contains hydroxyl group	Polyfunctional compound

TABLE 7 (*Continued*)

Peak Number*	Gas-Chromatographic Identification	Transesterification	Carbonyl Reactions	Oxidation	Partition	Conclusions
18	Methyl β-methyl-thiolpropionate	Methyl ester	Not a carbonyl	Contains thioether grouping	Not an alcohol	Methyl β-methyl-thiolpropionate
19	—	Ester of an alcohol higher than MeOH		Not readily oxidizable	Contains hydroxyl group	Ester of a higher alcohol
20	—	Not an ester				Alcohol
21	Ethyl β-methylthiol-propionate	Ethyl ester plus large amounts of the methyl ester		Contains thioether grouping	Not an alcohol	Ethyl β-methyl thiolpropionate
22	Methyl n-caprylate	Methyl ester		Not readily oxidizable		Methyl n-caprylate
23	—	Not an ester				—
24	—					—
25	n-Amyl n-caproate	Ester of a higher alcohol and caproic acid				n-Amyl n-caproate
26	—	Not an ester				—
27	—					—
28	—					—

* See Figure 1.

(b) *Transesterification.*—Pineapple oil (20 μl) was sealed with 150 μl of methanolic sodium methoxide solution (0·5 g in 100 ml methanol) in a glass ampoule and heated for 20 min at 100°. The reaction mixture was injected into the gas chromatograph without further treatment. Similar treatment with ethanolic sodium ethoxide yielded the ethyl ester derivatives.

(c) *Oxidation.*—Pineapple oil (20 μl) was shaken vigorously with 100 μl of a solution of equal volumes of acetic acid and 3% hydrogen peroxide for 10 min. After standing, the upper oily layer was injected into the gas chromatograph without further treatment.

(d) *Reactions of Carbonyl Compounds.*—(i) A saturated solution of dinitrophenylhydrazine (in 2N HCl) was added to 20 μl of pineapple oil until the production of a dinitrophenylhydrazone precipitate ceased. The mixture was allowed to stand and the upper oily layer injected into the gas chromatograph without further treatment.

(ii) Girard's P reagent (14 mg) was weighed into a small ampoule to which was added 4 μl acetic acid, 20 μl of pineapple oil, and 30 μl of ethanol. The ampoule was heated for 1 hr at 100°. The contents were then washed with a small quantity of saturated $NaHCO_3$ solution and the resultant upper oily layer was injected into the gas chromatograph.

(iii) The exit gas of the gas chromatograph was bubbled through 70 μl of carbonyl-free methanol as the required peak was eluted from the column. Subsequent testing of the methanol solution with Schiff's reagent revealed aldehydic compounds.

(g) *Partition of Alcohols.*—Pineapple oil (10 μl) which was dissolved in 100 μl of carbon tetrachloride was sealed into an ampoule with 100 μl of propyleneglycol and shaken vigorously for 5 min. The lower carbon tetrachloride layer was injected into the gas chromatograph without further treatment. Peak 5 was obscured by the carbon tetrachloride peak but was partitioned into propyleneglycol from isopentane using a similar technique.

V. Acknowledgments

This investigation forms part of the research programme of the Food Preservation Research Laboratory, Department of Agriculture and Stock, Brisbane. The author is deeply appreciative of the excellent direction and encouragement given him by Dr. M. D. Sutherland of the University of Queensland. The author also wishes to thank the C.O.D. Cannery, Brisbane, for their kind donation of the pineapple juice used, and Mr. R. Morgan for the initial stripping of the essence from the juice.

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INFLUENCE OF AERATION AND SUSPENDED MATERIAL ON HIGHER ALCOHOLS, ACETOIN, AND DIACETYL DURING FERMENTATION[1]

E. A. CROWELL and J. F. GUYMON[2]

Am. J. Enol. Vitiult. 14(4): 214-22, (1963)

During the past few years, much progress has been made on elucidation of the mechanism by which higher alcohols are formed during fermentation (26). Several intermediate steps must still be regarded as postulates, but a preponderance of experimental evidence indicates that during fermentation, aliphatic alcohols with molecular weight greater than that of ethanol are formed, principally from carbohydrate, by pathways common to those for the synthesis of their related amino acids (13). The older concept that the higher alcohols, collectively known as fusel oil, are derived from exogenous amino acids via the Ehrlich mechanism, must be considered as operable only to a minor extent, particularly in a medium such as grape must, which has relatively small amounts of amino acids available (3, 20). Much of the research that has led to new concepts of fusel oil production resulted from studies of the biosynthesis of valine and isoleucine by several microorganisms (2, 16). SentheShanmuganathan (23) and others have suggested that the real significance of fusel oil production is that it is a mechanism whereby yeast utilizes amino acids as a source of nitrogen. This view is supported by the fact, as originally demonstrated by Ehrlich (9), that when a fermenting medium is supplied with an ammoniacal nitrogen source such as ammonium sulfate, fusel oil production is reduced.

A knowledge of the mechanism of fusel oil formation is essential to rational attempts to control the levels formed in wines and, hence, in brandies. The need for practical means of control is illustrated by data of Guymon and Heitz (10) concerning the great variability in the fusel oil content of California wines. For example, 14 samples of chablis-type wine had a minimum of 210 ppm, a maximum of 307 ppm, and a mean of 255 ppm. For red wine, 25 samples of claret-type had 197 ppm minimum, 416 ppm maximum, and 283 ppm mean. In the majority of wine types tested, the range of values found was wide.

In brandy production, the fusel oil content of the product may be partially controlled by proper still operation provided that the operator is aware that an unusually high fusel oil level exists in the distilling material. Generally speaking, an ideal concentration appears to be 700 to 1000 ppm at 100° proof. Also, the brandy aging process may obscure the undesirability of a product high in fusel oil. The odor and flavor of oak extracted from the barrel tend to mask the oppressive properties of higher alcohols, but the aged product will nevertheless be heavy and full-bodied. Since grape musts often produce unexpectedly high levels of fusel oil, means for limiting the levels formed during fermentation are obviously desirable.

There are several conditions that are already known to affect the formation of fusel oil. For example, experiments in these laboratories show that Cabernet wines fermented at controlled temperature formed 365 ppm at 55°F, rose to a maximum of 385 ppm at 75°F, and dropped off to 345 ppm at 85°F. A temperature of 75°F appears to be optimum for other grape varieties, also.

The levels formed are highly dependent upon the strain of yeast used. Peynaud and Guimberteau (20) reported that yeast strain can influence the level by as much as fivefold. Castor and Guymon (5) found similar results when comparing fusel oils formed by three commonly used wine yeasts.

Guymon *et al.* (11) reported a comparison of fusel oil levels formed under near-aerobic and anaerobic conditions of fermentation. They found that aerated fermentations formed as much as five times the amount formed under anaerobic conditions. These results are somewhat contrary to those of Peynaud and Guimberteau (20) who reported that fusel oil production is slightly enhanced by anaerobic conditions. This conflict may arise from a difference in the degree of aerobic conditions used. In addition, Hough and Stevens (12) reported that anaerobic fermentations of a synthetic medium by *Saccharomyces carlsbergensis* formed approximately twice the fusel oil produced under aerobic conditions. Peynaud (20) selected a pH of 4.5 as most favorable for higher alcohol production.

We have observed that wines produced from extremely turbid grape musts invariably have fusel oil in excess of that from clarified juice of the same fruit. Also, wines fermented in contact with the skins for color extraction generally contain higher levels than do white wines.

Guymon *et al.* (11) suggested that this increase in the presence of pulp might be related to aerobic conditions of fermentation. The presence of the solid material was suggested as possibly providing a more active interface at which oxygen could be adsorbed, and hence provide the aerobic conditions more favorable to fusel oil formation. They also suggested, alternatively, that direct amino acid precursors of higher alcohols might be contained in the grape pulp or skins.

This paper describes recent studies to clarify the role of grape solids present during fermentation on fusel oil production.

METHODS AND MATERIALS

Yeast: Except for the experiment reported in table 1, for which a 1% volume of an actively growing culture was used, cells of *Saccharomyces cerevisiae* (Montrachet Strain #522) were pregrown for about 3 days in 1 liter of 10% Fleishmann's Diamalt sterile broth on a rotary shaker at 30°C. The cells were allowed to settle completely, were harvested by decantation and centrifugation, and were washed 3 times with sterile water. For inoculation, the cells were resuspended in a minimum volume of sterile water.

Analysis: Procedures for the determination of ethyl alcohol, reducing sugar, total fusel oil, and fusel oil composition have been described (11).

Diacetyl and acetoin were determined colorimetrically by a method described by Neish (18), which is a derivation of the Voges-Prokauer reaction. A red color develops when diacetyl reacts with a guanidino amino acid group in the presence of alkali. As used, diacetyl, creatine, and 1-naphthol in 2.5N NaOH are used as the reactants. The color from diacetyl forms almost immediately. Acetoin is slowly oxidized to diacetyl in the presence of the alkali. Therefore, the method specifies measurement of the color at 530 mμ after seven minutes for diacetyl and again after one hour, the increase representing the amount of acetoin. 2,3-Butanediol does not interfere.

Our analyses reported for acetoin and diacetyl are of 2:1 distillates of the fermented liquid. Acetoin is only moderately volatile, so that not all is recovered in the distillate. Neish (18) stated that only about 53.5% is recovered in a 2:1 distillate, but with our equipment we have found an 80% recovery of acetoin distilled from a 10% alcohol solution.

RESULTS

Effect of suspended matter and aeration on higher alcohol formation: A quantity of cold storage Emperor grapes was crushed in the usual Garolla-type stemmer-crusher. A portion of the must was lightly pressed to obtain normal "free run" white juice, which was cloudy with a normal amount of suspended colloidal material. The remainder of the must, containing skins and seeds, was run through

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[2] Department of Viticulture and Enology, University of California, Davis.

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TABLE 1

Effect of Suspended Matter and Aeration on Fusel Oil Formation in Emperor Grape Must[a]

Treatment	Ethanol (Vol. %)	Fusel Oil[b]	n-Propyl	Isobutyl	Active Amyl	Isoamyl	Total[c]
		(mg/100 ml of 2:1 distillate)					
Anaerobic-static							
Centrifuged	23.5	50	14	9	8	33	64
Free Run	21.4	51					
Pulped	23.5	62	10	9	12	41	72
Aerobic-shaken							
Centrifuged	21.7	94	14	28	13	61	116
Free Run	18.9	352	10	195	12	145	362
Pulped	20.0	364	15	177	17	170	379

[a] All results are expressed as amounts in 100 ml of the 2:1 distillate, or equal to 200 ml wine.

[b] Exclusive of n-propyl alcohol, colorimetric analysis.

[c] Inclusive of n-propyl alcohol.

pulper to reduce the skins to pulp as much as possible. Essentially only the seeds were eliminated from the pulped must. Then about one-half of the pulped must was centrifuged to remove the suspended solids and give a relatively clear juice.

Each of these three types of must was then divided into 1-liter portions and placed in 2-liter Erlenmeyer flasks. Flasks of each, in duplicate, were then fermented by two procedures: one static (essentially anaerobic) at room temperature, the other with vigorous continuous shaking on a rotary shaker at 25°C.

At the conclusion of fermentation, all samples were distilled (2:1 volumes) and analyzed for ethanol and colorimetrically for total fusel oil. Five of the six distillates were then concentrated by fractional distillation, drying, ether extraction, and solvent stripping for gas chromatographic analyses (6). The analytical data are given in table 1.

The influence of the suspended matter was to enhance the formation of higher alcohols to a very high level in the aerobic-shaken samples. The values of 379 mg of higher alcohols for the pulped sample and 362 for the cloudy free-run sample are about 7 times as much as in normal fermentations (25 to 35 mg per 100 ml of wine or 50 to 70 in the 2:1 distillate). The sample from which the pulp had been centrifuged was increased by aeration, but only to about 2 times the normal. The static or anaerobic samples can be regarded as normal.

As seen in the table, the large increase was composed essentially of isobutyl and isoamyl alcohols, the quantities of *n*-propyl and active amyl alcohol remaining low.

Comparison of fermentation of clarified grape juice with juice containing pulp: Cold-storage Emperor grapes were destemmed and crushed in a small screw-type press. In order to obtain a high amount of suspended matter the discharged solids were passed through the press a second time and the expressed liquid was combined with the first pressing. The must was allowed to settle overnight in the cold. By analysis, the must contained 20.2 g reducing sugar per 100 ml. The slightly turbid supernatant juice was decanted and centrifuged for clarification. The pulpy material remaining after decantation was mixed thoroughly for uniformity.

One-liter samples of the clear juice, the pulpy material, and a mixture containing equal parts of each were inoculated with washed cells of *Saccharomyces cerevisiae* (Montrachet Strain #522). Each sample re-

TABLE 2

Effect of the Presence of Grape Pulp on Fusel Oil Formation in Grape Must Fermentation

Treatment	Sugar Used (g/100 ml)	Ethanol (Vol %)	Fusel oil formed (mg/100 g of sugar used)	Diacetyl (ppm)	Acetoin (ppm)
Anaerobic					
Clear juice	17.5	9.6	120	1.5	8.5
Blend[a]	19.7	10.9	165	1.0	8.5
Pulpy juice	19.9	11.0	184	2.5	6.0
Aerobic		—			
Clear juice	20.1	10.2	21[illegible]	[illegible]	30[illegible]
Blend[a]	20.0	10.0	21[illegible]	[illegible]	37
Pulpy juice	20.0	10.2	230	45.0	27

[a] 50% clear juice + 50% pulpy juice.

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ceived 1/6 of the cells harvested from 1 liter of growth medium. The samples contained in 2-liter Erlenmeyer flasks were fermented at 20°C under anaerobic conditions provided by connecting the flask to a water trap. Aerobic conditions were provided by shaking the flask, covered only by a single milk-filter disk, at 240 rpm on a rotatory shaker.

At the termination of fermentation, the samples were analyzed directly for residual reducing sugar, and in a 2:1 distillate for ethyl alcohol, total fusel oil exclusive of n-propanol, diacetyl, and acetoin. The results are shown in table 2.

Concentrations of fusel oil have been expressed in terms of sugar used, to compensate for differences in the completeness of fermentation. Under anaerobic conditions, the results clearly show that the level of fusel oil formed rose sharply when grape pulp was present in the fermenting medium. Under aerobic conditions the fusel oil formed was markedly enhanced, but the influence of grape pulp was slight. Particularly striking were the amounts of acetoin and diacetyl found in the aerated samples. All aerated samples, including the clear juice, had distinct and rather unpleasant odors, often associated with pomace or pomace wash material.

Effect of grape skins on fusel oil production: Emperor grapes after stem removal were crushed as before. The skin fraction was again pressed, to remove as much liquid as possible, and then divided in half. One portion was washed three times with distilled water. The washed and unwashed skins were added to a medium of 20% glucose in *M*/15 phosphate, as follows: 1) glucose-phosphate medium alone, 2) glucose-phosphate medium plus 100 g washed skins per liter of medium, and 3) glucose-phosphate medium plus 100 g unwashed skins per liter of medium.

One liter of each, in 2-liter Erlenmeyer flasks, was fermented anaerobically and aerobically, as before. The samples were allowed to ferment for seven days and then analyzed. The results are shown in table 3.

Under anaerobic conditions, added grape skins greatly stimulated fusel oil production, but the stimulation did not appear to depend upon nutrients retained on the skins, which could be removed by repeated water washing.

Under oxidative conditions fusel oil levels were again enhanced, and isobutyl alcohol was particularly increased by the presence of grape skins, especially the unwashed skins, for which the fermentation was much more complete. High concentrations of acetoin and diacetyl accumulated, particularly in the flasks containing grape skins.

In addition to the results shown, all aerated samples contained large amounts of acetaldehyde. The washed-skins samples produced 550 ppm, and the unwashed 950 ppm. The lower values of ethyl alcohol for the aerated samples than for corresponding anaerobic samples probably reflect the oxidation of ethyl alcohol to acetaldehyde.

Effect of inert solids: Slightly turbid juice of Emperor grapes was centrifuged for clarification. To this medium was added two inert solids: 1) a coarse grade of Whatman cellulose powder, and 2) U.S.P. potato starch in levels of 20 g of solid to 1 liter of juice. Samples (250 ml) of the control juice, the juice with added solids, and the same juice with added solids but deaerated by applying a vacuum to the medium in the fermentation flask were inoculated with pregrown washed cells of *Saccharomyces cerevisiae* (Montrachet). Each flask received 1/6 of the cells from 5 liters of growth medium. The samples were fermented anaerobically at 20°C. The results are shown in table 4.

These results indicate that even inert solids added to grape must can enhance the amount of fusel oil formed. The observed drop in stimulation when the juice with added solids was deaerated by simply applying a vacuum suggests that air or oxygen contained on the solids may account for the increase. We have consistently found that even partially aerobic conditions increase the levels of higher alcohols.

TABLE 3

Effect of Grape Skins on Fusel Oil Production

Treatment	Sugar Used (g/100 ml)	Ethanol (Vol. %)	Fusel oil formed (mg/100 g sugar used)	(% isobutyl)	Diacetyl (ppm)	Acetoin (ppm)
Anaerobic						
Control	7.5	4.0	140	27.0	0.5	6.2
plus washed skins	10.7	5.1	205	23.0	0.5	3.0
plus unwashed skins	16.0	8.4	203	22.[illegible]	1.0	5.[illegible]
Aerobic						
Control	3.5	0.1	414	24.0	14.0	69.0
plus washed skins	7.5	3.1	522	37.0	225.0	1275.0
plus unwashed skins	15.5	6.2	343	53.0	350.0	2050.0

a Control medium contained 20 g glucose per 100 ml in *M*/15 KH_2PO_4.

TABLE 4

Effect of Inert Solids upon Fusel Oil Production

Solid Added	Fusel oil formed (ppm)	(mg/100 g sugar used)
None	168	95
Cellulose	227	118
Cellulose (deaerated)	185	101
Starch	262	140
Starch (deaerated)	207	112

DISCUSSION

As early as 1941, Dietrich and Klammerth (7) demonstrated that the production of fusel oil could be stimulated by the addition of animal charcoal. The data in table 4 clearly show that inert solid material added to grape juice can stimulate the formation of higher alcohols and that the degree of stimulation varies with the nature of the solid employed. That the deaerated samples showed a drop from the non-deaerated indicates that the added solid does contain sufficient adsorbed oxygen to provide the oxidative conditions favorable to higher alcohol production. It seems likely that adsorbed oxygen accounts for the stimulation of fusel oil formation if excessive amounts of insoluble solids, as pulp or skins, exist in the grape must. The results in tables 2 and 3 explain the large amounts of fusel oil that are separated from the distillation units in wine-industry operations after pomace residue is pulped in a disintegrator and refermented to recover residual sugar as wine spirits by distillation of the fermented slurry.

However, such data cannot account for the variability of the levels formed in similarly clarified juices fermented on different occasions. For example, a comparison of the levels formed by the separate clear-juice control samples fermented anaerobically and recorded in tables 2 and 4 shows that the first clear juice (table 2) formed 120 mg fusel oil per 100 g of sugar used, and the second only 95 (table 4). This represents a significant difference considering that the juice was from grapes of the same source and variety, clarified by similar techniques. There was a difference in the volume of yeast inoculant added to the two samples, with a net result that the first clear juice (Table 2) required 144 hours to ferment whereas the second juice (Table 4) required only 96 hours. One might reason that, during a fermentation prolonged because of insufficient yeast [illegible] lation present, the struggle for survival [illegible] growth by the yeast could be [illegible] by variable [illegible] fusel oil.

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Hough [illegible] (12) consider that, in a [illegible] fusel oil is formed primarily such as [illegible] amino acid[illegible] directly by from the [illegible] the Ehrlich mechanism, but that yeasts growing in grape juice relatively deficient in amino acids need to synthesize amino acids for protein synthesis, and that keto acid precursors formed in excess are transformed into higher alcohols. It must be noted that, during the fermentation of grape musts, fusel oil is derived mostly from carbohydrate, but it is very likely that the level and composition of existing free amino acids is a determining factor in the final level of fusel oil. This suggestion is consistent with work of Thorne (25) on the diversity of nutrient values of individual amino acids and mixtures thereof, and data of Castor (3) on the variation of amino acid composition in different grape varieties.

The results for diacetyl and acetoin (acetylmethylcarbinol) are included owing to their suggested presence from the pronounced odor of the aerated fermentation media. These compounds are normally present in trace amounts in wines and other fermented products, acetoin being the key compound, from which diacetyl is formed by direct oxidation, and 2,3-butanediol by reduction.

Ribéreau-Gayon and Peynaud (22) reported levels of 0.4 to 1.8 ppm of diacetyl, 2 to 84 ppm of acetoin, and 492 to 1485 ppm of 2,3-butanediol in French wines. Peynaud and Lafon (21) found higher levels of these compounds in Armagnac brandy (a mean of about 1 ppm of diacetyl, 6 ppm of acetoin, and 24 ppm of 2,3-butanediol at 100° proof) than in Cognac brandy (about 0.8 ppm diacetyl, 2.3 ppm of acetoin, and 3.0 ppm of 2,3-butanediol), which are reasonable results in view of the double-distillation method employed in Cognac production.

The origin of diacetyl in fermented products was once thought to be due entirely to bacterial action, but it now seems clear that yeasts produce acetoin and diacetyl (8, 14, 15, 17, 19). The mechanism of formation is also somewhat controversial. According to Wood (27), in yeast, one molecule of acetaldehyde combines with an acetaldehyde-enzyme complex to form acetoin, in contrast to the bacterial mechanism in which α-acetolactate, resulting from the net combination of two moles of pyruvate, is decarboxylated to acetoin. However, Lewis and Weinhouse (17) demonstrated that yeasts are also capable of transforming pyruvate into α-acetolactate and then acetoin. α-Acetolactate has been shown to be a key intermediate in the synthesis of valine, both by bacteria (2, 16) and by yeasts (17). Valine is the amino acid that yields isobutyl alcohol by the Ehrlich mechanism or that shares common synthetic pathways, via α-ketoisovaleric, with isobutyl alcohol formation (13, 26). α-Keto isovaleric acid is also the intermediate compound condensing with acetate, which leads to α-ketoiscaproic acid and hence to isoamyl alcohol (24).

The significance here is that our results show that conditions which favor acetoin and diacetyl formation, especially aerobic conditions, also result in increased fusel oil formation, particularly isobutyl alcohol and, to a lesser extent, isoamyl alcohol. Whether acetaldehyde plays a key role in the synthesis of acetoin and isobutyl alcohol, or merely that acetaldehyde formation is also favored by the same aerobic conditions that enhance acetoin and isobutyl alcohol, is not entirely clear.

However, it does appear that isobutyl alcohol and acetoin, from which diacetyl is derived, share a common intermediate, α-acetolactate, in yeast fermentation of carbohydrate. It would seem logical to expect to find diacetyl and acetoin in fermentations produced under oxidative conditions.

We have analyzed for these compounds in one sample of a typical baked sherry and four of sherry produced by the "submerged" flor process (1). The difference in levels between the two types was most apparent. The values found were:

Type	*Diacetyl (ppm)*	*Acetoin (ppm)*
Baked	1.8	4.3
Submerged flor	18.0	51.0
Submerged flor	17.5	57.5
Submerged flor	54.0	146.0
Submerged flor	160.0	450.0

Since neither the influence of these acetoinic compounds on sherry character nor their fate during aging has been established, the significance of these results is not clear.

SUMMARY

The formation of higher alcohols or fusel oil during the fermentation of grape musts was greatly increased under vigorously aerobic conditions compared to anaerobic fermentations. In one free-run must the increase was sevenfold, but it was usually about double the amounts formed under anaerobic conditions. Naturally occurring suspended solids also stimulate higher alcohol formation. Isobutyl and isoamyl alcohols accounted for most of the increase resulting from either aerobic conditions or the presence of suspended matter for which the amounts of *n*-propyl and active amyl alcohols were little changed. Both grape skins and inert solids stimulated higher alcohol formation. Evidence was obtained to indicate that the influence of suspended solids was attributable to adsorbed oxygen. Very high levels of acetoin and diacetyl were formed under aerobic conditions, and significant amounts of these compounds were found in some submerged culture "flor" sherries.

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ACETALDEHYDE AND RELATED COMPOUNDS IN FROZEN GREEN PEAS

JEAN JOSEPH DAVID[a] AND M. A. JOSYLN
Department of Food Technology, University of California, Berkeley, California

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The relation of glycolytic changes *(20)*, leading to the accumulation of acetaldehyde and alcohol, to enzyme activity, and off-flavor formation, in unscalded and underscalded frozen vegetable tissue has been pointed out by Joslyn *(12)*.

The separation and identification of acetaldehyde, ethanol, and other volatile carbonyl compounds present in frozen peas and the conditions governing their formation were investigated in some detail by David *(7)* during the period, 1945-1949. The data on the accumulation of aldehydes and alcohol has been reported elsewhere *(13)*. The chromatographic separation of 2,4-dinitrophenylhydrazones of carbonyl compounds occurring in fresh and frozen pea seeds, their characterization by absorption spectroscopy, and related observations are reported here.

MATERIALS AND METHODS

Preparation of material. Freshly harvested Laxton Progress peas which had been frozen and stored since 1937 at —18°C., and various lots of fresh green peas bought locally were used. They were shelled by hand, mixed, washed, scalded in water for 2 minutes or more at various temperatures, cooled and frozen and stored in sealed cans.

Anaerobiosis was usually carried out at room temperature on approximately 400 g. of peas held in a stoppered 500 ml. Erlenmeyer flask under nitrogen for 6 hours. When aldehydes and alcohol alone were to be determined, the peas were surface sterilized by placing them in a 1% solution of mercuric chloride for 10-15 minutes after which they were washed twice with boiled distilled water. When carboxylase activity was to be determined, sterilization was not carried out. No bacterial contamination occurred under these conditions.

The volatile aldehydes and alcohol were obtained by steam distillation of 100 g. of finely ground material, until 400 ml. distillate was obtained.

Pyruvic carboxylase activity. Carboxylase activity was determined on a suspension prepared by blending 100 grams of fresh, partially blanched or frozen peas with 100 ml. of 0.125 M acetate buffer of pH 4.6 for 2½ minutes in a Waring blendor *(25)*. The ground material was then strained through 2 layers of cheese cloth and its pyruvic carboxylase activity was determined immediately, at 29.7°C. and pH 5.06-5.07, using the usual Warburg manometric technique.

Formation and separation of 2,4-dinitrophenylhydrazones (DNPH).[b] The acetaldehyde and other carbonyl compounds were separated from pea tissue by steam distillation, converted to the DNPHs, chromatographically separated first on magnesium sulfate, then on powdered silicic acid, and identified by absorption spectroscopy as was done for apricot extracts *(18, 27)*.

Identification and quantitative determination of acetyl methyl carbinol and diacetyl.[c] The presence of acetyl carbinol (A.M.C.) and diacetyl was tested by a modification of

[a] Present Address: McGill University, MacDonald College, Food Technology Division, Montreal, Canada.

[b] Hereafter in this paper 2,4-dinitrophenylhydrazone will be abbreviated DNPH.

[c] Acetyl methyl carbinol or acetoin is abbreviated to AMC and diacetyl to DA in the text subsequently.

the Voges-Proskauer reaction *(23, 24)*. Acetyl methyl carbinol (A.M.C.) and diacetyl were determined quantitatively by a modification of the colorimetric method of White, *et al.* *(26)*, on an aliquot of steam distillate. Because of the presence of interfering substances, the clear filtrate resulting after addition of trichloroacetic or metaphosphoric acid could not be used. A.M.C. was oxidized before its determination by the procedure of Stotz and Raborg *(19)*. After cooling, 10 ml. of the oxidized mixtures were distilled and the diacetyl thus obtained was determined as above. When mixtures of A.M.C. and diacetyl were analyzed, A.M.C. was calculated as the difference between the oxidized and unoxidized samples.

Separation and identification of ethanol. Interfering carbonyl compounds present in the steam distillate were removed *(8, 9)* and the alcohol present was identified by the iodoform reaction and by the determination of the Duclaux constants of the acid obtained by oxidation with $K_2Cr_2O_7$ and concentrated H_2SO_4 *(17, 22)*.

RESULTS

Occurrence of acetaldehyde in frozen peas. The presence of acetaldehyde in fresh peas, as well as in peas submitted to anaerobiosis was shown by preparing the DNPH of acetaldehyde present in the distillate. The recrystallized DNPH from both preparations melted at a constant temperature of 167°C.[d] The same melting point was found for a preparation of the DNPH of pure acetaldehyde and for mixtures of the known with the unknown.

Supplementary evidence was supplied by a comparison of the absorption spectra of the unknown compound with that of the known derivative. Figure 1 shows that they are identical. A plot of the main band obtained on chromatography of the DNPH derivatives from fresh peas and of that obtained by the same procedure from peas submitted to anaerobiosis also gave curves similar to that of the pure acetaldehyde derivatives as can be seen from Figure 1. A mixed chromatograph of the known and unknown showed them to possess the same affinity for silicic acid since a separation of bands could not be observed.

On chromatographing the DNPHs derived from the carbonyl compounds present in frozen peas, one major component was always observed. Its absorption spectra corresponded closely to that of the acetaldehyde derivative except for slight differences observed occasionally which were probably due to the presence of non-volatile impurities in the eluting solvents. Mixed chromatographs did not show separation. These 2 observations were taken as evidence for the presence of acetaldehyde among the carbonyl compounds found in frozen peas.

Pyruvic carboxylase in peas. The pH optimum of pyruvic carboxylase in plant tissue has been reported to vary from 5.0 to 6.8 *(11, 21, 25)*. The data shown in Figure 2 indicate that for fresh pea tissue it is close to pH 5.15. It was found that a variation of as little as 0.14 of a pH unit produced about a 15% change in net evolution of carbon dioxide.

Shelled peas retain their carboxylase activity for a fairly long period of time when stored at 0°C. The CO_2 production, in μl per g. dry weight of pea seeds in 100 minutes was 1219.4, 1210.3 and 1105.9, respectively, immediately after shelling, after storage for 7 days and after storage for 10 days.

Freezing storage for a short period of time does not affect carboxylase activity. Raw peas stored at —18°C. for 15 days still showed about the same rate of carbon dioxide production as fresh peas. When frozen peas are placed at 0°C., several days after freezing, however, their ability to decarboxylate pyruvate disappears rapidly. Eleven days at 0°C. were sufficient to reduce the carbon dioxide production from 606.5 μl CO_2 per g. dry weight in 30 minutes for fresh peas to 143.7 μl for frozen peas stored at 0°C. The carboxylase activity of the raw peas after 11 years of freezing storage at —18°C., was about half of that of freshly frozen peas. The partially blanched samples did not show carboxylase activity after this long storage period.

Peas blanched at 54.5°C. for 2 minutes produced only 222.4 μl CO_2 per g. dry in 30 minutes as compared with 853.5 μl for the raw peas, while those blanched a

[d] The values reported in the literature on the melting point of acetaldehyde vary from 147-169 because of isomerism *(1, 2, 4, 5, 6)*.

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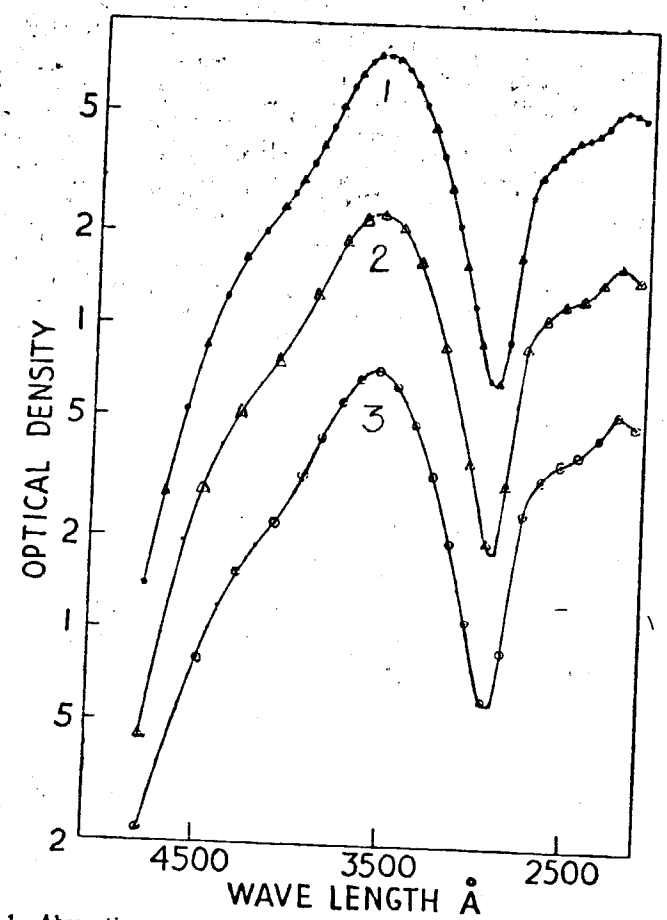

Figure 1. Absorption spectra of dinitrophenyl of pure acetaldehyde (curve 1) and of the acetaldehyde present in fresh pea seeds (curve 2) and of the acetaldehyde in peas held under anaerobic conditions. Solvent 95% ethanol.

produced only 30 μl in that same period of time, and those blanched at 71.1°C. did not produce any. When the peas of this series were allowed to respire in the absence of oxygen, the rate of carbon dioxide production increased for the raw peas and those blanched at 49° and 54.5°C. A decrease was observed for the 60°C. blanch.

An accumulation of aldehydes always occurred when partially blanched peas whose carboxylase activity was not completely destroyed were subjected to anaerobiosis. This increase in aldehyde content apparently is due to the production of acetaldehyde through the action of pyruvic carboxylase. The slight increase found for the peas blanched at 71.1°C. could also be due to the action of carboxylase present in a quantity not detectable by the Warburg technique.

Acetyl methyl carbinol and diacetyl. The bis-DNPH's of A. M. C. and D. A. were prepared, recrystallized from nitrobenzene and washed with alcohol and dried at 100°C. Their melting points were determined and found to be identical, which was expected, since from their structural formulas, an identical bis-DNPH should be obtained. They melted at about 315°C. after turning black around 300°C. This is similar to the reported melting point of the diacetyl bis DNPH *(14)*. Both compounds were practically insoluble in ethyl alcohol. They gave a blue violet color in alkaline alcoholic solution. Their

absorption spectra in chloroform were identical, as can be seen from Figure 3 (curves 1 and 2). A comparison of the spectra of the known derivatives with that of the unknown showed only minor discrepancies in the shorter wave lengths and therefore provided strong evidence in favor of the identity of the two compounds. This was further substantiated by the finding that a mixed chromatograph did not show separation into 2 bands.

The steam distillates from fresh peas and peas submitted to anaerobiosis when tested qualitatively were positive for acetyl methyl carbinol and diacetyl. Assuming that little oxidation of the A.M.C. occurs during the steam distillation, it can be stated that both A.M.C. and D.A. are naturally present in fresh peas. Experimental results on peas respiring in the absence of oxygen lead to the same conclusion.

During the preparation of the bis-DNPH derivatives of A.M.C. and D.A. it was observed that the diacetyl derivative precipitated out readily while that of A.M.C. took a longer time to form. In this last case, the solution always turned to a deep orange color before any cloudiness appeared. The purified derivatives, however, were identical as already mentioned. In order to obtain more complete data on these carbonyl com-

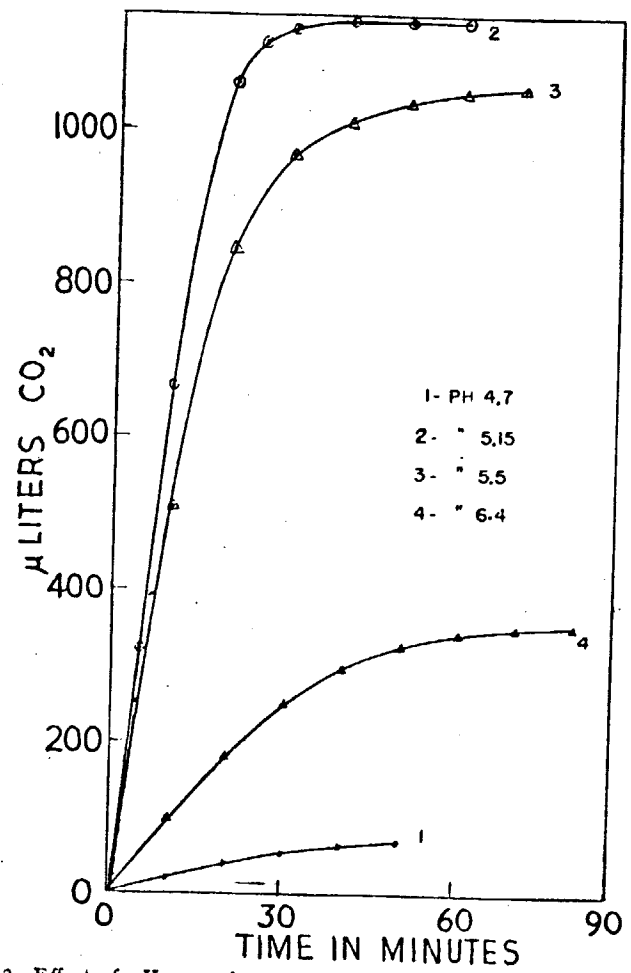

Figure 2. Effect of pH on carboxylase activity of peas. Microliters of CO_2 per gram dry weight of pea tissue in 0.125 M acetate buffer.

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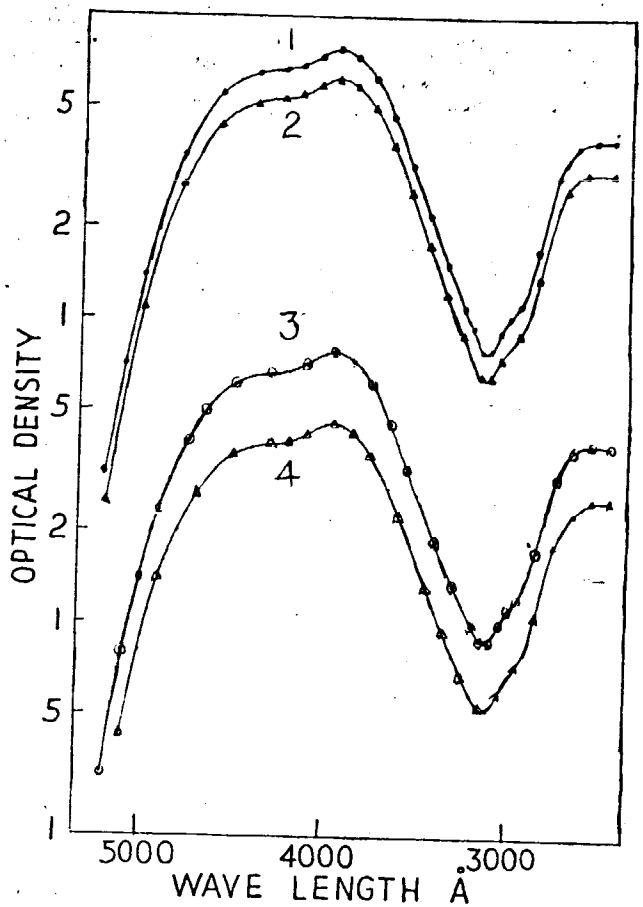

Figure 3. Absorption spectra of the bis-2-4 dinitrophenylhydrazones of acetyl methyl carbinol and diacetyl.

(1) Pure bis hydrazone of diacetyl; (2) pure bis hydrazone of acetyl methyl carbinol; (3) bis hydrazone obtained from fresh peas; (4) bis hydrazone obtained from fresh peas after anaerobiosis for 6 hours. Solvent: chloroform.

pounds, attempts were made to prepare their mono-DNPHs by adding the 2,4-dinitrophenylhydrazine in excess of the amounts required to form mono-derivatives from A.M.C. or D.A. A bright yellow precipitate could always be obtained readily with diacetyl. The A.M.C. preparations behaved very differently. As was the case for the bis-DNPH formation, the reaction mixture turned to an orange color on mixing the reagents and an orange precipitate came down slowly on standing. The diacetyl derivative could be recrystallized from an alcohol water mixture, melted at 175°C., and exhibited a deep red color in alkaline alcoholic solution. Its absorption spectrum, shown in Figure 4, was typical of a mono-DNPH. In comparison, the A.M.C. derivative was almost insoluble in alcohol and had to be recrystallized from nitrobenzene. It gave a blue violet color in alcoholic solution. Its identity with the bis-DNPH of A.M.C. was established by melting point determinations and spectral analysis.

Carboligase activity in peas. As can be seen from Table 1, peas possess a system capable of forming C_4 carbon chains from both pyruvate and acetaldehyde but pyruvate is a better substrate for this synthesis. Our results in which differential analyses were

TABLE 1

A. M. C. and diacetyl formation from added substrate by a pea preparation *

	Diacetyl + A.M.C	Diacetyl	A.M.C.
Peas bought locally November 5, 1948	μg	μg	μg
1. Fresh peas, no substrate	455	425	30
2. Fresh peas + 200 mg. pyruvate	1,895	1,895	20
3. Fresh peas + 200 mg. acetaldehyde	890	525	320
4. Fresh peas + 100 mg. pyruvate + 100 mg. acetaldehyde	882	775	107

*Formation of D.A. and A.M.C. in 100 gm. pea slurry containing 0.1% merthiolate as preservative incubated for 16 hours with pyruvate, acetaldehyde, or mixture of the two.

made indicate that in all the experiments diacetyl was present in larger amounts and that it was the only compound formed from added pyruvate. If acetaldehyde is used as substrate, the total production of the C_4 compounds is decreased to about half but a larger amount of A.M.C. is produced. Pyruvate apparently depresses the production of A.M.C. When both substrates are added together, the total weight being the same as

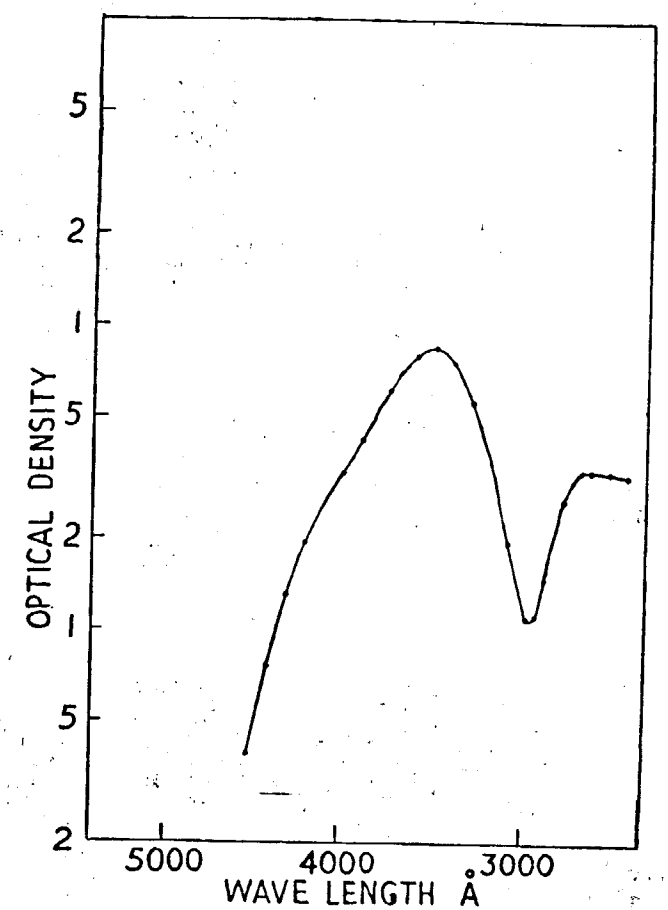

Figure 4. Absorption spectra of mono hydrazone of diacetyl in alcohol.

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when they are used alone, the total amount of C_4 compounds does not vary greatly from that of acetaldehyde alone but the quantity of A.M.C. formed decreases considerably.

When pyruvate alone was used as substrate, only about 1% of it was converted to diacetyl. If A.M.C. originates mostly from acetaldehyde, one could expect a larger amount of it when pyruvate is added since acetaldehyde should be formed by decarboxylation. The pH of the slurry should be around 6.6 and it has been demonstrated in an earlier section that the rate of decarboxylation of added pyruvate is quite slow at pH 6.4. Although formed, acetaldehyde was probably not present in great enough concentration to favor A.M.C. formation. In addition, pyruvate has been found to exert an inhibitive action on the synthesis of A.M.C. from acetaldehyde when both substrates are added simultaneously. In this experiment pyruvate was always present in a much larger concentration than was acetaldehyde.

Occurrence of other carbonyl compounds. Several hydrazones were found to be present in both fresh peas and peas subjected to anaerobiosis. In order of their development on silicic acid after preliminary separation on magnesium sulfate, these were: (1) unknown 1; (2) acetaldehyde hydrazone; (3) unknown 2; (4) diacetyl hydrazone; (5) unknown 3; (6) unknown 4.

Unknown 1, obtained from fresh peas before and after anaerobiosis, and in peas scalded at 60°C. and 71.1°C. and stored for 8 years at −18°C., gave a red color in alkaline solution. It showed a maximum absorption at 3750A and a minimum at 3000A, indicating that the parent carbonyl compound is unsaturated and that it has a general formula of the type $XHN=C-C=C$ *(3, 16)*.

Unknowns 2 and 3 were present in larger amounts in peas subjected to anaerobiosis for 6 hours than in peas respiring in air. The diacetyl derivative appeared between these 2 unknowns on chromatography with silicic acid, using 80% benzene in petroleum ether as the eluant. The general appearance of the spectra of unknown 2 in peas before and after anaerobiosis suggests that they are identical compounds. Likewise, unknown 3 was apparently the same in both cases due to the close resemblance to both spectra and similar affinity for the adsorbent. The general characteristics of unknowns 2 and 3 resemble those of diacetyl bis-DNPH. Their ability to give a blue color in alkaline solution suggests α-dicarbonyl compounds or compounds capable of producing bis-DNPHs in which the two DNPHine molecules are in a 1,2 position.

The blue color in alkaline solution exhibited by unknown 4 and its broad, main band showing a peak of 4400A again indicated a bis-DNPH of the α di-keto type.

Unknowns 2, 3 and 4 were easily soluble in alcohol which was used as the solvent for spectral analysis differing therefore in that respect from the diacetyl derivative.

TABLE 2

Identification of the acid obtained by oxidation of the alcohol present in fresh peas before and after anaerobiosis for 6 hours by the ordinary Duclaux method

Before anaerobiosis	After anaerobiosis for 6 hours	Duclaux no. for acetic acid
6.72	6.80	6.8
7.00	7.15	7.1
7.39	7.42	7.4

TABLE 3

Identification of the acid obtained by the oxidation of the alcohol present in fresh peas submitted to anaerobiosis for 6 hours and in unblanched frozen peas, by a modified Duclaux procedure

Fresh peas after anaerobiosis for 6 hours	Unblanched frozen peas stored at −18°C. 11 yrs.	Duclaux constants for acetic acid
15.4	15.7	15.7
32.4	32.8	32.7
51.1	51.1	51.4
72.2	72.8	72.6

Identification of alcohol in peas. The acid resulting from the oxidation of the steam distillate free from carbonyl compound was tested first by the ordinary Duclaux procedure and then by a modification of this procedure. Tables 2 and 3 give the results obtained by these 2 procedures. They show that ethyl alcohol is apparently the only alcohol present in peas.

SUMMARY

Acetaldehyde was identified in the steam distillate of fresh peas, fresh peas subjected to anaerobiosis, and frozen peas.

The presence of pyruvic carboxylase was demonstrated in peas. It was most active at pH 5.1 and and was not inhibited by freezing. Pea carboxylase was inactivated by heating peas at 60°C. for 2 minutes.

Acetyl methyl carbinol and diacetyl were identified in the steam distillate of fresh peas, fresh peas subjected to anaerobiosis, and frozen peas. In addition, at least 4 other carbonyl compounds were present, but these occurred in quantities too small to permit their identification.

Diacetyl was shown to accumulate in presence of added pyruvate and but little was formed in presence of added acetaldehyde, indicating that diacetyl was formed preferentially from pyruvate.

Acetyl methyl carbinol was formed largely from added acetaldehyde rather than added pyruvate, but in amounts considerably below those of diacetyl.

Ethyl alcohol was the only alcohol present in the steam distillate of peas.

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Volatile Flavor of Strawberry Essence. I. Identification of the Carbonyls and Certain Low Boiling Subtances

51098

K. P. DIMICK AND BENJAMIN MAKOWER

Western Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Albany 10, California

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In aqueous distillates of Marshall strawberry puree, acetaldehyde, acetone, 2-hexenal, biacetyl, methanol, ethanol, and esters containing acetic and n-butyric acids were identified. An oil fraction separated by further distillation of the aqueous essence amounted to about 13% of the total essence and was about one-third free fatty acids: n-caproic, n-valeric, n-butyric, isobutyric, and acetic. The fatty-acid-free oil fraction seemed to contain all of the characteristic strawberry aroma. The amount of carbonyls and the total amount of essence as measured by carbon analysis were not correlated with the flavor potency of the essence, as judged by a taste panel.

A systematic study of the identities and amounts of the volatile flavoring compounds in strawberries is being made at the Western Utilization Research Branch. The purpose is to determine why some varieties of strawberries have more flavor than others. The method consists of identification and measurement of the volatile chemical compounds responsible for the quality, quantity, and potency of flavor. From this knowledge an objective chemical test may be developed for assaying the flavor potency. The volatile substances from fruit are a mixture of organic compounds varying in their amounts, solubility, and volatility. The low concentration of these volatile substances and the instability of the fruit increase the problems of separation and analysis. These organic substances also vary markedly in their flavor intensity.

Previous work by Coppens and Hoejenbos *(2)*, using an ether extract of 445 kg. of strawberry juice, showed the following compounds to be present: acetic acid, cinnamic acid, *n*-caproic acid, ethanol, biacetyl, ethyl acetate, iso-amyl alcohol, a formic ester, a butyric ester (probably ethyl), a caproic ester (ethyl or methyl), acetic esters, a benzoic ester, n-hexanol, ethyl salicylate, borneol, α-terpineol, terpin, and an unidentified acid and ketone. Other constituents reported as probably present were acetyl methyl carbinol, acetophenone, iso-fenchyl alcohol. McGlumphy *(6)* has confirmed acetophenone, biacetyl, cinnamic acid, and acetic acid. In addition he identified *n*-butyraldehyde, cis-terpinol hydrate, and probably ethyl acetoacetate.

MATERIALS AND METHODS

The 4 major steps in the procedure for this investigation are: (A) preparation of a puree or juice of the fruit, (B) removal

of the flavors from the puree or juice by distillation, (C) concentration of the dilute flavor solutions, and (D) performing appropriate analyses of the various constituents.

The equipment used for separating the volatile flavor from the fruit and for concentrating the essence solutions consists of a continuous laboratory vacuum flash evaporator which will remove 50% of the water from a puree or 70% from a juice in a single pass at about 100° F. (37.8° C.), and an all-glass continuous bubble plate distillation column. This equipment was described in detail elsewhere *(3, 4)*. By means of this distillation column up to 2,000-fold essence solutions have been prepared.[a] Since the volatile constituents are organic, the recovery of essence was followed by means of a total carbon analysis of the various distillates *(1)*. Approimately 80% of the total strawberry volatiles are removed when 40% of the water is stripped from a puree, and practically no loss occurs in the concentration step with the still.

An analysis of the carbonyl content of the different varieties of berries was made on the aqueous concentrate mentioned above by a modification of the methods described by Lappin and Clark *(5)* and Neuberg and Strauss *(7)*. This method depends on the formation of the 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives of the carbonyl compounds, and the formation of an intense colored complex when potassium hydroxide is added to an alcoholic solution of these derivatives. Monocarbonyl derivatives form a red color, whereas dicarbonyl derivatives, such as biacetyl 2,4-DNPH, form a blue color.

The procedure is as follows: To each ml. of 40-fold essence is added 0.4 ml. of reagent (500 mg. of DNPH reagent in 50 ml. of 40% sulfuric acid) in a glass-stoppered bottle. This mixture is heated for 20 minutes on a steam bath and then cooled for one hour. The precipitated derivatives are then filtered on a Celite[b] coated sintered-glass funnel. The filter cake is washed with 2 or 3 ml. of water. The monocarbonyl derivatives which are soluble in alcohol are then washed from the funnel with about 20 ml. of hot alcohol and the filtrate is made up to 25 ml. with alcohol. The dicarbonyl derivatives are then washed from the funnel with a minimum of hot nitrobenzene and the filtrate is made up to 15 ml. with nitrobenzene. Nitrobenzene is the only good solvent known for this type of compound. Aliquots of the two filtrates are made up to 9 ml. with ethanol and one ml. of 2 N KOH is added. The intensity of the colors developed are estimated with a Klett-Summerson colorimeter and a 540 mμ (green) filter for the red solutions and a 590 mμ (orange) filter for the blue solutions. The amount of material is estimated from standard curves prepared from stock solutions of acetone and biacetyl 2,4-DNPH derivatives.

For analysis of the individual carbonyls, the alcohol-soluble 2,4-DNPH derivatives were separated with a silicic acid chromatographic column. Petroleum ether (b.p. 60° to 70° C., 140° to 158° F.)-ethyl ether 90/10 was used as the developing solvent. Three bands were separated and the material composing each band was identified as the 2,4-DNPH derivatives of 2-hexenal, acetone, and acetaldehyde. Identification was made on the assumption that with authentic samples there was no lowering of melting point and that ultraviolet absorption spectra and X-ray diffraction patterns were identical. The 2,4-DNPH derivatives of the carbonyls melted as follows: 2-hexenal 293 to 295° F. (145 to 146° C.), acetone 255° F. (124° C.), acetaldehyde 315° F. (157° C.), bis-biacetyl 615° F. (324° C.).

RESULTS AND DISCUSSION

The amounts of total carbonyls, acetone, 2-hexenal, and biacetyl are given in Table 1. The amount of acetaldehyde can be estimated as the difference between the total amount of carbonyls and those listed. There is no correlation between the total or individual carbonyl content of the essence and the flavor intensity.

A striking difference was found in the 2-hexenal content of the essence from frozen and unfrozen whole strawberries (Table 2). This aldehyde, sometimes called "leaf aldehyde," is apparently produced enzymatically by the fruit when pureed, as shown by the fact that either freezing or heating the whole berry will prevent the appearance of this aldehyde in the essence, while heating or freezing the puree from untreated whole berries will not affect the 2-hexenal content. Nye and Spoehr *(8)* have shown this compound to be produced in large amounts in crushed leaves of *Ailanthus glandulosa* (Chinese "tree of heaven"). Heating or crushing the leaves in the absence of oxygen prevented the formation of 2-hexenal. White *(11)* reported its presence in the essence from apple juice. The relative importance of 2-hexenal to the flavor of the fruit is yet to be determined.

TABLE 1

Carbonyl content and flavor intensity of the aqueous essence from various varieties of strawberries, 1948 season

Variety	Carbon	Carbonyls total	P.P.M. in puree			Flavor intensity
			Acetone	Biacetyl	2-hexenal	
Britemore	57	5.65	2.34	.82	trace	455
Sovereign	59	8.47	0.78	.76	trace	472
Marshall	75	2.6	1.56	.51	.038	600
Red Heart	55	19.2	8.55	1.30	trace	880
Corvallis	92	6.75	1.87	.59	trace	1104
President	81	11.9	2.07	1.48	trace	1300
Ettersburg	66	6.1	5.2	1.88	trace	2200

TABLE 2

2-hexenal content p.p.m. in frozen and unfrozen Marshall strawberries

Year	Frozen	Unfrozen
1948	0.038	
1949	0.066	7.2
1950	.....	6.1

On the basis of results thus far, neither total essence as measured by carbon analysis nor carbonyl content seemed related to the flavor strength of the berries. Therefore, it became desirable to investigate other chemical components of the essence. Since freezing was shown to cause alterations, fresh Marshall berries were used for a systematic general analysis of the essence.

Eight hundred pounds of strawberries obtained from the State of Washington were cleaned, stemmed, pureed, and processed immediately. The stripped essence from the puree was concentrated 850-fold. Total-carbon and carbonyl analyses were made on a 50-fold essence, since separation of an oil occurred when the concentration reached about 300-fold.

The major portion of the organic volatiles was separated from the 850-fold product by distillation in a fraction which boiled below 190° F. (88° C.). This fraction contained no typical strawberry aroma. The remainder of the essence was collected with a large amount of water in a fraction boiling between 203° and 212° F. (95° and 100° C.). This fraction contained a

[a] The word fold as used here means essentially the extent of concentration. In this example it would take 2,000 g. of puree to make 1 g. of 2,000-fold essence.

[b] Mention of specific products does not constitute endorsement by the Department of Agriculture over others of a similar nature not mentioned.

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small amount of a water-insoluble oil and possessed a powerful aroma of strawberries. The organic components of this fraction were separated from the water by an isopentane extraction and appeared as a light-yellow oil after removal of the isopentane.

An over-all analysis of the strawberry essence is shown in Table 3. The amount of essence is shown as parts per million of carbon and is also calculated as ethyl alcohol. The amount of the 4 carbonyl compounds mentioned before — acetaldehyde, acetone, 2-hexenal, and biacetyl—is shown, and their percentages of total

TABLE 3
Analysis of fresh Marshall strawberries

Description	P.P.M. in original puree	Percent of total essence
Essence (as carbon)	42	100
Essence (as ethanol)	80	100
Acetaldehyde	4.9	6.1
2-Hexenal	7.2	9.0
Acetone	2.7	3.4
Biacetyl	0.21	0.26
Ethanol	45.5	57.0
Methanol	4.7	5.8
Esters (calc. as ethyl acetate)	9.4	11.7
Acids of esters: acetic, *n*-butyric		
Water Insoluble Oil	7.5	9.5
Free Fatty Acids	3.00	3.7
n-caproic	1.5	1.9
n-valeric	0.8	1.0
n-butyric-isobutyric	0.52	0.7
Acetic	0.12	0.15

essence are listed. Two alcohols, methanol and ethanol, were separated by distillation and measured. They were identified by boiling point and mixed melting point of their 3,5-dinitrobenzoate derivatives, which showed no depression with authentic samples: ethanol 3,5-DNB (m.p. 92° C.) methanol, 3,5-DNB (m.p. 106° C.) As may be seen, ethanol accounts for more than half of the total essence.

The amounts of esters calculated as ethyl acetate were determined by saponification of the low-boiling fraction. Only acetic and *n*-butyric acids were found in this ester group. The organic acids were separated by the chromatographic procedure described by Ramsey and Patterson *(9, 10)* and identified by X-ray diffraction pattern of their crystalline sodium salts. This method of identifying the acids was found to be very satisfactory, provided care was used in drying the samples. All of the sodium salts were dried at 20% relative humidity and were transferred to the capillaries in a dry box. The iso-acids of butyric, valeric, and caproic were found to crystallize with one molecule of water under these conditions.

The highly flavored oil fraction amounting to about 13% of the total essence was about one-third free fatty acids: *n*-caproic, *n*-valeric, *n*-butyric, isobutyric, and acetic. These acids, in the concentration found in the fruit, cannot be detected by taste in distilled water. The remainder of the oil fraction, although amounting to only about 7.5 p.p.m. in the fruit, has such a powerful flavor intensity that the aroma of strawberry can be clearly recognized in a concentration of 0.1 p.p.m. This is about one-tenth of a gram per ton of fruit. The composition of this oil is presently under investigation.

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SUMMARY

Different varieties of strawberries vary in their flavor intensity. The amount of total organic volatiles does not measure the strength of flavor, nor does the carbonyl content seem related to flavor intensity. Freezing or heating the whole fruit reduces the 2-hexenal content of the essence to a trace. In all the volatile organic compounds called essence we have a major portion (90% or greater) which in themselves are not reminiscent of strawberries. The typical strawberry aroma appears to reside in an oil fraction amounting to about 7% of the total essence. It may turn out, however, that the well-balanced flavor will require the presence of these less aromatic constituents. This will have to be determined after a complete analysis of the oil has been made.

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SMOKE

(0454)

Composition of Hickory Sawdust Smoke. Low-Boiling Constituents

ROBERT C. DOERR,
A. E. WASSERMAN, and
WALTER FIDDLER

Meat Laboratory,
Eastern Utilization Research and
Development Division,
Agricultural Research Service,
U. S. Department of Agriculture,
Philadelphia, Pa.

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Thirteen volatile components derived from hickory wood smoke have been separated by gas-liquid chromatography. Acetaldehyde, acetone, methyl formate, methyl acetate methyl alcohol, biacetyl, and acetol were each identified by both gas-liquid chromatographic retention time and infrared spectrum. Carbon monoxide, carbon dioxide, and methane were identified by infrared only, while furan and/or propionaldehyde, 2-methylfuran, isopropyl alcohol, ethanol and/or isovaleraldehyde, methyl vinyl ketone, and crotonaldehyde were identified tentatively by GLC retention time. The formation of methyl acetate and methyl formate and the decrease in concentration of methyl vinyl ketone occur as secondary reactions in the smoke condensate upon standing. The nature and extent of these chemical changes have not been reported previously.

Wood smoke, as used for the processing of foodstuffs, is a complex mixture of many classes of compounds. The authors have reported the identification of a number of phenolic and furyl compounds present in a fraction of the hickory sawdust smoke (*1*). Although others have claimed that the smoky aroma of foodstuffs exposed to smoke is due to the phenols (*5, 11*), volatile components such as acids, alcohols, and carbonyls are present also. These may modify the effect of the phenols in the development of the characteristic aroma.

The acids, from C_1 to C_{10}, have been identified (*2, 1-5, 6*), as well as a number of ketones and aldehydes (*3, 6, 7, 8, 10*). A number of alcohols also have been reported (*3, 7, 12*). However, as observed for the phenols, the number of components reported by most of the previous investigators has been limited, and only a few of the compounds have been identified in more than one smoke preparation. The most extensive study of low boiling smoke components has been that of Hoff and Kapsalopoulou (*6*), who identified 20 compounds by the combined use of a syringe microtechnique—in which classes of compounds were removed from the sample—and gas-liquid chromatography (GLC). Variations in the parameters of producing smoke and the differences in the techniques of isolating and identifying the components make it difficult to extrapolate the results reported in the literature to other smoke preparations. Since studies of meat processing are likely to involve a knowledge of smoke composition and reactions, this laboratory is engaged in the development of reproducible methods of smoke generation which can be used to study the effects of variables such as combustion temperature, air flow, sawdust source, and others on the chemical composition of smoke in order to determine optimum parameters for the most desirable smoke. The data reported in this paper present further information on the composition of controlled, reproducibly generated hickory sawdust smoke consisting of the volatile components present in smoke preparations, as well as some chemical changes which occur upon standing.

Experimental

The smoke used in this study was generated in a laboratory apparatus (Figure 1) in which various parameters of smoke generation could be controlled. The smoke generator was constructed of a borosilicate glass tube 12 inches long by 2¼ inches in diameter and fitted with stainless steel ends. The cylinder was wrapped with a 2-foot × 1-inch, 110-volt, 192-watt flexible heating tape and the rate of heating controlled with a Variac. The sawdust charge was placed in the vertically positioned generator over a 5-inch layer of borosilicate glass wool covering the bottom plate. Three thermocouples were fitted into the apparatus to measure the temperatures at the center and at the periphery of the sawdust bed as well as the temperature of the smoke 1 inch above the sawdust bed. Twenty-gram quantities of hickory sawdust having a moisture content of about 7% were heated to temperatures ranging between 250° and 450° C. Air was passed through the generator at a constant flow of 800 ml. per minute.

The smoke samples were obtained in two ways: Whole smoke condensate was collected by passing the smoke first through a water-cooled condenser and an cooled trap, then through two dry ice acetone traps. The condensate was

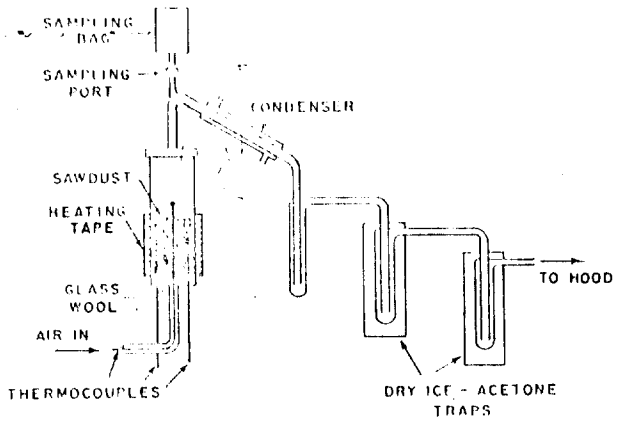

Figure 1. Smoke generator and traps for the production and collection of smoke and smoke condensates

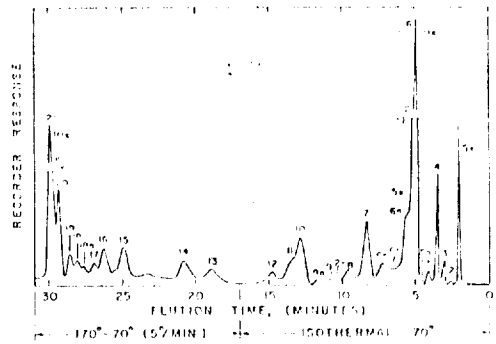

Figure 2. Gas-liquid chromatogram of the volatile components present in hickory sawdust smoke condensate

combined and had a total volume of about 5 ml. Smoke vapor was collected adjacent to the exit port of the generator in a polyethylene bag. The sampling port was approximately 12 inches from the hot reaction zone. About 3 minutes after heavy smoke evolution began, a portion of the smoke was diverted into the bag for a period of 1 minute. The total heating time was 45 minutes. Heavy smoke was visible for about 15 minutes during this period.

The whole smoke and the condensate were subjected to gas-liquid chromatography. Components of the smoke were separated using a Perkin-Elmer Model 800 gas chromatograph with a flame ionization detector and fitted with a 4 to 1 ratio stream splitter. A single column 10½ feet long by ¼ inch in diameter, packed with 30% Carbowax 20M on 60- to 80-mesh Gas Chrom P was used. The operating conditions were: isothermal heating at 70° C. for 17 minutes, then programmed heating at a rate of 5° per minute to 170° C., helium carrier gas flow at 100 ml. per minute and the injection port and detector temperatures at 210° and 200° C., respectively. Sample size used for analysis was 10 μl. of smoke condensate and 5 ml. of whole smoke.

As only one type of column was used for the gas chromatographic studies, the chromatograms of the components were arbitrarily divided into fractions to simplify the analysis of the smoke. Thus, the term "volatile fraction" as used in this paper refers to that portion of the smoke components up to and including the peak that elutes at 30 minutes, which includes virtually all the aliphatic aldehydes, alcohols, and ketones. As this classification is based on the elution time of the components, the authors recognized that with another column and under different operating conditions the composition of their volatile fraction could vary.

Preliminary identification of the various smoke components was made by comparing the retention times with those of known compounds. Many of the individual components were trapped in dry ice chilled capillary tubes that were attached to the exit port of the gas chromatograph. The tubes were rinsed with approximately 4 μl. of carbon tetrachloride and the solutions transferred to 0.1-mm. path length NaCl type D microcavity cells obtained from the Barnes Engineering Co., Stanford, Conn. The infrared spectra were obtained on a Perkin-Elmer Model 421 spectrophotometer fitted with a beam condenser. Several of the low-boiling components of the volatile portion were extremely difficult to trap and transfer at dry ice temperatures. For these compounds, the GLC effluent was led directly into a 7.5-cm. path length stainless steel infrared gas cell equipped with Irtran windows. The cell could be cooled by direct contact with dry ice. Valves on the cell ports permitted the trapping of the desired peak. The cell served both as a trap and an analytical chamber and eliminated sample losses due to transfer operations.

The hickory sawdust used in this work was obtained as such from Koch Supplies, Inc. and is the same as used in commercial smoking operations. Authentic samples of chemicals used for the identification of smoke components were purchased from commercial sources.

Results and Discussion

Component Identification. Figure 2 is a representative chromatogram of the volatile components of whole smoke condensate analyzed 30 minutes after collection. The identification of the components, both positive and tentative, is listed in Table I. Identification of a component is considered positive only when both the GLC elution time and the infrared spectrum agree with those of an authentic sample. The volatile composition of the smoke from successive runs was qualitatively reproducible. The major components of the condensate, based on peak area, are acetaldehyde, acetone, methanol, biacetyl, and acetol. The following were tentatively identified, based on GLC retention time: peak 5, furan and/or propionaldehyde; peak 5a, 2-methylfuran; peak 6a, isopropyl alcohol; and peak 6b, ethanol and/or isovaleraldehyde. Peak 11 was tentatively identified as crotonaldehyde based on retention time and the fact that it formed a hydrazone when bubbled through 2,4-

Table I. Low-Boiling Components of Hickory Smoke

Peak No.	Identity	GLC	IR	Other
A	Carbon monoxide, carbon dioxide, methane		+	
1	Acetaldehyde	+	+	
2	Methyl formate[a]			
3	Furan and/or propionaldehyde	+		
4	Acetone, methyl acetate[a]	+	+	
5				
5a	2-Methylfuran	+		
6	Methanol	+	+	
6a	Isopropyl alcohol	+		
6b	Ethanol and/or Isovaleraldehyde	+		
6c	Methyl vinyl ketone	+		
7	Biacetyl	+	+	Odor
8–10				
11	Crotonaldehyde	+		Positive DNP reaction
12–20				
21	Acetol	+	+	

[a] These two substances form upon standing.

[illegible] compound.

Attempts at trapping the remaining minor components were unsuccessful since these compounds were present in too low a concentration to yield recognizable infrared spectra. To complicate the identification of the minor components further, water (which does not produce a signal with the flame ionization detector) eluted during a large part of the 70° C. isothermal program and was trapped in the capillary collecting tube together with the desired substance. When the tube was rinsed with carbon tetrachloride, distribution of the compound between the water and the solvent could occur, reducing the amount of material available for infrared analysis. This would decrease the infrared absorption intensities to a point where spectral interpretation would become virtually impossible.

While the collection of smoke condensate is a convenient procedure for the study of smoke components, food products are generally exposed to whole smoke during processing. To determine, therefore, whether there were any differences between the composition of whole smoke and the condensate, the whole smoke was collected in a polyethylene bag and a 5.0-ml. volume introduced into the gas chromatograph by means of a gas-tight syringe. Figure 3 is a representative chromatogram of this material. Comparison of the chromatograms of the whole smoke and condensate (Figure 2) reveals several apparent differences. Peak A is a major constituent of the whole smoke but is not present in the condensate. This peak consists of several gases noncondensable at dry ice–acetone temperature at the partial pressures found in wood smoke. The gases comprising peak A were trapped in a 7.5-cm. path length infrared gas cell and the spectrum recorded. Since small molecules in the gas phase yield relatively simple yet extremely characteristic spectra, positive identification of the components of peak A was possible even though several compounds were present together. Substances identified were methane, carbon monoxide, and carbon dioxide. Peak 3 (furan and/or propionaldehyde) is a major component of the whole smoke but is only a minor constituent of the condensate; peak 5a (2-methylfuran) is present also in greater relative concentration in the smoke. On the other hand, acetone (peak 4), methanol (peak 6), and biacetyl (peak 7) are major components of the condensate but not of the whole smoke. The minor components between peaks 10 and 21_3 which appear in the condensate, were not detected in the whole smoke.

Although quantitative calculations from the data are not possible, the differences in relative composition between the whole smoke fraction and the smoke condensate appear to be real.

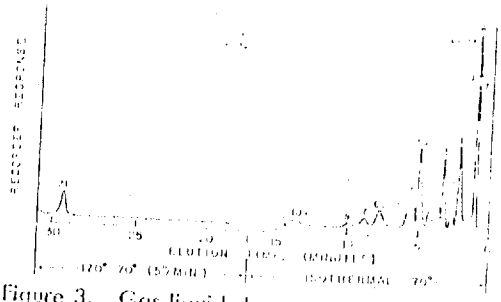

Figure 3. Gas-liquid chromatogram of volatile components present in hickory sawdust smoke

Esterification Studies. During the study of the composition of the low-boiling components of smoke, the condensates were stored at room temperature (25° C.) in tightly stoppered bottles. When they were used after several weeks of storage, some interesting changes had occurred. These changes are shown in Figure 4, *A*, *B*, and *C*.

Figure 4*A* is a chromatogram of the whole smoke condensate 2 hours after generation. Figure 4*B* shows the same condensate after 20 hours storage. There was a considerable increase in the concentration of peak 2. This was trapped subsequently in an infrared gas cell and identified as methyl formate.

After 20 hours of storage, a shoulder appeared on the left side of the acetone peak (arrow on peak 4, Figure 4*B*). After 72 hours (Figure 4*C*), this component had increased in concentration to such an extent that the acetone now appeared only as a shoulder (arrow in Figure 4*C*). Identification of this major component as methyl acetate was accomplished by trapping the eluting peaks in a gas cell and obtaining an infrared spectrum of the mixture. The infrared spectral band characteristics of acetone were subtracted from the spectrum of the mixture. The resultant spectrum was identical to the authentic sample of methyl acetate.

Figure 4 also shows the disappearance of methyl vinyl ketone (peak 6*C*). After 72 hours its disappearance is virtually complete.

A quantitative study was made of the formation of methyl formate and methyl acetate in the whole smoke condensate at 25° C. as a function of time. The values were obtained with GLC, using 10-μl. samples of smoke condensate at intervals over a period of 700 hours. A known amount of *p*-dioxane was added at the start as an internal standard to eliminate variations in sampling technique and gas-liquid chromatographic operating parameters. Results are shown in Figure 5. Molar concentrations of methyl formate and methyl acetate were obtained by injecting known quantities of authentic compounds into the gas chromatograph and calculating the corresponding peak areas. During the first 20-hour period the concentration of peak

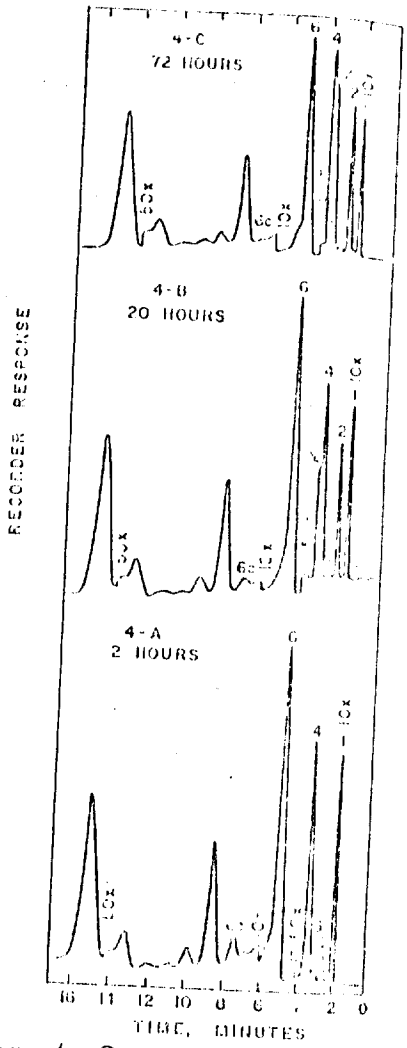

Figure 4. Gas chromatograms of the low-boiling components present in hickory sawdust smoke condensate over a period of time

Curve A, 2 hours; curve B, 20 hours; curve C, 72 hours

4 remained constant, indicating that peak 4 was due entirely to acetone, and no appreciable amount of methyl acetate was present. After 20 hours, peak 4 contained a mixture of methyl acetate and acetone. To obtain the concentration of methyl acetate alone, the concentration of the mixture was corrected for the quantity of acetone present in the

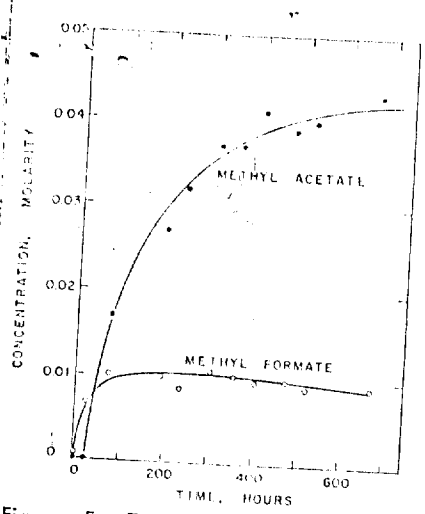

Figure 5. The formation of methyl acetate and methyl formate in whole smoke condensate as a function of time

initial period. Figure 5 also indicates that the rate of formation of methyl formate was extremely rapid and seemed to reach equilibrium after 70 hours. Methyl acetate, on the other hand, continued to form at a significant rate and had a concentration 4½ times that of methyl formate, even after a 700-hour period. Even though trace amounts of methyl acetate and methyl formate may be present in fresh smoke, the major portion results from secondary reactions which occur upon standing.

The formation of methyl acetate and methyl formate was followed in model experiments, using a mixture of methanol, formic acid, and acetic acid. The concentrations of the compounds used in the mixture were the same as those found in the smoke condensate. The quantities of methyl acetate and methyl formate formed and the rates of formation were comparable with those observed in the condensate.

The identification of methyl formate indicated the presence of formic acid in whole smoke condensate. The authors had been unable to detect this acid directly by the use of GLC. Positive identification of formic acid by GLC has not been accomplished because it decomposes on the hot metal inlet system of the apparatus (*9*). However, in other investigations on the composition of wood smoke, the presence of formic acid in substantial quantities has been established by the use of other methods (*4, 5, 7, 8*).

Correlation studies between secondary reaction products and the organoleptic quality of food products treated with the condensate have not been made. The known odor qualities of the esters reported here, however, suggest the possibility that they may contribute to the development of a more mellow aroma of the smoke condensate upon standing.

Acknowledgment

The authors thank E. Wierbicki for the translation of reference *12*.

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Changes in the Volatile Carbonyls of Potato Chips During Storage

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Theodore P. Dornseifer and John J. Powers

Food Technology Department, University of Georgia, Athens, Georgia

SUMMARY

Steam distillate of potato chips was treated with 2,4-dinitrophenylhydrazine reagent, and the resulting derivatives were fused with α-ketoglutaric acid in a closed system. The regenerated carbonyls were separated with gas-liquid chromatography. The following compounds were tentatively identified from retention-time data: ethanal, propenal, 2-propanone, *n*-butanal, 2-pentenal, 2,3-butanedione, 2-hexenal, *n*-heptanal, and 2-heptenal. 2,3-butanedione was positively identified.

INTRODUCTION

Prevention or postponement of off-flavor is of great economic importance to potato-chip processors. Many studies have been reported dealing with the darkening phenomenon (Danehy and Pigman, 1954; Ross, 1948) and with fat decomposition (Carlin *et al.*, 1954; Melnick, 1957), both of which affect flavor, but the identification of flavor components has not been reported. Fats constitute 35-50% of the total weight of potato chips and are of significant importance to flavor. This is demonstrated by regional consumer demand for chips fried in a blend of fat or in cottonseed, corn, or peanut oil (Brown, 1960).

We chose to work with the volatile carbonyl compounds because they have long been recognized as being responsible for the off-flavor of autoxidized or reverted fats and oils (Lea, 1953).

Isolation and identification of the volatile products formed in oxidation or reversion of fats have been the subject of considerable research in the past few years (Buttery, 1961; Chang *et al.*, 1961; Day *et al.*, 1960; Ellis *et al.*, 1961; Forss *et al.*, 1960; Gaddis and Ellis, 1957; Gaddis *et al.*, 1959, 1960; Hoffmann, 1961; Pippen *et al.*, 1957; Yu *et al.*, 1961).

In almost every study the carbonyls were converted to 2,4-dinitrophenylhydrazones (DNPH's), and column or paper chromatography was used for separation. The separations obtained with these techniques are very slow and often incomplete. We therefore used the exchange reaction with α-ketoglutaric acid (Ralls, 1960) to regenerate the original carbonyls, which were then separated by gas-liquid chromatography.

MATERIALS AND METHODS

Fresh potato chips were obtained from the production lines of the Frito Lay Company, Atlanta, Georgia. The packaged chips were held at 2°C in the dark until used.

One series of trials (treatment A) consisted of chips stored 24 hr at 85°C. A second series (treatment B) was stored 3 months at room temperature. Control lots were stored at 2°C in the dark.

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Table 1. Comparison of retention times[a] of known carbonyl compounds with those for carbonyls obtained from fresh and stored potato chips.

Peak	Fresh A	Stored A	Fresh B I	Fresh B II	Stored B I	Stored B II	Pure Compounds	
A	2.5	....	....	2.5	2.5	2.5	Ethanal	2.5
B	2.8	....	....	....	....	....	Propanal	3.1
C	2.9	3.9	....	....	3.9	3.6	Propenal	3.9
D	4.5	....	4.4	4.5	4.5	4.4	2-Propanone	4.2
E	5.2	5.2	....	....	....	....	*n*-Butanal	5.0
F	6.0	6.0	6.4	6.0	6.4	6.4	Ketoglutaric acid[b]	6.1
G	....	8.6	....	....	8.4	....	2-Butanone	6.2
H	9.5	....	9.5	9.4	....	....	2-Pentenal	8.3
I	....	....	11.9	....	11.9	11.7	2,3-Butanedione	9.5
J	11.8	11.9	11.5	11.7	11.8	11.4	2-Hexenal	12.6
K	18.4	18.3	18.0	18.4	18.0	17.7	*n*-Heptanal	15.4
							2-Heptenal	18.4
							2-Heptanone	23.5

[a] Retention times are given in minutes from injection. Column 30% LAC-446 on 60–80-mesh Chromosorb 75°C, argon 12.5 cc/min. Chart speed 12 in./hr.

[b] Probably a decomposition product of the ketoglutaric acid used in regeneration of the carbonyls.

Distillation of volatiles. The apparatus consisted of a 50-quart pressure cooker with "snap-in" fittings for attachment to the vacuum line, 4 hollow-wall glass traps, and a vacuum pump. The two traps immediately preceding the pump were packed with ethanol-solid CO_2; the remaining two traps were packed with crushed ice. Twenty pounds of chips were packed into the cooker, 10 L of distilled water were added, and a vacuum (3 mm Hg) was drawn on the system. A hot plate was used to regulate the rate of distillation (500 ml per hour). Approximately 2 L of distillate was collected from each run.

Preparation of DNPH's. The reagent (2 g of 2,4-dinitrophenylhydrazine per liter of 2*N* HCl) was added to the distillate in the ratio 1:10. The solution was brought to a boil, removed from the heat, and allowed to stand overnight. It was then filtered, and the crystals were washed with distilled water and dried in a vacuum desiccator.

The distillates from all four traps in treatment A were combined before preparation of the derivatives. In treatment B the distillates in the wet ice traps and in the ethanol-solid CO_2 traps were reacted separately.

Regeneration and chromatography. The method of Ralls (1960) was modified in that regeneration was done in 20 cm glass tubing drawn to a closed capillary approximately 3 inches long. The DNPH's and α-ketoglutaric acid (1:3 by weight) were thoroughly mixed, and approximately 1 mg of the mixture was placed in each tube. The wide mouth of the tube was sealed with a rubber septum. Regeneration was brought about by heating the tube for 10 seconds in a hot oil bath (200°C). A 2 ml gas-tight syringe was used to remove vapor from the tube and inject it into the gas chromatograph. The volume of vapor injected could not be measured because it was necessary to flush the gas syringe one time with air prior to injection in order to keep the peaks on scale.

The column was a $\frac{1}{4}$-in.×4 ft column of 30% LAC-446 on 60–80-mesh chromosorb. Column temperature was 75°C, and the carrier gas was argon at a flow rate of 12.5 cc/min. A β-ionization detector was used; radium D was the source.

Infrared spectroscopy. Infrared spectra were determined on a Beckman, Model IR-5 spectrometer, using the KBr pellet technique.

RESULTS AND DISCUSSION

The potato chips used were sampled by ourselves and other workers in the laboratory. The chips stored at 2°C in the dark were considered fresh and acceptable, whereas those stored 24 hr at 85°C and those stored 3 months at room temperature were described variously as rancid, reverted, stale, and/or old.

The distillates obtained had a strong potato-chip aroma that was almost completely destroyed on addition of 2,4-dinitrophenylhydrazine reagent.

Fig. 1 compares fresh (control) and heated chips; Fig. 2 compares fresh (control) and aged chips.

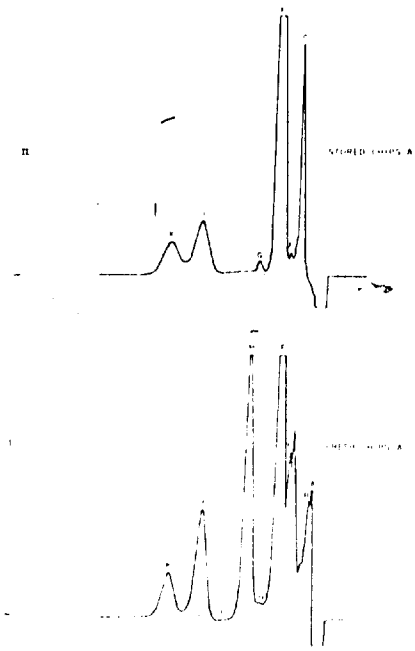

Fig. 1. Chromatograms of heated (stored) potato chips and fresh chips. Column $\frac{1}{4}$-in.×4 ft LAC-446 on 60–80-mesh chromosorb. Argon flow rate 12.5 cc/min, column temp. 75°C.

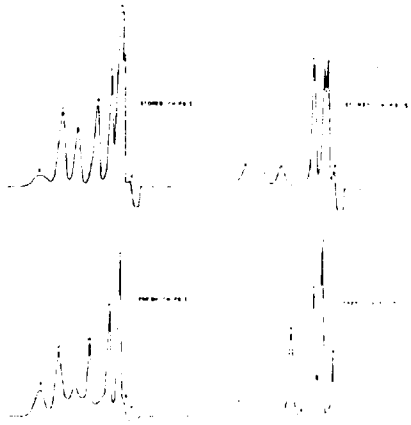

Fig. 2. Chromatogram of chips stored 3 months (upper row) and fresh chips. I refers to ethanol-solid CO_2 trap; II refers to wet ice trap. Column conditions same as in Fig. 1.

From retention time data presented in Table 1 and Fig. 1, we tentatively identified peak A, C, D, E, G, H, I, J, and K as respectively ethanal, propenal, 2-propanone, *n*-butanal, 2-pentenal, 2,3-butanedione, 2-hexenal,

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2-heptanal, and 2-heptenal. From Fig. 4 it should be noted that peak H might also contain 2-pentanone and peak I might contain 2-hexanone.

Ethanal and 2,3-butanedione, which have pleasing aromas, decreased in storage whereas 2-propenal, which has a sharp pungent aroma, increased greatly. We believe these compounds play an important role in the flavor of potato chips.

Flavor compounds differ according to the batch. Propenal and 2-butanal were present in the control lot of treatment A but were lost in storage. 2-hexenal was not present in the chips used in treatment A but was present in those of treatment B and increased during storage.

2,3-Butanedione was positively identified. The derivative was isolated from the fresh chip DNPH's by taking advantage of the insolubility of polycarbonyl DNPH's in hexane. The material was dissolved in hexane and filtered. The residue after washing twice with hexane was dissolved in chloroform and this solution filtered by gravity. An orange band that migrated toward the edge of the paper during filtering was cut out and eluted with chloroform. This material was evaporated to dryness and regenerated. The retention time of the peak corresponded to that of 2,3-butanedione. By repeating the filtering process 10 times, enough material was obtained to prepare a KBr pellet. Fig. 3 shows the infrared spectra of this material and of pure 2,3-butanedione bis DNPH.

It should perhaps be noted that in the presence of excess 2,4-DNPH reagent 2,3-butanedione bis DNPH may be formed from 3-hydroxy-2-butanone DNPH. While the characteristic aroma of 2,3-butanedione could be detected in the steam distillate, it is possible that some 3-hydroxy-2-butanone was also present. It could well be that 3-hydroxy-2-butanone due to its ready autoxidation is the precursor of the 2,3-butanedione.

$$CH_3\text{-}CHOH\text{-}CO\text{-}CH_3 + \tfrac{1}{2}\,O_2 \longrightarrow CH_3\text{-}CO\text{-}CO\text{-}CH_3 + H_2O$$

Peak F is probably a decomposition product of the α-ketoglutaric acid. A peak having the same retention time was noted on every chromatogram and the vapor above pure α-ketoglutaric acid heated to 200°C gave a peak having the same retention time.

Ralls (1960), using a four-filament thermal-conductivity cell detector, found that 2,3-butanedione was not regenerated sufficiently to give a recorded response. Also, he did not record a response for α-ketoglutaric acid. That we have been able to record peaks for these two compounds is no doubt due to the much greater sensitivity of the β-ionization detector.

The identification of 2,3-butanedione is interesting since Chang *et al.* (1961), working with soybean oil in the early stages of reversion, isolated a fraction characterized by a strong "buttery" odor. Also, Daubert and O'Connell (1953) described the first flavor developed from a bland, freshly deodorized soybean oil as "buttery" or "mild beany."

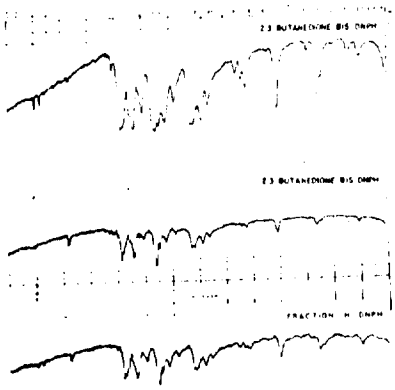

Fig. 3. Infrared spectra of 2,3-butanedione bis DNPH of fraction H. The middle curve shows the pure compound diluted with KBr to correspond with the concentration of fraction H.

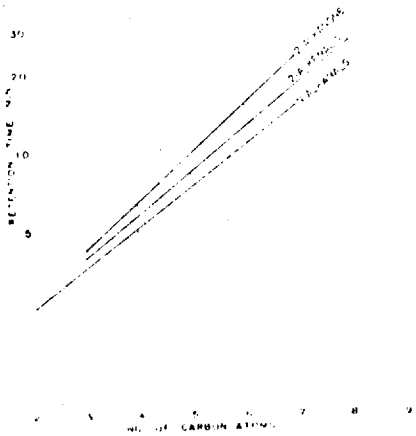

Fig. 4. Plot of retention times of *n*-alkanals, 2-alkanals, and 2-alkanones vs. number of carbons in the molecule.

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0477

German Translation

Gas Chromatographic Determination of Diacetyl and 2,3-Pentanedione in Beer

B. Drews, G. Barwald and H.-J. Niefind (Institute of Fermentation Industry and Biotechnology, Berlin)

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I. Introduction

Two procedures are mainly used for the quantitative determination of the vicinal diketones diacetyl (2,3-butanedione) and 2,3-pentanedione in beer, which are formed as metabolic products in alcoholic fermentation. In the first method, these vicinal diketones are swept out from the beer by a gas stream and determined together in a chemical reaction, e.g. by oxime formation with dimethylglyoxime followed by formation of an Fe(II)-DMG complex, having an absorption peak at 530 nm (1,2). In the second method, the two compounds are determined separately by using thin-layer chromatography (1,3) and gas chromatography (4,5).

For technical brewing plant control purposes, which is primarily concerned with diacetyl (an undesirable product in beer), a simple method for the separate quantitative determination of vicinal diketones would be very advantageous. In this article, a gas-chromatographic method for the separation and quantitative determination of diacetyl and 2,3-pentanedione in beer is described, based on the selective identification of these products by means of an electron capture detector. Further, commercial beers are investigated for their diacetyl and 2,3-pentanedione

content, and the formation and elimination of these products during primary and secondary fermentation in the brewery is checked. The colorimetric method, modified by Drews, Specht, Barwald and Trenel (2), for determining these products is used for comparison purposes.

Method

A. Preliminary Experiments

At first, preliminary experiments were necessary to find out what types of columns and industrial conditions are needed for the separation of diacetyl and 2,3-pentanedione, using gas chromatography and an electron capture detector. For this purpose, test mixtures were prepared from 96% alcohol, with a content of 0 to 0.5 mg/l diacetyl or 2,3-pentanedione. Aqueous samples could not be separated, since water greatly affects the sensitivity of the electron capture detector. Insertion of a precolumn for catching water, e.g. a glycerol column, also proved to be impractical since a portion of the diketones was retained with the water.

Conversion of water to acetylene in a precolumn filled with calcium carbide was also unsuccessful, because the acetylene band in the chromatogram was so large that the diketones, which were eluted later, were completely masked. Thus, both the direct injection of beer and the use of a beer distillate had to be rejected as unsuitable for the determination by means of the electron capture detector.

Diketone test mixtures were separated by gas chromatography by using the following procedures:

Basic instrument: F & M scientific gas chromatograph, model 810, with ^{3}H-electron capture detector and double-flame ionization detector.

Recorder: 2-channel-Moseley, Model 7128A, 1 mγ full deflection.

Separation columns:

(a) Poropak Q, 80-100 mesh, 2.4 m long, 1/8 in. internal diameter, steel.

(b) 10% Carbowax 20 M on Chromosorb p-NAW 80 to 100 mesh, 3.0 m long, 3 mm internal diameter, glass.

(c) 5% nonylphenoxy polyethyleneoxyethanol (1 gepal) on Chromosorb W-NAW 80-100 mesh, 4.2 m long, 4 mm internal diameter, copper or steel.

(d) 5% Igepal on chromosorb W-NAW 80-100 mesh, 3.0 m long, 3 mm internal diameter, glass.

(e) 0.5% Igepal on glass powder, 3.0 m long, 3 mm internal diameter, glass.

Quantitative analyses should preferably be carried out under isothermal plant conditions, since the electron capture detector is sensitive to temperature changes. The properties of various stationary phases for the gas-chromatographic separation of ethanol, diacetyl and 2,3-pentanedione are listed in Table 1.

Table 1

Suitability of various stationary phases for the gas-chromatographic separation of ethanol, diacetyl and 2,3-pentanedione

1 Stationäre Phase	2 Eigenschaften	3 Betriebsbedingungen	4 Geeignet
Porapak Q, 80 -- 100 mesh	a zu lange Retentionsvolumina	(up to) bis 200 C bis 75 ml He/min	nein -(No)
10 % Carbowax 20 M (on) - auf Chromosorb P	b gute Trennung ohne Tailing	70 C 50 ml He/min	ja -(Yes)
5 % Igepal (on) - auf Chromosorb W	c gute Trennung ohne Tailing	70° C 35 ml He/min	ja -(Yes)
0,5 % Igepal (on) - auf Glaspulver	d zu kurze Retentionsvolumina	7 bei Raumtemperatur 35 ml He/min	nein -(No)
25 % Squalan (on) - auf Chromosorb G	e teilweise Absorption der VDK	70 C	nein -(No)
10 % Silicongummi SE 30 (on) - auf Chromosorb W	f gute Trennung bei 2 m × ¼" Ø	70° C 35 ml He/min	ja -(Yes)
5 desgleichen, aber ⅛" Ø	g schlechte Trennung		nein -(No)
10 % Diisodecylphthalate (on) - auf Chromosorb W	h gute Trennung, aber Tailing bei Pentandion-(2,3)	100 C 70° C	8 bedingt ja
5 % Carbowax 1540 6 auf Teflon-Pulver	i gute Trennung ohne Tailing	35 ml He/min	

1. Stationary phase
2. Properties
3. Plant conditions
4. Suitable
5. same, but 1/8" internal diameter
6. on Teflon-Powder
7. at room temperature
8. conditional

a. Retention volumes too long
b. Good separation without tailing
c. Good separation without tailing
d. Retention volumes too short
e. Partial absorption of vicinal diketones
f. Good separation at 2 m x 1/4 in. internal diameter
g. Poor separation
h. Good separation, but tailing of 2,3-pentanedione
i. Good separation, without tailing

The phases suitable for a quantitative determination of diacetyl and 2,3-pentanedione in beer are listed under the heading "Suitable " in Table 1.

From these preliminary separation tests, we can see that neither synthetic resins (Porapak) nor nonpolar liquid phases (squalan) are suitable for the quantitative gas chromatographic determination of diacetyl and 2,3-pentanedione in beer. In addition, separation depends on the geometrical structure of the column, since the samples contain primarily ethanol; therefore, a column with 1/8 in. internal diameter is not suitable for separation because it is overcharged with ethanol. The detection sensitivity for diacetyl and 2,3-pentanedione in a quantitative analysis, when 1 μl of sample liquid is injected, is equal to 10^{-8} of the compound.

The separation efficiencies of the recommended columns are shown in Figures 1-3. The oven temperature was uniformly equal to 70°C. The carrier gas passage (throughput) was set at 50 ml He/min for the Carbowax 20 M column, and at 35 ml He/min for the Igepal and SE 30 columns. The electron capture detector was operated at 180°C and the injection unit at 140°C. The chart speed was equal to 1/2 in/min.

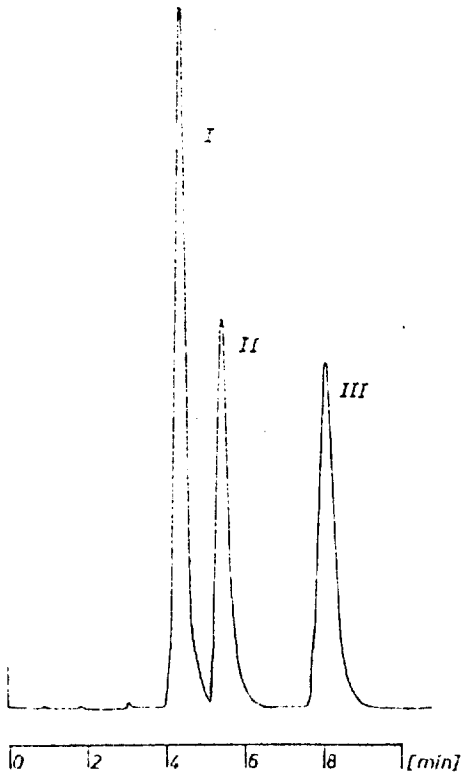


Fig. 1. Separation of ethanol (peak I), diacetyl (peak II) and 2,3-pentanedione (peak III) on a 3 mm x 3 mm internal diameter glass column, filled with 10% Carbowax 20 M on Chromosorb P-NAW 80-100 mesh at 70°C and 50 ml He/min.

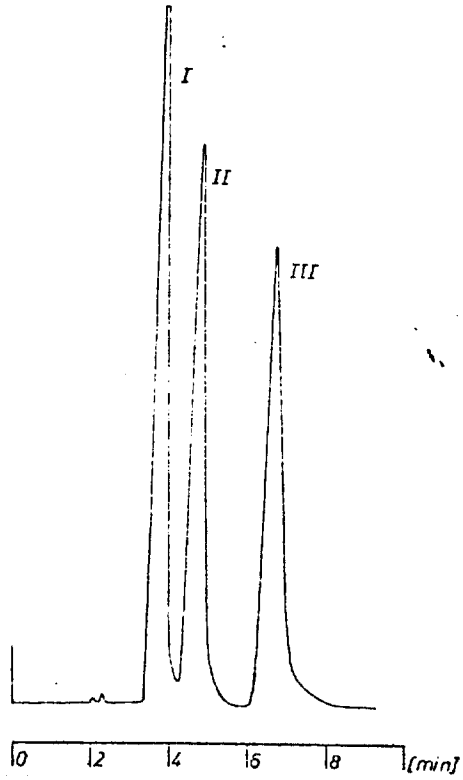


Fig. 2. Separation of ethanol (I), diacetyl (II) and 2,3-pentanedione (III) on a 3 mm x 3 mm internal diameter glass column, filled with 5% nonylphenoxypolyethyleneoxyethanol (Igepal) on Chromosorb W-NAW 80-100 mesh at 70°C and 35 ml He/min.

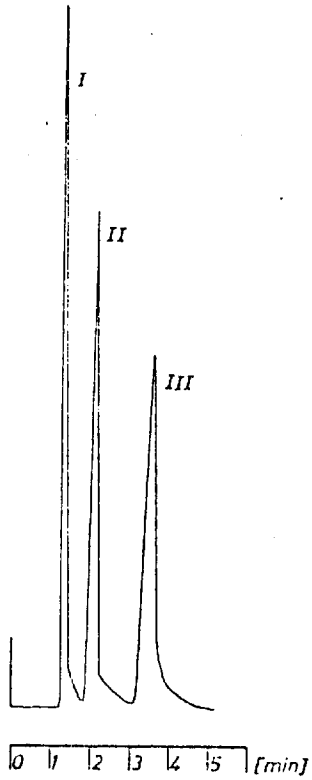


Fig. 3. Separation of ethanol (I), diacetyl (II) and 2,3-pentanedione (III) on a 2.44 m x 1/4 in. internal diameter steel column, filled with 10% silicone rubber SE 30 on Chromosorb W 60-80 mesh at 70°C and 35 ml He/min.

Copper is unsuitable as a column material, since complex formation between copper and diketones appears to be possible. When copper columns were used, the peak areas for diacetyl and 2,3-pentanedione were about 30% smaller than in similarly operated steel or glass columns. The results of separations on copper columns were not reproducible, and for this reason only glass or steel columns can be used for such analyses. Since glass proved to be too costly as a working material in view of the high breakage ratio during replacement of the columns, steel was successfully used (Remanit pipes, DEW Remanit 1880 ST, Wst. Nr. 4541 from the firm "Deutsche Edelstahlwerke AG, Krefeld).

B. Working Procedure

A 30 g sample of non-decarbonated beer is weighed into a diacetyl-

separation (stripping) glass, as described in the colorimetric diacetyl determination method (2), and the diketones are distilled off at 65°C for 2 hours with a nitrogen or carbon dioxide stream; 3 ml of carbonyl-free 96% alcohol is placed into the receiver. The receivers are placed into an ice bath. After stripping, the tips of the delivery tubes leading to the receivers are washed with alcohol, and the receivers are filled at the same time up to the 5 ml mark. The receivers are closed with ground glass stoppers, shaken and stored in a cool environment until ready for gas-chromatographic analysis.

For the gas-chromatographic determination, 1-2 µl of the thus treated sample is removed from the receiver with a Hamilton syringe and injected into the injection unit. The analysis time is 8 min for the following operating conditions:

Injection unit temperature 140°C

Oven temperature 70°C, isothermal

Electron capture detector 180°C (in simultaneous operation with a flame ionization detector (t=200°C), at a sample splitting of 1:1)

Separation column: 4.2 m x 0.4 cm internal diameter, filled with 5% Igepal on Chromosorb W-NAW 80-100 mesh, steel column

Without a precolumn

Carrier gas: Helium, 35 ml/min

Chart speed: 1/2 in/min

Detector gas EED: methane-argon (5:95), 100 ml/min

Detector gases FID: hydrogen, 50 ml/min; synthetic air mixture, 500 ml/min

The band areas (peak areas) are calculated by planimetry or by the

approximation method (7).

C. Quantitative Evaluation

For the quantitative determination of diacetyl and 2,3-pentanedione, calibration mixtures were prepared and treated as described in section II B. These mixtures consisted of 4% alcohol solutions (carbonyl-free alcohol and bidistilled water) to which increasing amounts of diacetyl and 2,3-pentanedione were added (both supplied by Fluka and tested for purity by gas chromatography) up to a dosage of 0.5 mg/l. In the measuring interval used (16 x 10 to 64 x 10), the detector readings were linear and reproducible with maximum deviations of ± 5%. Since the substances present in the mixture are completely vaporized under the experimental conditions used, quantitative evaluation was carried out as follows:

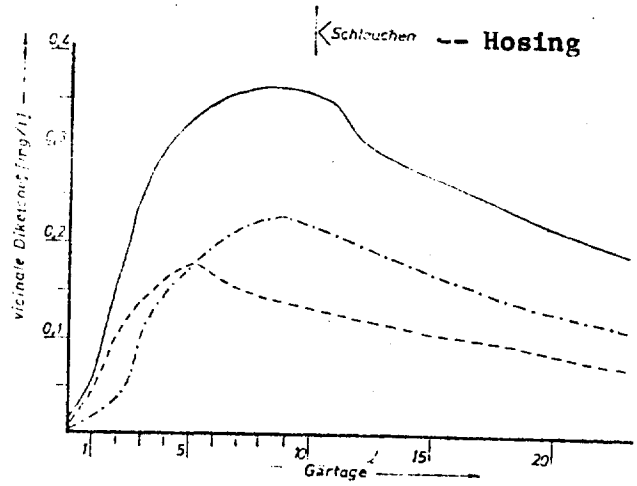
$$\text{mg St of sample} = \frac{\text{Band area St X mg St in calibration mixture}}{\text{Band area St of calibration mixture}}$$

where St = diacetyl or 2,3-pentanedione

III. Studies on Beer

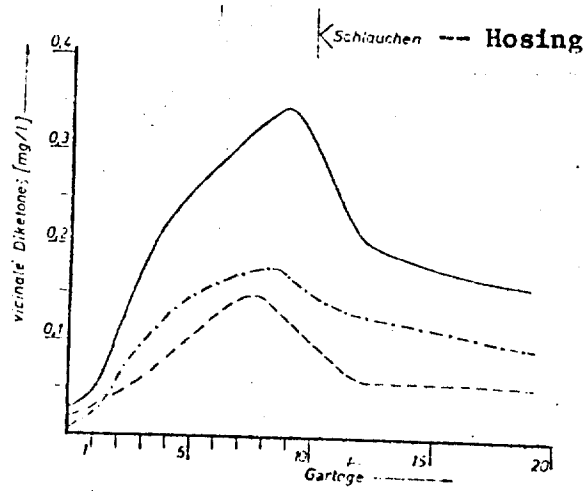
Two industrial fermentation batches were studied in regard to the formation and reduction of vicinal diketones. Figure 4 shows the diacetyl and 2,3-pentanedione contents of a Pilsener beer, determined by gas chromatography and spectral photometry. The main fermentation was carried out for 10 days at an initial temperature of 6°C rising to a maximum of 9.5°C. The diacetyl 2,3-pentanedione peak, determined by spectral photometry, was formed relatively late, between the 6th and 9th day, and corresponded to a content of only 36 mg diketone/l beer, which was due to the cold fermentation process. After a total of 23 days (13 days of secondary fermentation), the diketone content fell below the 0.2 mg/l

threshold. The separately plotted (determined) curves for diacetyl and 2,3-pentanedione did not have a synchronous course. It can be clearly seen that the diacetyl peak at 0.18 mg/l beer was already reached on the 5th day of fermentation. On the other hand, the maximum content of 2,3-pentanedione was determined only on the 8th and 9th day.



(Fermentation days)

Fig. 4. Formation and reduction of vicinal diketones in an industrial fermentation batch (Pilsener beer). Diacetyl determination - - -; 2,3-pentanedione determination -. -. -, by gas chromatography. Joint determination of vicinal diketones by spectral photometry: ———.



(Fermentation days)

Fig. 5. Industrial fermentation of a light beer: diacetyl - - - -; 2,3-pentanedione -. -. -., both by gas chromatography. Spectral photometric determination of vicinal diketones ———.

In a second industrial fermentation batch of a light full beer under similar conditions, with 9.0°C as the maximum fermentation temperature, the results shown in Figure 5 were obtained. A sharply expressed diketone peak was obtained by spectral photometry on the 9th day of fermentation. Reduction of vicinal diketones below the limit value of 0.2 mg/l took place very rapidly. In this fermentation batch, the diacetyl peak at 0.15 mg was measured between the 7th and 8th, also preceding the 2,3-pentanedione peak at 0.18 mg/l beer, measured on the 8th and 9th day. During beer storage, diacetyl was reduced more rapidly by yeast than 2,3-pentanedione.

Figures 6-8 show three gas chromatograms obtained by the method described here. In bock beer (Fig. 7), the diacetyl and 2,3-pentanedione content is almost the same, whereas in Pilsener beer (Fig. 8) the diacetyl content is definitely greater. Fig. 6 shows a sample from a main fermentation batch carried out during industrial tests. A selection of the beers studied is given in Table 2. The highest content of diacetyl (0.25 mg/l) and of 2,3-pentanedione (0.09 mg/l) was found in a March beer. This beer was unsuitable for sale in view of a clearly noticeable diacetyl odor already in the cold. From the figures given in Table 2 we can see that most finished beers contain somewhat more diacetyl than 2,3-pentanedione. The table also gives threshold values, average values and standard deviations for diacetyl and 2,3-pentanedione. It is therefore advisable to further pay particular attention to diacetyl, in view of its intense aroma, within the framework of industrial brewing control, in order that beer defects may be recognized prior to filtration and overcome by appropriate measures.

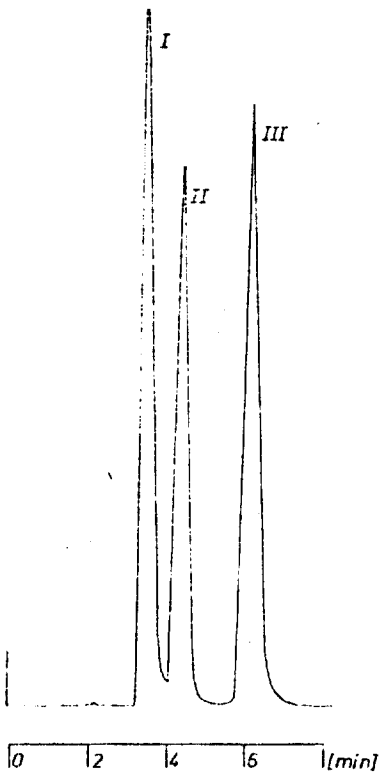


Fig. 6. Presence of diacetyl (II) and 2,3-pentanedione (III) on the 6th day of main fermentation: gas chromatogram.

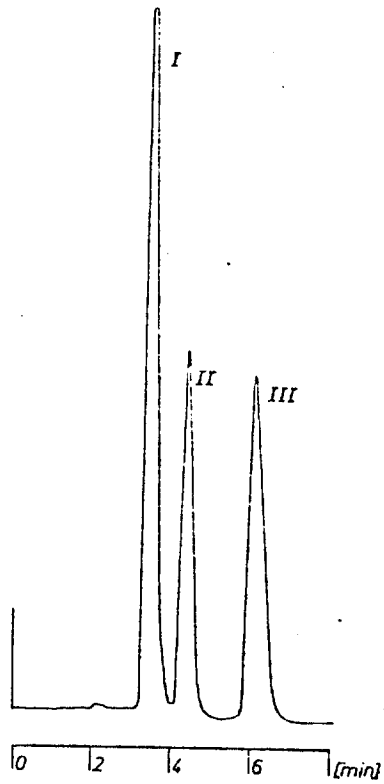


Fig. 7. Gas chromatogram of diacetyl (II) and 2,3-pentanedione (III) in a bock beer.

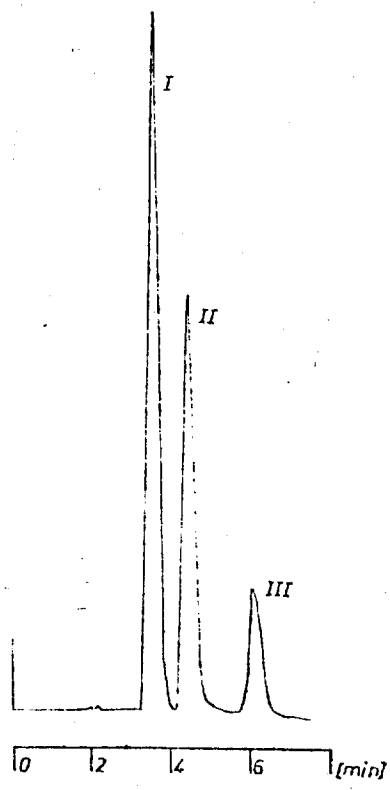


Fig. 8. Gas chromatogram of diacetyl (II) and 2,3-pentanedione (III) in a Pilsener beer.

Table 2

Diacetyl and 2,3-pentanedione content of German beers

1 Lfd Nr.	2 Biertyp	Diacetyl mg/l	3 Pentandion-(2,3) mg/l	4 Stammwürze %
1	Export	0,045	0,035	12,65
2	"	0,05	0,03	12,12
3	"	0,03	0,02	12,55
4	5 Märzen	0,015	0,01	13,83
5	"	0,25	0,09	13,50
6	Pilsener	0,03	0,02	12,10
7	"	0,04	0,01	11,98
8	"	0,035	0,025	12,04
9	"	0,06	0,035	12,31
10	"	0,035	0,03	12,25
11	6 Bockbier, hell	0,035	0,025	16,45
12	"	0,045	0,055	16,65
13	"	0,035	0,040	16,48
14	7 Bockbier, dunkel	0,045	0,035	16,80
15	"	0,055	0,025	16,72
16	"	0,07	0,04	17,04
17	8 Altbier	0,025	0,015	11,95
18	"	0,055	0,015	12,05
19	Kölsch	0,02	0,015	11,12
20	"	0,015	0,01	11,59
9 Schwellenwert		0,20	0,90	--
10 Mittelwert		0,067	0,039	--
11 Standardabweichung		0,063	0,021	—

1. Item #
2. Beer type
3. 2,3-pentanedione
4. Original wort
5. March
6. Bock beer light
7. Bock beer dark
8. Old beer
9. Threshold value
10. Average value
11. Standard deviation

Summary

Gas chromatographic determination of diacetyl and 2,3-pentanedione in beer

A gas chromatographic method for the determination of the diketone compounds diacetyl and 2,3-pentanedione is described. This method is based on the selective identification of these compounds by an electron capture detector.

The diketones are volatilised in a gas stream from the beer at 65°C and passed into carbonyl-free 96% alcohol in an ice bath; 1-2 ul of the resultant mixture are used for gas chromatography. The separation of the compounds is carried out on the columns: Parapak Q 10%, Carbowax 20 M, and 5% nonylphenoxypolyethylenoxyethanol (Igepal), the last one being a Chromosorb W-NAW 80-100 mesh. The best separation is achieved on a column 4.2 m. long filled with 5% Igepal at 70°C. Copper is not suitable as a material for the columns and therefore glass or stainless steel is used.

Several commercial beers are tested for their diacetyl and 2,3-pentanedione content, and in addition the variations in the concentrations of these compounds during a primary and a secondary fermentation in a brewery are followed. The values obtained are compared with those found in the colorimetric diacetyl determination.

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Gaschromatographische Bestimmung von Diacetyl und Pentandion-(2,3) im Bier

0477

Von B. Drews, G. Bärwald und H.-J. Niefind

Institut für Gärungsgewerbe und Biotechnologie, Berlin

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I. Einleitung

Für die quantitative Bestimmung der vicinalen Diketon-Verbindungen Diacetyl (Butandion-(2,3)) und Pentandion-(2,3) im Bier, die bei der alkoholischen Gärung als Stoffwechselprodukte entstehen, gibt es im wesentlichen zwei Arbeitsweisen. Bei der ersten werden diese vicinalen Diketone mit einem Gasstrom aus dem Bier getrieben und gemeinsam in einer chemischen Reaktion erfaßt, z. B. durch Oximieren mit Dimethylglyoxim und nachfolgender Bildung eines Fe(II)-DMG-Komplexes, der bei 530 nm ein Absorptionsmaximum zeigt (1,2). Bei der zweiten werden die beiden Verbindungen getrennt bestimmt, was durch den Einsatz der Dünnschichtchromatographie (1,3) und der Gaschromatographie (4,5) gelingt.

Für die brautechnologische Betriebskontrolle, die sich vornehmlich auf das im Bier unerwünschte Diacetyl erstreckt, ist eine einfach zu handhabende Methode zur getrennten quantitativen Erfassung der vicinalen Diketone sehr vorteilhaft.

In der vorliegenden Arbeit wird eine gaschromatographische Methode zur Trennung und quantitativen Bestimmung von Diacetyl und Pentandion-(2,3) im Bier beschrieben, die auf der selektiven Anzeige dieser Stoffe durch einen Elektroneneinfang-Detektor beruht. Weiterhin werden Biere des Handels auf ihre Gehalte an Diacetyl und Pentandion-(2,3) untersucht sowie die Bildung und Eliminierung dieser Stoffe bei der Haupt- und Nachgärung im Betrieb verfolgt. Die von Drews, Specht, Bärwald und Trénel (2) modifizierte kolorimetrische Bestimmung dieser Stoffe wird zum Vergleich herangezogen.

II. Methodik

A. Vorversuche

Zunächst mußte durch Vorversuche geklärt werden, welche Säulen und Betriebsbedingungen für die Gaschromatographie mit dem Elektroneneinfang-Detektor zur Trennung von Diacetyl und Pentandion-(2,3) führen. Dazu wurden Testgemische aus carbonylfreiem 96%igem eff-Sprit hergestellt, mit einem Gehalt an Diacetyl oder Pentandion-(2,3) von jeweils 0 bis 0,5 mg/l. Wäßrige Proben ließen sich nicht auftrennen, da das Wasser die Empfindlichkeit des Elektroneneinfang-Detektors stark beeinträchtigt. Auch das Vorschalten einer das Wasser zurückhaltenden Vorsäule, z. B. einer Glycerinsäule, erwies sich als unzweckmäßig, weil ein Teil der Diketone mit dem Wasser zurückgehalten wurde.

Die Umsetzung des Wassers in einer mit Calciumcarbid gefüllten Vorsäule zu Acetylen führte gleichfalls nicht zum Erfolg, denn die Acetylenbande des Chromatogramms war so groß, daß die Diketone, die später eluiert werden, völlig überdeckt wurden. Somit mußten sowohl die direkte Einspritzung des Bieres als auch die Verwendung von Bierdestillat als ungeeignet für die Bestimmung mittels Elektroneneinfang-Detektors verworfen werden.

Die Diketon-Testgemische wurden in folgenden Versuchsanordnungen gaschromatographisch getrennt:

Grundgerät: F & M Scientific Gaschromatograph, Modell 810 mit ^{3}H-Elektroneneinfang-Detektor und Doppelflammenionisations-Detektor.

Schreiber: 2-Kanal-Moseley, Modell 7128 A, 1 mV Vollausschlag.

Trennsäulen:

a) Porapak Q, 80—100 mesh, 2,4 m lang, 1/8" ϕ_i, aus Stahl
b) 10% Carbowax 20 M auf Chromosorb P-NAW 80 bis 100 mesh, 3,0 m lang, 3 mm ϕ_i, aus Glas
c) 5% Nonylphenoxypolyäthylenoxyäthanol (Igepal) auf Chromosorb W-NAW 80—100 mesh, 4,2 m lang, 4 mm ϕ_i, aus Kupfer bzw. aus Stahl
d) 5% Igepal auf Chromosorb W-NAW 80—100 mesh, 3,0 m lang, 3 mm ϕ_i, aus Glas
e) 0,5% Igepal auf Glaspulver, 3,0 m lang, 3 mm ϕ_i, aus Glas.

Isotherme Betriebsbedingungen sind für quantitative Analysen in jedem Fall vorzuziehen, da der Elektroneneinfang-Detektor temperaturempfindlich reagiert.

In der Tabelle 1 sind die Eigenschaften verschiedener stationärer Phasen zur gaschromatographischen Trennung von Äthanol, Diacetyl und Pentandion-(2,3) zusammengestellt. Die für eine quantitative Bestimmung von Diacetyl und Pentandion-(2,3) aus Bier geeigneten Phasen werden in der Rubrik „geeignet" angegeben.

Aus den Trennversuchen ist ersichtlich, daß sich für die quantitative gaschromatographische Bestimmung von Diacetyl und Pentandion-(2,3) aus Bier weder Kunstharze (Porapak) noch unpolare flüssige Phasen (Squalan) eignen. Die Trennung hängt außerdem von der Geometrie der Säule ab, da sich in den Proben überwiegend Äthanol befindet; deshalb ist zur Trennung eine Säule mit ⅛" Innendurchmesser wegen Überladung durch Äthanol nicht geeignet. Die Nachweisempfindlichkeit für Diacetyl und Pentandion-(2,3) beträgt bei der quantitativen Analyse und einer Einspritz-

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Tabelle 1

Eignung verschiedener stationärer Phasen zur gaschromatographischen Trennung von Äthanol, Diacetyl und Pentandion-(2,3)

Stationäre Phase	Eigenschaften	Betriebsbedingungen	Geeignet
Chrapak Q, 80 — 100 mesh	zu lange Retentionsvolumina	bis 200° C bis 75 ml He/min	nein
10 % Carbowax 20 M auf Chromosorb P	gute Trennung ohne Tailing	70° C 50 ml He/min	ja
5 % Igepal auf Chromosorb W	gute Trennung ohne Tailing	70° C 35 ml He/min	ja
0,5 % Igepal auf Glaspulver	zu kurze Retentionsvolumina	bei Raumtemperatur 35 ml He/min	nein
25 % Squalan auf Chromosorb G	teilweise Absorption der VDK	70° C	nein
10 % Silicongummi SE 30 auf Chromosorb W	gute Trennung bei 2 m × ¼" Ø	70° C 35 ml He/min	ja
desgleichen, aber ⅛" Ø	schlechte Trennung		nein
10 % Diisodecylphthalat auf Chromosorb W	gute Trennung, aber Tailing bei Pentandion-(2,3)	100° C 70° C	bedingt
5 % Carbowax 1540 auf Teflon-Pulver	gute Trennung ohne Tailing	35 ml He/min	ja

menge von 1 µl Probenflüssigkeit jeweils 10^{-8} des Stoffes. In den Abbildungen 1 bis 3 sind die Trennleistungen der empfohlenen Säulen dargestellt. Die Ofentemperatur hatte dabei einheitlich 70° betragen. Der Trägergasdurchfluß wurde bei der Carbowax 20 M-Säule auf 50 ml He/min, bei der Igepal- und SE 30-Säule auf 35 ml He/min eingestellt. Der Elektroneneinfang-Detektor wurde bei 180° C und der Einspritzblock bei 140° C betrieben. Der Papiervorschub hatte ½"/min betragen.

Kupfer ist als Säulenmaterial ungeeignet, denn eine Komplexbildung zwischen Kupfer und Diketonen scheint denkbar. Bei Benutzung von Kupfersäulen waren die Peakflächen für Diacetyl und Pentandion-(2,3) um etwa 30 % kleiner als bei gleicherweise betriebenen Stahl- oder Glassäulen. Die Ergebnisse der Trennungen auf Kupfersäulen waren nicht reproduzierbar. Deshalb kommen nur Glas- oder Stahl-

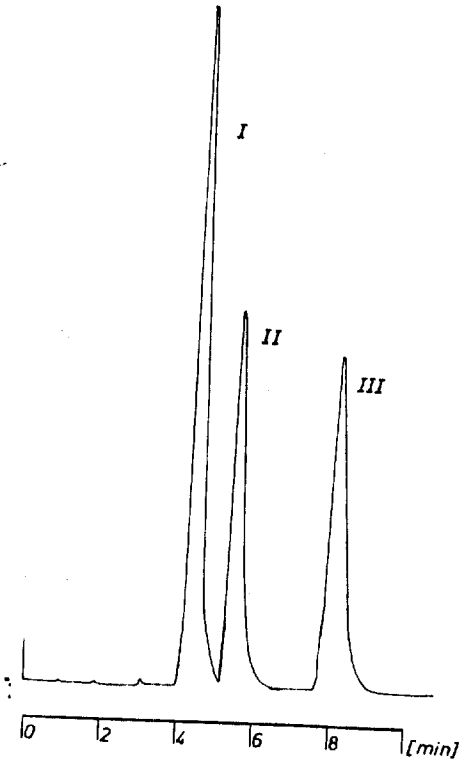

Abb. 1: Trennung von Äthanol (Peak I), Diacetyl (Peak II) und Pentandion-(2,3) (Peak III) auf einer 3 m × 3 mm Øi Glassäule, gefüllt mit 10 % Carbowax 20 M auf Chromosorb P-NAW 80-100 mesh bei 70° und 50 ml He/min

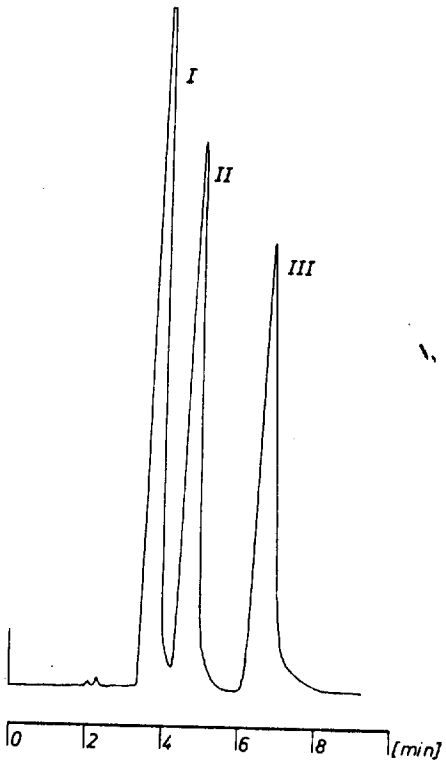

Abb. 2: Trennung von Äthanol (I), Diacetyl (II) und Pentandion-(2,3) (III) auf einer 3 m × 3 mm Øi Glassäule, gefüllt mit 5 % Nonylphenoxypolyäthylenoxyäthanol (Igepal) auf Chromosorb W-NAW, 80-100 mesh bei 70° C und 35 ml He/min

säulen für diese Analysen in Frage. Da Glas sich als Werkstoff wegen der hohen Bruchquote beim Auswechseln der Säulen als zu teuer erwies, wurde mit gutem Erfolg Stahl (Remanitrohre, DEW Remanit 1880 ST, Wst. Nr. 4541 der Fa. Deutsche Edelstahlwerke AG, Krefeld) als Werkstoff verwendet.

B. Arbeitsvorschrift

30 g nicht entkohlensäuertes Bier werden in ein Diacetyl-Abtriebsglas, wie bei der kolorimetrischen Diacetylbestimmung beschrieben (2), eingewogen, und die Diketone werden bei 65° mittels Stickstoff- oder Kohlendioxidstrom zwei Stunden lang ausgetrieben. In das Vorlageglas werden 3 ml carbonylfreien 96%igen elf-Sprits gegeben. Die Vorlagegläser befinden sich in einem Eisbad. Nach dem Über-

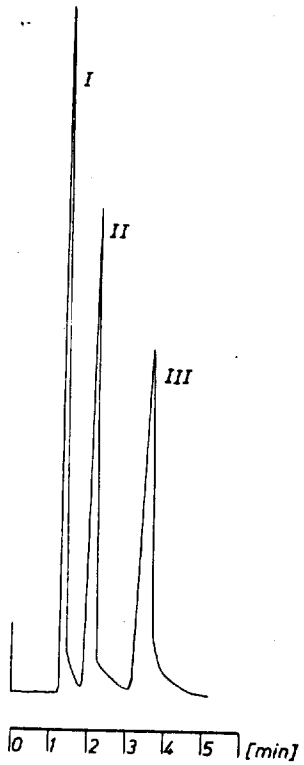

Abb. 3: Trennung von Äthanol (I), Diacetyl (II) und Pentandion-(2,3) (III) auf einer 2,44 m × ¼" Øi Stahlsäule, gefüllt mit 10 % Silicongummi SE 30 auf Chromosorb W 60-80 mesh bei 70° C und 35 ml He/min

treiben werden die Spitzen der in die Vorlagegläser führenden Einleitungsröhrchen mit dem Sprit abgespült, wobei gleichzeitig bis zur 5-ml-Marke aufgefüllt wird. Die Vorlagegläser werden mit Schliffstopfen verschlossen, umgeschüttelt und bis zur gaschromatographischen Analyse kühl aufbewahrt.

Für die gaschromatographische Bestimmung werden mit einer Hamilton-Spritze 1 bis 2 µl der so behandelten Probe aus dem Vorlageglas entnommen und in den Einspritzblock injiziert. Für die folgenden Betriebsbedingungen ergibt sich eine Analysenzeit von 8 Minuten:

Einspritzblock-Temperatur	140°
Ofen-Temperatur	70° isotherm
Elektroneneinfang-Detektor	180°

(im Simultan-Betrieb mit Flammenionisationsdetektor [t = 200°] bei einer Probenteilung 1 : 1)

Trennsäule	4,2 m × 0,4 cm Øi, gefüllt mit 5 % Igepal auf Chromosorb W-NAW 80—100 mesh, Stahlsäule
ohne Vorsäule	
Trägergas	Helium, 35 ml/min
Papiervorschub	½"/min
Detektorgas EED	Methan-Argon (5 : 95) : 100 ml/min
Detektorgase FID	Wasserstoff : 50 ml/min synthetische Luft : 500 ml/min

Die Bandenflächen werden durch Planimetrieren oder nach Näherungsverfahren (7) ermittelt.

C. Quantitative Auswertung

Für die quantitative Bestimmung von Diacetyl und Pentandion-(2,3) wurden Eichgemische hergestellt und diese gemäß Abschnitt II. B. behandelt. Die Eichgemische bestanden aus 4%igen Spritlösungen (carbonylfreier eff-Sprit und bidestilliertes Wasser), denen steigende Mengen an Diacetyl und Pentandion-(2,3) (beide von Fluka; gaschromatographisch auf Reinheit geprüft) bis zu 0,5 mg/l zudosiert wurden. Die Detektoranzeige war in den verwendeten Meßbereichen von 16 × 10 bis 64 × 10 linear und reproduzierbar mit maximalen Abweichungen von ± 5 %. Da die im Gemisch vorhandenen Substanzen unter den Versuchsbedingungen vollständig verdampfen, wurde bei der quantitativen Auswertung wie folgt verfahren:

$$\text{mg St der Probe} = \frac{\text{Bandenflächest} \times \text{mg St in der Eichlösung}}{\text{Bandenflächest der Eichlösung}}$$

worin St = Diacetyl oder Pentandion-(2,3) bedeutet.

III. Untersuchungen an Bier

Es wurden zwei Betriebsgärungen in bezug auf Bildung und Reduzierung der vicinalen Diketone untersucht. In der Abbildung 4 sind die gaschromatographisch und spektralphotometrisch ermittelten Diacetyl- und Pentandion-(2,3)-Gehalte eines Pilsener Bieres aufgezeigt. Die Hauptgärung wurde von 6° Anstelltemperatur auf maximal 9,5° C zehn Tage lang geführt. Das spektralphotometrisch ermittelte Diacetyl-Pentandion-(2,3)-Maximum wurde verhältnismäßig spät zwischen dem 6. und 9. Tage gebildet, hatte aber nur, durch die kalte Gärführung bedingt, 36 mg VDK/l Bier betragen. Nach insgesamt 23 Tagen (13 Tage Nachgärung) wurde die 0,2-mg/l-Schwelle unterschritten. Die separat bestimmten Diacetyl- und Pentandion-(2,3)-Kurven verliefen nicht synchron. Es ist deutlich zu erkennen, daß das Diacetyl-Maximum mit 0,18 mg/l Bier bereits am 5. Gärtage erreicht wurde. Hingegen wurde der größte Gehalt an Pentandion-(2,3) erst am 8. und 9. Gärtag ermittelt.

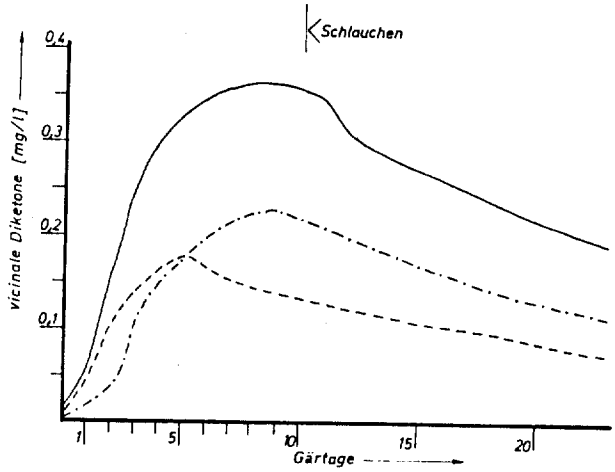

Abb. 4: Bildung und Reduzierung der vicinalen Diketone bei einer Betriebsführung (Pilsener Bier). Bestimmung des Diacetyls — — — und Pentandion-(2,3) —.—.— durch Gaschromatographie; gemeinsame Bestimmung der VDK ———— durch Spektralphotometrie

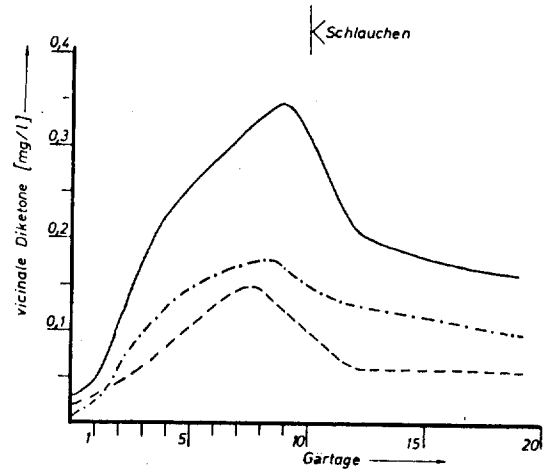

Abb. 5: Betriebsgärung eines hellen Bieres : Diacetyl ————, Pentandion-(2,3) —.—.—, beide gaschromatographisch bestimmt. Spektralphotometrische VDK-Bestimmung ————

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In einer zweiten Betriebsgärung eines hellen Vollbieres unter ähnlichen Bedingungen mit 9,0° C als maximaler Gärtemperatur wurden die in Abbildung 5 dargestellten Ergebnisse erzielt. Mit der spektralphotometrischen Methode wurde am 9. Gärtage ein scharf markiertes VDK-Maximum erhalten. Die Reduzierung der vicinalen Diketone erfolgte sehr schnell unter den Grenzwert von 0,2 mg/l. Auch in dieser Gärung lag das Diacetyl-Maximum mit 0,15 mg zwischen dem 7. und 8. Tage vor demjenigen des Pentandion-(2,3), das am 8. und 9. Tage mit 0,18 mg/l Bier ermittelt wurde. Bei der Lagerung des Bieres wurde das Diacetyl schneller als das Pentandion-(2,3) von der Hefe reduziert.

In den Abbildungen 6 bis 8 werden drei Gaschromatogramme dargestellt, die aus der beschriebenen Methode resultieren. Bei dem Bockbier (Abb. 7) sind der Diacetyl- und Pentandion-(2,3)-Gehalt nahezu gleich, während beim Pilsener (Abb. 8) das Diacetyl deutlich überwiegt. Abbildung 6 zeigt eine Probe bei der Hauptgärung aus den Betriebsversuchen. Eine Auswahl der untersuchten Biere wird in der Tabelle 2 angegeben. Der höchste Gehalt an Diacetyl mit 0,25 mg/l und 0,09 mg Pentandion-(2,3)/l wurde in einem Märzen-Bier gefunden. Das Bier war durch einen bereits in der Kälte deutlich merkbaren Diacetylgeruch nicht verkaufsfähig. Aus den in der Tabelle angegebenen Zahlen ist zu ersehen, daß die meisten fertigen Biere etwas mehr Diacetyl als Pentandion-(2,3) enthalten. Die Tabelle enthält außerdem Schwellenwerte, Mittelwerte und Standardabweichungen für Diacetyl und Pentandion-(2,3) im Bier. Deshalb ist es angebracht, weiterhin dem aromaintensiven Diacetyl im Rahmen der brautechnologischen Betriebskontrolle eine besondere Beachtung zu widmen, damit Bierfehler bereits vor dem Filtrieren erkannt und durch entsprechende Maßnahmen beseitigt werden können.

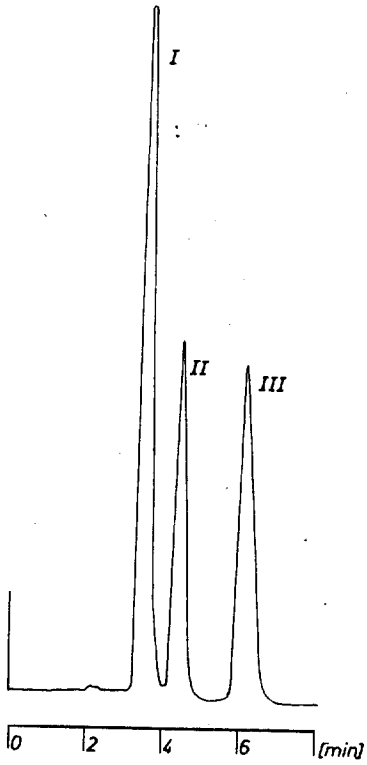

Abb. 7: Gaschromatogramm von Diacetyl (II) und Pentandion-(2,3) (III) eines Bockbieres

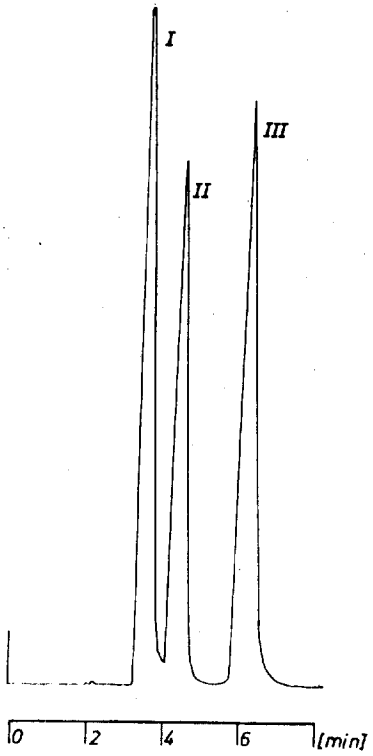

Abb. 6: Vorkommen von Diacetyl (II) und Pentandion-(2,3) (III) am 6. Tage der Hauptgärung: Gaschromatogramm

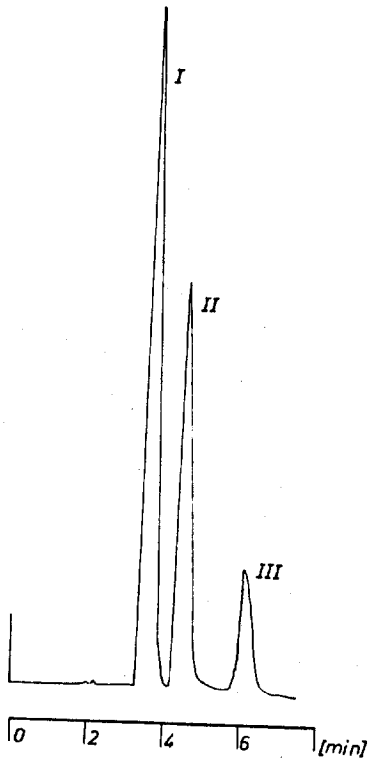

Abb. 8: Gaschromatogramm von Diacetyl (II) und Pentandion-(2,3) (III) eines Pilsener Bieres

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Tabelle 2
Diacetyl- und Pentandion-(2,3)-Gehalte von deutschen Bieren

Lfd. Nr.	Biertyp	Diacetyl mg/l	Pentandion-(2,3) mg/l	Stammwürze %
1	Export	0,045	0,035	12,65
2	„	0,05	0,03	12,12
3	„	0,03	0,02	12,55
4	Märzen	0,015	0,01	13,83
5	„	0,25	0,09	13,50
6	Pilsener	0,03	0,02	12,10
7	„	0,04	0,01	11,98
8	„	0,035	0,025	12,04
9	„	0,06	0,035	12,31
10	„	0,035	0,03	12,25
11	Bockbier, hell	0,035	0,025	16,45
12	„	0,045	0,055	16,65
13	„	0,035	0,040	16,48
14	Bockbier, dunkel	0,045	0,035	16,80
15	„	0,055	0,025	16,72
16	„	0,07	0,04	17,04
17	Altbier	0,025	0,015	11,95
18	„	0,055	0,015	12,05
19	Kölsch	0,02	0,015	11,12
20	„	0,015	0,01	11,59
Schwellenwert		0,20	0,90	—
Mittelwert		0,067	0,039	—
Standardabweichung		0,063	0,021	—

IV. Zusammenfassung

Es wird eine gaschromatographische Methode zur Bestimmung der vicinalen Diketon-Verbindungen Diacetyl und Petandion-(2,3) im Bier beschrieben, die auf der selektiven Anzeige dieser Stoffe durch einen Elektroneneinfang-Detektor beruht.
Die vicinalen Diketone werden im Gasstrom aus dem Bier bei 65° ausgetrieben und in carbonylfreiem 96%igem eff-Sprit im Eisbad aufgefangen. Es werden 1 bis 2 µl des aufgefangenen Gemisches für die gaschromatographische Bestimmung verwendet. Die Trennung der Stoffe wird auf den Säulen: 10 % SE 30, 10 % Carbowax 20 M und 5 % Nonylphenoxypolyäthylenoxyäthanol (Igepal), letztere auf Chromosorb W — NAW 80 — 100 mesh, vorgenommen. Die beste Trennung wird auf einer 4,2 m langen, mit 5 % Igepal gefüllten Säule bei 70° erzielt. Kupfer ist hierbei als Säulenmaterial ungeeignet. Es wird daher Glas oder Stahl als Werkstoff eingesetzt.
Mit der beschriebenen Methode wurden zahlreiche Biere des Handels auf ihre Gehalte an Diacetyl und Pentandion-(2,3) untersucht sowie eine Haupt- und Nachgärung im Betrieb verfolgt. Die erhaltenen Werte werden mit denjenigen der kolorimetrischen Diacetyl-Bestimmung verglichen.

Summary

Gas chromatographic determination of diacetyl and pentane-2,3-dione in beer

A gas chromatographic method for the determination of the diketone compounds diacetyl and pentane-2,3-dione is described. This method is based on the selective identification of these compounds by an electron capture detector.
The diketones are volatilised into the gas stream from the beer at 65 °C and passed into carbonyl-free 96 % spirit in an iced water bath. 1–2 µl of the resultant mixture are used for gas chromatography. The separation of the compounds is carried out on the columns: Parapak Q 10 % Carbowax 20 M and 5 % nonylphenoxypolyethylenoxyethanol (Igepal), the last one being a Cromosorb W-NAW 80-100 mesh. The best separation is achieved on a column 4.2 metres long filled with 5 % Igepal at 70° C. Copper is not suitable as a material for the columns and therefore glass or stainless steel is used.
Several commercial beers are tested for their diacetyl and pentane-2,3-dione content and in addition the variations in the concentrations of these compounds during a primary and a secondary fermentation in a brewery are followed. The values obtained are compared with those found in the colorimetric diacetyl determination.

Résumé

Détermination par chromatographie en phase gazeuse du diacétyl et du 2,3 pentanedion

On décrit une méthode par chromatographie en phase gazeuse pour la détermination dans la bière des deux cétones voisines, le diacétyl et le 2,3 pentanedion; cette méthode est basée sur l'indication sélective de ces substances par un détecteur à captation d'électrons.
Ces cétones voisines sont déplacées de la bière par un courant gazeux à 65° et captées dans de l'alcool éthylique à 96 %, sans composés carbonyls, au bain de glace. Un à deux µl du mélange obtenu sont utilisés pour l'analyse par chromatographie en phase gazeuse. On réalise la séparation des composés sur les colonnes Porapak Q, 10 % de Carbowax 20 M et 5 % de nonylphénoxypolyéthylèneoxyethanol (Igepal); ce dernier est fixé sur du Chromosorb W-NAW 80—100 mesh. La meilleure séparation est obtenue à 70° sur une colonne de 4,2 m chargée de l'Igepal à 5 %. Des colonnes en cuivre ne conviennent pas; il faut du verre ou de l'acier.
De nombreuses bières du commerce ont été examinées par cette méthode quant à leur teneur en diacétyl et en 2,3 pentanedion; on a également suivie une bière industrielle aux stades de la fermentation principale et secondaire. Les valeurs obtenues sont comparées à celles de la détermination du diacétyl par colorimétrie.

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[BC 361; Diacetyl. Gärungsnebenprodukte]

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KARAM E. EL'ODÉ, THEODORE P. DORNSEIFER,
ELIZABETH S. KEITH, and JOHN J. POWERS
Department of Food Science, University of Georgia, Athens, Georgia 30601

Effects of pH and Temperature on the Carbonyls and Aromas Produced in Heated Amino Acid–Sugar Mixtures

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SUMMARY

Solutions of glycine, glutamic acid, lysine, methionine, and phenylalanine adjusted to pH 6.5 were reacted separately with glucose, fructose, maltose, and sucrose at 100 and 180°C. Some of the aromas generated were reminiscent of foods, whereas others were distinctly unpleasant. Ethanal, propanal, acetone, isobutanal, acrolein, butanal, 2-butanone, 2,3-butanedione, crotanal, 2-pentanone, and heptanal were identified as end products in one or more of the 20 mixtures. The glutamic acid–glucose mixture was also heated at 100 and 180°C after being adjusted to pH 5.0 and 8.0. Of the compounds above, isobutanal and heptanal were not formed. Pentanal was formed at 100° but not at 180°C. A synthetic potato medium containing glucose and glutamic acid heated at 100°C yielded ethanal, propanal, acetone, butanal, 2-butanone, 2,3-butanedione, and crotonal. A potato extract heated at 180° differed from the synthetic potato medium heated at 100° in the products formed. Not formed were 2-butanone and 2,3-butanedione; pentanal, heptanal and hexen-1-al were formed. In all, 85 carbonyls were detected in the various mixtures or substrates.

INTRODUCTION

The reactions between individual amino acids and sugars have frequently been studied with reference to color changes, aroma, and taste (Beacham and Dull, 1951; [illegible], 1965; Lento *et al.*, 1960a,b; Herz and Shallenberger, 1960; Rohan and Stewart, 1965; Wiseblatt and Zoumut, 1963), but the identities of the compounds formed have not been as extensively studied. Some of them, being volatile, may logically be expected to affect the flavor of foods. The present work was undertaken to: 1) separate the products formed when sugars and amino-acids in a model system were reacted under the influence of temperature; 2) study the effect of pH on the carbonyl compounds formed; and 3) evaluate organoleptically the aroma of the reaction products.

REVIEW OF LITERATURE

Browning occurs in both acidic and alkaline media. The rate of the reaction and the extent of browning increase greatly as the pH increases (Boretti, 1944; Wolfrom *et al.*, 1947; Haugaard *et al.*, 1951; Katchalsky and Sharon, 1953; Underwood *et al.*, 1959). The rate of browning also increases with an increase in temperature (Hendel *et al.*, 1955; Hannan and Lea, 1951; Lea *et al.*, 1951). Zabrodskii and Vitkovaskaya (1954) stated that increasing the temperature increased the quantity of sugars combining with the same quantity of amino acid.

EXPERIMENTAL

Selection of the sugars and amino acids. Selection of the sugars and amino acids used in the pure chemical phases was based on identification of sugars and amino acids present in fresh potato tubers. A water extract of Katahdin variety of potato was used for analysis. A unidimensional chromatographic technique, employing 1-butanol–acetic acid–water (BAW) (4:1:5 v/v) and 80% phenol in water (4:1) was used for the identification of sugars and amino acids. The dry chromatograms were sprayed with 0.32% anisidine to identify sugars and 0.25% ninhydrin in acetone to spot amino acids, followed by heating for 3-5 min in hot-air ovens for development of the color.

Of the amino acids identified, glycine, glutamic acid, lysine, phenylalanine, and methionine were separately reacted with the sugars identified (glucose, fructose, maltose, and sucrose). The amino acids selected were chosen to represent five major classes of amino acids: acidic, basic, neutral, aromatic, and sulfur-containing.

Collection of carbonyl compounds. The reaction between each sugar and amino acid was carried out at 100 and 180°C. The sugars and amino acids (0.2*M* each) were dissolved in 100 ml of 0.028*M* phosphate buffer at pH 6.5. The flasks containing the solution of sugar and amino acid were heated for 60 min in a water bath at 100°C. Each flask was connected to another flask containing dinitrophenylhydrazine (DNPH) reagent. These connections were kept airtight to ensure that all the volatiles bubbled into the reagent. At the end of the heating time, the flasks were removed and cooled to room temperature and poured in the reagent flask to collect traces of carbonyls still in the reaction mixture. The DNPH derivatives were collected according to the procedure of Dornseifer and Powers (1963, 1965), filtered

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through Whatman No. 2 filter paper, washed thoroughly with distilled water, and dried in a desiccator containing phosphopentoxide as a desiccant.

For the reaction at 180°C, the sugar and amino acid solution was placed in a special tube capable of withstanding high pressure. The tube was made of a borosilicate glass with an approximate pressure tolerance of 325 psi. A hypodermic syringe inserted through a rubber septum was used to fill the tubes to prevent the loss of solution from back pressure generated by air bubbles. The tubes were connected to a vacuum pump. After complete evacuation was obtained, the tubes were sealed in an oxygen flame and heated in an oil bath for 30 min. At the end of the heating period, the tubes were removed and rapidly cooled to room temperature, and the solutions were poured into DNPH reagent and kept overnight. The crystals were collected and dried as described above.

Effect of pH on the carbonyl compounds generated. Glucose and glutamic acid solutions were prepared as before. Two 100-ml portions were adjusted to pH 5.0, 6.5, and 8.0. One flask at each pH was heated for 60 min at 100°C. The second 100-ml portions were also heated at each pH level, but at 180°C in the borosilicate tubes. The carbonyl compounds were collected as described above.

Generation of carbonyl compounds in potato media. A synthetic potato medium was formulated as closely as possible to diced frozen potatoes (Watt and Merrill, 1963). The mixture was placed in a round-bottom flask and connected to a series of traps. The first two traps contained diluted HCl, and the last three traps DNPH reagent. The mixture was heated 60 min at 100°C. Nitrogen gas was bubbled through the mixture to carry the carbonyls formed to the traps containing the DNPH reagent.

A potato extract was prepared by pureeing a small amount of water containing 130 ppm sodium bisulfite with diced potatoes, followed by filtration under vacuum with a Buchner funnel and Whatman No. 2 filter paper. This extract was heated for 30 min at 180°C. The carbonyl compounds were collected and treated as previously described.

Regeneration of the carbonyl compounds. The method of Ralls (1960) as modified by Dornseifer and Powers (1963, 1965) was used to regenerate the carbonyls. The vapors generated in the tube were withdrawn with a gas-tight syringe and injected directly into the gas chromatograph. An F & M gas chromatograph, model 609, with a flame ionization detector was used. The best of several columns tried were two 10-ft columns, one consisting of 15% butanediol succinate on Diatoport P, and the second of 20% polyethylene glycol 600 on firebrick. The column temperature was 75°C; nitrogen gas flow rate, 57 ml/min; sensitivity range 1; and attenuation either 15 or 32.

RESULTS AND DISCUSSION

Sugars and amino acids present in potatoes. Identified as being present in the water extract of the Katahdin potatoes were glucose, fructose, maltose, sucrose, glycine, aspartic acid, glutamic acid, proline, lysine, serine, histidine, methionine, valine, and phenylalanine (Table 1).

Habib and Brown (1957) found glucose, fructose, maltose, and sucrose in the Katahdin potatoes they examined; Schwimmer *et al.* (1954) also reported that potatoes contain glucose, fructose, and sucrose, but did not detect the presence of maltose.

Slack (1948), Hirsch *et al.* (1952), Katayama *et al.* (1956), and Furuholmen *et al.* (1964) found arginine, cystine, isoleucine, threonine, tyrosine, and tryptophan, which we did not. The differences are probably a result of the variations in variety of potato examined and of cultural or storage conditions. The composition of potato tubers undergoes changes of varying degrees from year to year, and conditioning of the tubers results in some difference in amino acid content of potatoes, particularly the basic ones (Habib and Brown, 1957).

Table 1. The R_f values of individual sugars and amino acids in the water extract of Katahdin potato.

Compounds	Solvent	
	BAW[a]	PW[b]
Sugars		
Fructose	0.21	0.55
Glucose	0.17	0.40
Maltose	0.10	0.33
Sucrose	0.12	0.37
Amino acids		
Aspartic	0.16	0.19
Glycine	0.19	0.41
Glutamic	0.26	0.31
Proline	0.38	0.90
Lysine	0.45	0.80
Serine	....	0.40
Histidine	0.18	0.70
Methionine	0.53	0.79
Valine	0.39	0.73
Phenylalanine	....	0.90

[a] BAW = Butanol-acetic acid-water solvent.
[b] PW = Phenol-water solvent.

Table 2. Aromas generated at pH 6.5 by the reaction of various sugars and various amino acids at 100 and 180°C.

	Glycine		Glutamic		Lysine		Methionine		Phenylalanine	
	Aroma	Color	Aroma	Color	Aroma	Color	Aroma	Color	Aroma	Color
At 100°C										
Glucose	Caramelized sugar NO	DB	Old wood small P	Y	Baked sweet potato NO	DB	Overcooked sweet potato NO	B	Rancid caramel candy UP	DY
Fructose	Caramel smell UP	DR	Too weak to describe	YO	Frying butter O	O	Chopped cabbage O	Y	Stinging smell VO	LY
Maltose	Too weak to describe	YO	Too weak to describe	YO	Burned wet wood UP	YO	Overcooked cabbage O	YO	Sweet caramel P	YO
Sucrose	Weak NH_3 O	LY	Caramel smell P	LY	Rotten raw potato UP	LY	Burned wood smell UP	LY	Sweet caramel smell UP	LY
At 180°C										
Glucose	Burned candy NO	DB	Chicken tray smell O	DB	Burned fried potato NO	DB	Cabbage smell O	DB	Caramel smell NO	DB
Fructose	Beef broth P	DR	Chicken manure O	DR	Fried potato NO	DR	Bean soup smell NO	BR	Dirty dog smell VO	DR
Maltose	Beef broth NO	DR	Baked ham NO	DR	Stale potato O	DR	Harsh horse-radish UP	OR	Sweet caramel P	YO
Sucrose	Beef broth NO	DR	Charred meat NO	DR	Boiled meat P	DR	Overcooked cabbage VO	BR	Chocolate smell P	BR

ABBR: P = pleasant; O = objectionable; NO = not objectionable; UP = unpleasant; and VO = very objectionable; Y = yellow; O = orange, B = brown; BR = black red; DR = dark red; DB = dark brown; LY = light yellow; OR = orange red; and YO = yellow orange.

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Sensory evaluation of aromas generated. Table 2 summarizes the sensory evaluation of the aromas evolved from reacting sugars and amino acids at 100 and 180°C. The pH of all the mixtures was 6.5. The aroma was described by 3 judges with previous experience in evaluating the aroma of foods and chemical mixtures. As anticipated, various odors were produced. The aromas developed when sugars and amino acids were heated at 180°C were different from those produced at 100°C. The aromas produced by the interaction of sugars and amino acids ranged from pleasant to objectionable. Glutamic acid gave a meaty-type odor with the majority of the sugars examined. Methionine was always associated with a sharp and distinctive odor which resembled overcooked cabbage, especially at the higher temperature. This may be attributed to the sulfur compounds released during the reaction. The volatile odors emitted from the reaction of lysine and the sugars were associated with a potato smell. The aroma given off by phenylalanine was described, in most cases, as sweet caramel.

Relative reactivity of individual sugars and amino acids. The amino acids varied greatly in the ease with which they reacted with each sugar. In general, glycine showed the greatest and phenylalanine the least reactivity toward the four sugars used. When the weight of the DNPH crystalline derivatives was considered as a criterion for reactivity, sucrose showed the greatest reactivity, followed by glucose, fructose, and maltose (Table 3). When browning was considered the measure of reactivity, maltose and sucrose were less reactive than glucose and fructose, regardless of the amino acids. The amount of browning varied widely as influenced by temperature, type and the chain length of the amino acid, and kind of sugar present. Hydroxylation of the C-2 position of an aldose is essential for a significant degree of browning (Haugaard *et al.*, 1951; Wolfrom *et al.*, 1947). The Amadori rearrangement is a key reaction for browning (Hodge, 1953). These, coupled with the fact that an elapse of time is required for hydrolysis to occur, might account for the low intensity of browning of the disaccharides. Lysine produced the greatest increase in color formation, followed by glycine, methionine, glutamic acid, and phenylalanine.

Volatile carbonyls formed. Table 4 shows the volatile carbonyls obtained from the reaction of the sugars at 100 and 180°C with the five amino acids. The aldehydes and ketones were identified tentatively by comparing their relative retention times with those of authentic carbonyls on two columns.

A total of 85 compounds were detected. Identified in the 20 combinations of the four sugars and five amino acids were ethanal, propanal, isobutanal, butanal, acrolein, crotonal, heptanal, acetone, 2-butanone, 2,3-butanedione, and 2-pentanone. Because of the unavailability of many authentic carbonyls, the identity of the other compounds could not be determined.

The number of peaks on both columns was significantly greater when the reaction was made to occur at 180°C as compared with 100°C. The volatiles formed from the reactions between the four sugars and methionine at 180°C could not be identified. The instrument did not respond properly when the volatiles from these reactions were injected. In every attempt to resolve them, drift in the base line to the upper part of the chart occurred, apparently from a poisoning effect of the sulfur compounds on the detector.

Table 3. Weight in grams of DPNH derivatives collected from 100 ml of solution at 100 and 180°C.

	Glucose		Fructose		Maltose		Sucrose	
Amino Acid	100°C	180°C	100°C	180°C	100°C	180°C	100°C	180°C
Glycine	.....	0.7010	0.3248	0.8090	.	1.314	0.5499	1.3880
Glutamic acid	.....	0.7780	0.2396	0.6500	0.1490	0.5395	0.4650	1.2048
Lysine	.....	0.4185	0.3752	0.4196	0.2258	0.9240	0.3825	0.9420
Methionine	.....	0.9776	0.2862	1.3462	0.2392	.	0.3365	.
Phenylalanine	0.2707	0.6560	0.1503	0.5640	0.2300	0.7970	0.3285	0.9594

Table 4. Volatile carbonyl compounds formed by reacting various sugars and amino acids at pH 6.5 at 100 and 180°C.

	Glucose					Fructose					Maltose					Sucrose				
	Gly.	Glut.	Lys.	Meth.	Phy.	Gly.	Glut.	Lys.	Meth.	Phy.	Gly.	Glut.	Lys.	Meth.	Phy.	Gly.	Glut.	Lys.	Meth.	Phy.
At 100°C																				
Ethanal	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propanal	+	+	+	+	+	(+)	(+)	+	+	+	+	(+)	(+)	+	(+)	+	+	+	+	+
Acetone	--	--	--	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Isobutanal	--	-	--	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+
Acrolein	+	+	+	(+)	(+)	--	(+)	-	-	-	-	-	-	-	-	(+)	(+)	-	-	-
Butanal	-	(+)	(+)	+	--	--	--	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Butanone	+	+	+	(+)	+	-	--	-	-	-	-	-	-	(+)	-	-	--	-	-	-
2,3-Butanedione	+	(+)	+	+	+	(+)	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+
Crotanal	+	-	-	(+)	-	(+)	(+)	+	(+)	(+)	-	-	-	+	-	-	-	-	-	-
2-Pentanone	-	-	-	-	-	--	-	-	+	-	-	-	-	-	-	-	--	-	-	-
At 180°C																				
Ethanal	-	-	-		-	+	+	+	+		+	+	+		+	+	(+)	(+)	(+)	
Propanal	+	+	+		+	+	+	+	+		+	+	+		+	(+)	+		(+)	
Acetone	+	+	+		+	--	-	-	-		+	+	+		+	+	+		+	
Isobutanal	-	-	-		-	+	+	+	+		+	+	+		(+)	+	+		+	
Acrolein	-	-	-		-	(+)	(+)	--	(+)		(+)	(+)	(+)		(+)	-	-		(+)	
Butanal	+	+	+		+	(+)	-	(+)	-		-	-	-		-	-	-		-	
2-Butanone	+	+	+		(+)	(+)	+	(+)	+		+	(+)	+		(+)	+	+		+	
2,3-Butanedione	-	-	-		(+)	(+)	(+)	(+)	+		+	-	-		+	(+)	+		-	
Crotonal	+	(+)	+		+	(+)	(+)	(+)	+		-	-	-		+	-	-		+	
2-Pentanone	+	(+)	+		+	(+)	+	+	+		(+)	+	+		(+)	-	-		(+)	
Heptanal	-	-	(+)		(+)	-	--	--	(+)		-	-	-		-	-	-		-	

+ Detected on two columns; (+) Detected on one column; -- Not detected. Gly = glycine; Glut = glutamic acid; Lys = lysine; Meth = methionine; Phy = phenylalanine.

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Table 5. Volatile carbonyl compounds formed by reacting glucose and glutamic acid at 100 and 180°C at pH 5.0, 6.5, and 8.0.

	At 100°C at pH:			At 180°C at pH:		
Compound	5.0	6.5	8.0	5.0	6.5	8.0
Ethanal	+	+	+	+	–	+
Propanal	+	+	+	+	+	+
Acetone	+	–	+	(+)	+	+
Acrolein	–	+	–	(+)	–	(+)
Butanal	–	(+)	–	+	+	(+)
2-Butanone	+	+	+	+	+	+
2,3-Butanedione	+	(+)	+	+	–	+
Pentanal	+	–	+	–	–	–
Crotonal	–	–	(+)	(+)	(+)	(+)
2-Pentanone	–	–	–	–	(+)	–

\+ Appeared on two columns
(+) Appeared on one column
– Not detected

Effect of pH on the volatile carbonyls formed. Table 5 shows the volatile carbonyls generated by the reaction of glucose and glutamic acid at pH 5.0, 6.5, and 8.0 at 100 and 180°C. The amount of browning produced at 100°C increased moderately as the pH increased toward the alkaline range. At 180°C, the respective colors for pH 5.0, 6.5, and 8.0 were orange, deep red, and black. The number of carbonyl compounds produced by the glucose–glutamic acid system at the three pH levels was almost equal at 100°C. When the systems were heated at 180°C, the number of volatiles was greater and increased more drastically as pH increased.

Volatile carbonyls generated in the potato substrates. Table 6 shows the volatile carbonyls formed when the synthetic potato medium was heated at 100°C and the potato extract at 180°C. The difference in the heat treatment was due to the fact that heating the synthetic potato medium at 180°C formed a caramelized mass devoid of liquid. The potato extract was heated at 180°C to simulate the process of frying potato chips. The volatiles isolated confirmed that the sugars and amino acids reacted in mixed systems and that many of the same carbonyls were formed.

For the potato substrates as for the amino acid–sugar mixtures, the number of carbonyls formed increased as the temperature was raised. As already pointed out, the total yield of carbonyls was greater at 180 than at 100°C (Table 3), and browning was greater at the higher temperature. Hodge (1953) has reviewed possible browning mechanisms and end products formed. This study was not designed to elucidate the mechanism of carbonyl formation. However, since all the various amino acid–sugar combinations tended to yield carbonyls differing by only one carbon length, breakdown of the compounds appears to have been a step-wise degradation. The Wohl degradation or thermal decarboxylation (Roberts and Caserio, 1964) might account for the decrease of chain length by one unit. The data suggest that coupling of compounds might have also occurred. Lysine and phenylalanine with glucose or fructose yielded heptanal (Table 4). Of the four compounds, none of the initial reactants contained over 6 carbon units except phenylalanine. An aldol condensation could account for the increase in chain length.

Even though only 14 carbonyls were identified, 85 were detected as judged by gas chromatographic retention times. Some of the carbonyls identified are fairly odoriferous; the others are quite mild. The odors listed in Table 2 probably did not come from the carbonyls alone. In the heating of the amino acid–sugar mixture, noncarbonyl compounds very likely were formed

Table 6. Volatile carbonyl compounds generated upon heating a synthetic potato medium for 60 min at 100°C and a potato extract for 30 min at 180°C.

Carbonyl compound	Synthetic potato medium	Potato extract
Ethanal	+	+
Propanal	+	+
Acetone	+	–
Isobutanal	–	–
Acrolein	–	–
Butanal	+	+
2-Butanone	+	–
2,3-Butanedione	+	–
Crotonal	+	+
2-Pentanone	–	–
Pentanal	–	+
Heptanal	–	+
Hexen-1-al	–	+

A plus sign shows the compound was present, a minus sign shows it was absent.

may have contributed to the odors by the three judges. The fact particular types of odors were produced amino acid–sugar mixtures is im- both basically and for applied rea- That there are differences in the odor by different combinations has been for some time. This study is im- as a first step in relating the com- of the volatiles to odor differences conditions which result in the generation

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EFFECT OF GROWTH OF *PSEUDOMONAS PUTREFACIENS* ON DIACETYL AND FLAVOR OF BUTTER[1]

P. R. ELLIKER AND B. E. HORRALL

Purdue Agricultural Experiment Station, Lafayette, Indiana

The cheesy and putrid defects[2] of butter are among the most serious manufacturing problems confronting the butter industry of Indiana. Numerous reports emphasize the significance of this problem elsewhere. Studies in this laboratory indicate that in the surrounding region the putrid defect and perhaps in most cases the pronounced cheesy defect are due to species of *Pseudomonas putrefaciens*. Certain of the less pronounced cheesy defects, however, appear to be due to other related water bacteria capable of some lipolytic and proteolytic action on milk and butter constituents.

During preliminary investigations on the nature and cause of these defects it was repeatedly noted in butter containing neither salt nor starter that the first apparent change produced by *Ps. putrefaciens* was loss of the typical full, clean odor. The butter at this stage appeared to have completely lost its normal odor and instead was characterized by a distinctly flat and in some cases by a slightly "oxidized-like" odor.

When butter was made with added diacetyl, the occurrence of cheesy or putrid defects was invariably preceded by almost complete destruction of all diacetyl aroma. The obvious conclusion was that *Ps. putrefaciens* and possibly related organisms destroyed the compounds essential to high aroma of butter. The ability of *Ps. putrefaciens* to destroy added diacetyl in milk and butter was therefore studied.

HISTORICAL

The subject of cheesy, also sometimes referred to as putrid, rabbito, surface taint, fetid, or limburger defect in butter has been reviewed (2, 3, 5, 6, 7, 14, 23). These papers, with many others, indicate the widespread occurrence of this general type of defect.

Considering the tremendous volume of research on production of aroma compounds in dairy products and other foods, comparatively few reports are available on the effect of microorganisms on diacetyl. Neuberg and Nord (13) and Nagelschmidt (12) demonstrated the ability of yeasts to reduce diacetyl to 2, 3-butylene glycol. Michaelian and co-workers (10) noted a

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[2] The defects referred to in this paper have, in every case, been produced by bacteria growing in the butter after its manufacture and should not be confused with the cheesy defect of cream which may be carried into butter.

rapid decrease of diacetyl in a well-ripened butter culture when neutralized to a low acidity and held at a temperature favorable for development of citric acid fermenting streptococci. Subsequent studies (4) showed that *Streptococcus citrovorus* and *Streptococcus paracitrovorus* reduce diacetyl to acetylmethylcarbinol and 2, 3-butylene glycol. Wiley *et al.* (21) reported that betacocci produced diacetyl rapidly and then actively destroyed it. Virtanen and Kontio (20) investigated the action on diacetyl of various microorganisms isolated from butter. Diacetyl and acetylmethylcarbinol were added to sterile milk and respective lots inoculated and incubated at 19–21° C. for periods up to 160 hours. *B. punctatum* destroyed about 90 per cent of both compounds. *B. vulgatus* destroyed 30 to 50 per cent of the diacetyl but only 9 per cent of the carbinol. A mixture of yeasts destroyed 30 to 40 per cent of the diacetyl and as much as 30 per cent of the acetylmethylcarbinol. Certain strains of the colon-aerogenes group, green fluorescent bacteria and a number of species of aerobic spore formers are able to destroy acetylmethylcarbinol (22).

Slatter and Hammer (18) and Prill and Hammer (16) observed both increases and decreases in the diacetyl and acetylmethylcarbinol content of unsalted butters made from sweet cream with a butter culture. Salt appeared to prevent significant increase or decrease of diacetyl and acetylmethylcarbinol. However, in one lot of butter containing only 0.75 per cent salt there occurred a relatively rapid disappearance of these compounds. The increases and decreases were attributed to aroma bacteria from the starter. Toth (19) reported that in butter prepared from sour cream, diacetyl increased for some days during storage and later decreased. In butter from sweet cream a constant decrease took place during storage. According to Mohr *et al.* (11) the diacetyl content of butter during storage may remain constant or may decrease. In one trial they noted a pronounced decrease of diacetyl in unsalted but not in salted butter stored at 10° C. for 10 days. The diacetyl content of salted and unsalted butters decreased at about the same rate when the butters were stored at −20° C. Bunger (1) reported increases followed by decreases of diacetyl in butter.

Some investigators (8, 9, 17, 23) have indicated that diacetyl has an inhibitory effect on bacteria. The inhibiting effect of diacetyl on growth of various organisms in sterile skim milk has been observed by us. However, the inhibiting concentrations were higher than would occur in average butter.

EXPERIMENTAL

The apparent ability of putrid butter organisms to destroy diacetyl was noted both in commercial salted butter and in unsalted laboratory samples. Since in laboratory churnings the usual percentage of salt appeared to inhibit development of *Ps. putrefaciens*, no salt was added to the experimental butter. Under commercial conditions the salt sometimes provides

less assurance of complete inhibition of this organism than under laboratory conditions.

Sterilized cream in 2000-ml. quantities was churned at about 7.2° C. (45° F.) in sterile "Dazey" churns. The butter was inoculated with *Ps. putrefaciens* by adding a sufficient quantity of a 24-hour beef infusion broth culture to provide about 1,000,000 cells per ml. of wash water. After one washing with either sterile or contaminated water, the butter was worked in the churns with large, sterile, metal spoons. A volume of water equal to the original volume of cream was used. This appeared to adequately wash the butter granules and the large number of organisms in the water provided sufficient inoculation of the butter.

Diacetyl in the form of starter distillate was added to the butter after washing and partial working. Five per cent of starter was added a short time before churning to some lots of cream. The starters used did not appear to have a high aroma, but the butters prepared from them always had a desirable aroma. After thorough working, the butter was placed in

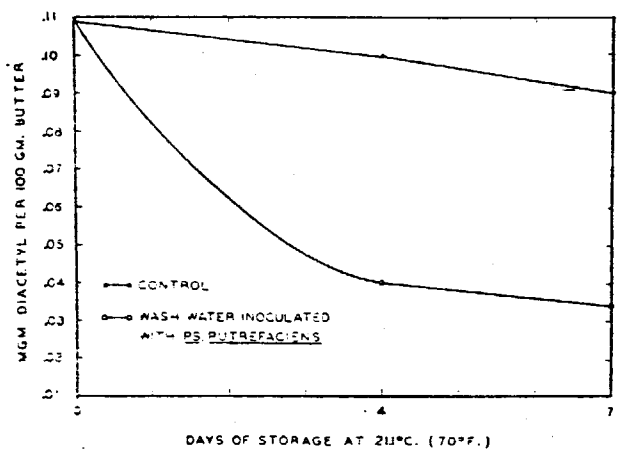

FIG. 1. Effect of growth of *Pseudomonas putrefaciens* on diacetyl added in the form of starter distillate to butter.

approximately 100-gram amounts in sterile 4-ounce sample jars and stored at 21.1° C. (70° F.). Samples were examined for odor and flavor, daily, and for diacetyl content on the day of preparation and again after 4 and 7 days of storage. The method for determining diacetyl was essentially that of Prill and Hammer (15).

Figures 1 and 2 present a summary of the results of several experiments. The analyses substantiated earlier observations on loss of aroma in butter due to growth of *Ps. putrefaciens*. Within 7 days at 21.1° C. the diacetyl content was reduced from 0.109 mgm. to 0.034 mgm. per 100 gm. In the sterile control only a slight drop occurred during the 7-day period.

The diacetyl contents of the starter butters were comparatively low. One

evident reason for this was the fact that the starter culture employed had a low diacetyl content. Also the fact that the starter was added only shortly before churning undoubtedly resulted in the butter carrying over less diacetyl from the starter than would have been true if starter and cream had been in contact for a period of several hours.

The starter butter containing no *Ps. putrefaciens* showed a slight increase in diacetyl. Such increases were noted in other experiments. In some samples containing starter plus added starter distillate, marked decreases occurred. This might be expected in the light of other reported investigations. When *Ps. putrefaciens* was present, the diacetyl in starter butter decreased slightly. The main purpose of adding starter in this study was to determine the effect of *Ps. putrefaciens* on diacetyl in butter when its activity was inhibited by starter bacteria. Cultures of *Streptococcus lactis* added in the same manner as starter cultures also effectively inhibited development of *Ps. putrefaciens*.

The aroma and clean, full flavor of both starter and uninoculated starter distillate samples were maintained throughout the storage period. Samples of inoculated butter containing starter distillate became flat and lost all aroma in 24 to 48 hours and within several hours thereafter became definitely putrid.

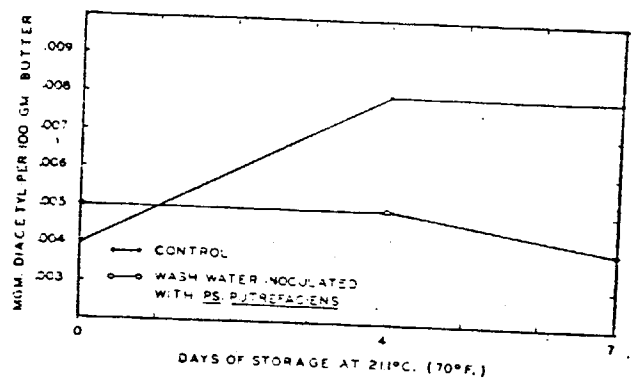

FIG. 2. Effect of *Pseudomonas putrefaciens* on diacetyl in butter made with starter.

Results of other experiments showed comparatively rapid destruction of diacetyl at refrigeration temperatures when active development of *Ps. putrefaciens* occurred in the butter. In one such trial the diacetyl content fell from 0.080 mgm. per 100 grams of butter to 0.040 mgm. in 4 days at 7.2° C. and in the corresponding sample at 21.1° C. it fell to 0.025 mgm. in 2 days.

DISCUSSION

In general the results indicated relatively rapid destruction of diacetyl in butter under conditions favorable to the growth of *Ps. putrefaciens*. The rapid disappearance at 7.2° C. (45° F.) coincided with observations on commercial samples that developed a cheesy or putrid defect under normal

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storage and retail conditions. It is probable that *Ps. putrefaciens* rapidly destroyed the diacetyl in the aqueous phase. Diacetyl in the fat phase may have been sufficiently protected so that little was affected by the organisms. This explanation might account for the complete lack of aroma following growth while one-third to one-fourth of the original diacetyl still remained intact in the butter.

The diacetyl contents of starter butters, both control and inoculated, were low. Nevertheless the butter had a definite, pleasant aroma. It is possible that the aroma in the starter butter was enhanced or maintained by continual slow production of diacetyl during storage of the butter not only in the control sample but also in that where *Ps. putrefaciens* was slightly active. In the non-starter butter containing *Ps. putrefaciens* the opposite condition may have been true, namely, the organisms destroyed all diacetyl that might have been able to contribute to the aroma of the butter.

It has been suggested that diacetyl may be the component that enables butter starters to inhibit development of *Ps. putrefaciens* in butter (23). The low diacetyl contents in the starter butter, however, suggest that it is the activity of the lactic acid bacteria (probably lactic acid production) rather than presence of diacetyl that inhibits action of *Ps. putrefaciens* in butter made with starter. The ability of *Streptococcus lactis* alone to inhibit *Ps. putrefaciens* in butter further substantiates this conclusion.

One fact that should be borne in mind is that commercial butters present a somewhat different problem than laboratory churned samples. For example neither salt nor starter definitely insures the inhibition of *Ps. putrefaciens* in commercial butter. Various factors such as methods of working, printing, storage and distribution influence the activity of the microorganisms involved. However, the close correlation between changes occurring in commercial and laboratory samples definitely indicates a similarity in the sequence of changes taking place under the two different conditions.

One of the most troublesome angles in this problem, particularly to the creameryman who has been without the technical advice of a control laboratory, has been the failure to recognize the nature of the defect caused by *Ps. putrefaciens* and related bacteria. The cheesy and putrid defects sometimes have not been recognized by the butter manufacturer or handler except where they have progressed to an advanced stage. Possibly the term surface taint would be more suggestive for this defect; however, the so-called cheesy or putrid defect is usually not confined to the surface. Many commercial samples received in this laboratory or picked up in retail outlets appear to run through the whole series of changes to the final cheesy or putrid state only after periods considerably longer than they ordinarily would be stored in the household refrigerator. This means that such samples, when in the hands of the creameryman, wholesaler and retailer, and often the final purchaser, show only a flat, disagreeable odor and flavor difficult to identify. Many creamerymen are aware that their butter is off

in flavor but recognize it only as flat. Part of a recent large shipment was returned to one manufacturer with the complaint that it was "flat and metallic." This butter had a pleasing, full flavor with high aroma at the time of shipment from the creamery. The cause of this "flat" flavor was *Ps. putrefaciens*. This fact was indicated by bacteriological examination and also because the "flat" flavor was followed by the development of a typical putrid flavor. This type of flavor defect seems more common in commercial butter than is generally realized. Results to be published later indicate that species other than *Ps. putrefaciens* and the aroma bacteria may destroy diacetyl in butter.

The "metallic" flavor mentioned above may have been confused with the tallowy flavor reported by Toth (19) and others to accompany reduction of diacetyl in butter. In such occurrences the fat apparently is oxidized. When the masking effect of a strong aroma has been removed by the action of microorganisms or other factors, a tallowy or oxidized flavor might become more apparent. On the other hand our studies and those of others (23) indicate that an oxidation of certain compounds in the butter may possibly be due to *Ps. putrefaciens*. The flavor developed would undoubtedly be masked by the pronounced cheesy or putrid flavor following active growth of the organism.

SUMMARY

Observations on both commercial and laboratory samples of butter in which *Ps. putrefaciens* developed showed that the first apparent stage of decomposition by this organism was a complete loss of typical butter aroma. The butter became flat in odor and flavor. This stage was then followed by development of typical cheesy or putrid flavors.

Chemical analyses indicated that during a 7-day storage period at 21.1° C. the diacetyl content of the sterile control butter samples remained fairly constant. More than half the diacetyl was destroyed under the same conditions in similar lots of butter contaminated with *Ps. putrefaciens*.

The diacetyl content of butter prepared with starter and contaminated with *Ps. putrefaciens* remained relatively stable over a 7-day storage period at 21.1° C., although some destruction of diacetyl occurred.

Results and observations indicate that the ability of *Ps. putrefaciens* and other organisms to produce a flat-flavored butter is perhaps more prevalent than is commonly realized.

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Determination of Diacetyl

DUANE T. ENGLIS, EMILY J. FISCH[1], AND SHIRLEY L. BASH
University of Illinois, Urbana, Ill.

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Dimethylglyoxime shows an intense absorption peak near 226 mμ in the ultraviolet region of the spectrum. The absorption by the compound follows Beer's law and serves as a basis for a direct determination of diacetyl in starter distillates or distillates from other food products after its conversion to dimethylglyoxime, but without the supplementary formation of a metal complex. The results obtained with this spectrophotometric procedure compare favorably with those obtained with the gravimetric method in which the nickel compound is formed. The spectrophotometric procedure is of high sensitivity. Concentrations of 0 to 10 p.p.m. may be easily estimated. The method should be advantageous for laboratories equipped with instruments for work in the ultraviolet range.

DIACETYL has long been recognized as a constituent occurring in small amounts in vinegar, fermented cane juice, cultured milk products, and a number of other food materials. Because the amount normally present is small, relatively large samples are usually necessary to furnish enough of the compound to make possible a quantitative determination. The widely used method of Barnicoat (*2*) involves a steam distillation of the diacetyl, its conversion to dimethylglyoxime, precipitation with a nickel salt, and final gravimetric estimation as the nickel compound. Colorimetric methods have also been employed. As an alternative procedure, Barnicoat has dissolved the nickel precipitate in chloroform and estimated the material by a standard series comparison with similarly prepared solutions of known concentration.

Stotz and Raborg (*8*) suggested a sensitive method based upon an assumed tetravalent nickel dimethylglyoxime compound. Okac and Polster (*5*) confirmed the sensitivity of the method, but differed as to the nature of the colored compound. Mohler and Almasy (*4*) detected diacetyl by the specific absorption of the ferric complex. Prill and Hammer (*6*) employed the rose-red ferrous complex for the quantitative microdetermination of diacetyl. They reported that it was possible to detect as little as 0.001 mg. of diacetyl per 5 ml. of solution and to measure conveniently 0.01 to 0.5 mg. per 10 ml. (1 to 50 p.p.m.) of solution. More recently, Speck (*7*) has developed a method for diacetyl based on the formation of a purple dye with chromotropic acid in the presence of concentrated sulfuric acid. This method may be used for quantities from 0.03 to 0.10 mg. per ml. (30 to 100 p.p.m.), which is not quite as sensitive as that of Prill and Hammer. A considerable number of substances interfere or similarly respond to the reagents.

Diacetyl has only a moderate absorption in the ultraviolet region of the spectrum (*3*). However, when converted to the dimethylglyoxime it has intense absorption. This property appeared to offer promise as a means of determination of diacetyl in various food products without the necessity of conversion to a metallic compound.

[1] Present address, University of California, Los Angeles, Calif.

Table I. Absorbance of Aqueous Dimethylglyoxime Solutions at 226 mμ

Mg. per 100 Ml. Dimethylglyoxime	Diacetyl	Absorbance, 1-Cm. Cell
1.130	0.838	1.348
0.565	0.419	0.655
0.451	0.335	0.540
0.226	0.168	0.265

EXPERIMENTAL

A Beckman Model DU spectrophotometer and a Cary Model 11 recording instrument were employed for the study. In order to establish the absorption curve of pure dimethylglyoxime, the reagent-grade material was first dried at 100° to 105° C. for 1 hour. A carefully weighed portion was then dissolved in water, and the solution was examined with the Cary spectrophotometer. In this aqueous solution an absorption peak (Figure 1) was observed at 226 mμ and the calculated molecular extinction coefficient was 13,400.

A similar experiment with 95% ethanol as the solvent yielded an absorption curve of similar nature, but the peak was shifted slightly (about 2 mμ) toward the longer wave-length side and was of higher intensity. The molecular extinction coefficient for the compound in the ethanol solution was of the order of 18,300.

Because of the greater intensity of absorption in the ethanol solvent, some experiments were carried out in the early stages of the investigation in which the oximation reactions and the final spectrophotometric evaluations were made in ethanol solutions. Satisfactory results were obtained, but no marked advantage was apparent over the use of aqueous solutions. As a consequence, all data are for aqueous solutions. The experimental results on concentration and absorbance relationships for the aqueous solutions (Table I) show good conformance to Beer's law. The wave length for the absorption maximum of dimethylglyoxime is subject to change with pH. The peak observed at 226 mμ at pH 5.4 for the aqueous solution shifted to a position near 260 mμ when the solution was made basic with potassium hydroxide to a pH of 10.6. However, the solution buffered with sodium acetate at pH 7.00 showed no displacement of the peak from its position at pH 5.4.

For the preparation of the glyoxime compound, Vizern and Guillot (*9*) specify the use of 1 ml. of 10% hydroxylamine hydrochloride and 1.7 ml. of 0.1 *N* sodium hydroxide for each milligram of diacetyl present. Preliminary tests with the spectrophotometer showed that the reagents themselves absorb strongly.

For both sodium acetate and hydroxylamine hydrochloride, an absorption maximum is indicated below 210 mμ, which is beyond the working range of the instrument. With the high concentration of these reagents which are employed, the side band of the oximation reagent mixture entirely obliterated the characteristic peak of the dimethylglyoxime. However, when the reagents were mixed with pure dimethylglyoxime and compared to a blank solution containing the same quantities of reagents, the characteristic dimethylglyoxime curve was observed. A similar observation was made when diacetyl was converted to the glyoxime and the solution compared to a blank containing the same amounts of reagents. It was evident from this experiment that the quantity of reagent consumed in the formation of the reaction product, dimethylglyoxime, was insignificant in comparison to the excess of reagent left in solution.

The addition of potassium hydroxide to the hydroxylamine hydrochloride caused a shift in the absorption toward the longer wave-length region. Hence, control of pH in the determination is necessary. In actual practice, this acetate buffer has accomplished this control very satisfactorily, and no difficulty has been experienced with the pH factor.

Other preliminary experiments employing the conditions of conversion specified by Vizern and Guillot served to indicate a slow rate of reaction and apparent incomplete conversion on some occasions. The modified Barnicoat method (*2*) calls for equal amounts by weight of sodium acetate and hydroxylamine hydrochloride as the converting reagents. The final step involves a concentration to a small volume, so that a heat treatment is applied which probably contributes to the completeness of the reaction. Prill and Hammer (*6*) use hydroxylamine acetate and prescribe heating at 85° C. for 1 hour to form the dioxime. In the present work, experiments on conversion were carried out in which the quantities of reagents, pH, and periods of reaction were varied.

PROCEDURE

Weigh out a sample of such size as to contain 0.1 to 1.0 mg. of diacetyl. Remove the diacetyl from the food product by distillation in the usual way. Convert the diacetyl with 20 ml. of 10% hydroxylamine hydrochloride and 10 ml. of 40% sodium acetate. Heat the reaction mixture for 1 hour at 85° C. according to the directions of Prill and Hammer, or allow to stand overnight at room temperature. Dilute the solution to 100 ml. and determine the absorbance at 226 mμ, using for a comparison solution one containing identical quantities of reagents in a final volume

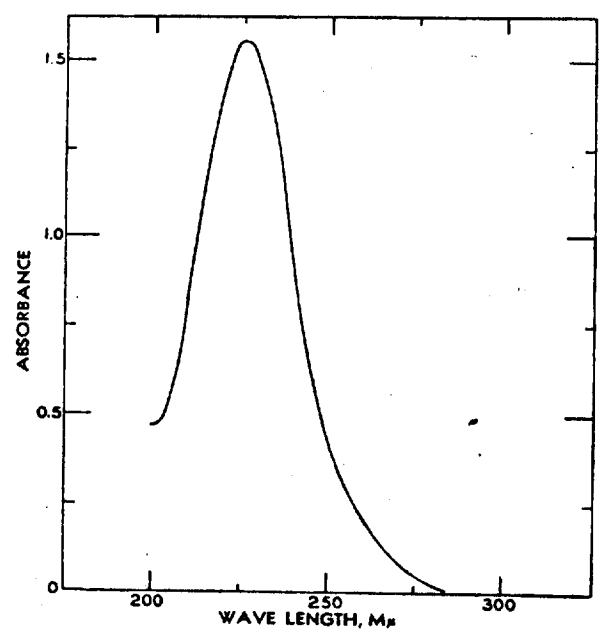

Figure 1. Absorption Curve for Dimethylglyoxime

1.38 mg. per 100 ml. in water

equal to that used for the sample. Refer to the prepared working curve based on spectrophotometric evaluation of standard solutions of pure dimethylglyoxime (as shown in Table I) for indication of the diacetyl content.

COMPARISON OF SPECTROPHOTOMETRIC AND GRAVIMETRIC METHODS

In order to test the proposed method, samples of starter distillates from cultured milk were secured from a commercial source. These had been analyzed by the producer, but no information wa furnished as to the method used. Upon their arrival, the samples were stored in a refrigerator, and considerable time elapsed after the receipt of the samples before they were subjected to examination. Although slight changes may have taken place during storage, the conditions for preservation were such as to assure rea-

the final procedure was developed, but data for a final comparison of methods are given for one sample.

The sample was analyzed first by the Barnicoat gravimetric method in which the precipitated nickel dimethylglyoxime was filtered out on a tared sintered-glass crucible, washed with ether, dried, and finally weighed. The spectrophotometric analysis was carried out as previously described. The results are given in Table II. Slightly higher values are to be noted for the spectrophotometric method. However, in view of the small amounts of material being determined the results are in reasonably good agreement.

Several advantages may be cited for the spectrophotometric method. It requires less time to make a determination, it is more sensitive, and it is free of errors resulting from solubility losses which are inherent in the gravimetric method.

Table II. Determination of Diacetyl in Starter Distillate

Gravimetric[a]

Nickel Dimethylglyoxime, Grams	Diacetyl, Mg. In portion analyzed	Diacetyl, Mg. Per ml. of original sample
0.1251	74.6	37.3
0.1249	74.4	37.2
0.1246	74.3	37.1
0.1251	74.6	37.3
0.1247	74.0	37.0
0.1247	74.3	37.0

Spectrophotometric[b]

Absorbance, 1-Cm. Cell at 226 mμ	Diacetyl, Mg. Per 100 ml. of portion analyzed	Diacetyl, Mg. Per ml. of original sample
Series A[c] 1.26	0.798	39.8
1.25	0.792	39.6
1.24	0.785	39.2
1.31	0.830	41.5
1.24	0.785	39.2
Series B[d] 1.25	0.792	39.6
1.28	0.806	40.3
1.26	0.796	39.8
1.27	0.804	40.2

[a] Equivalent of 2 ml. of starter subjected to analysis.
[b] Equivalent of 1 ml. of starter diluted to 50 ml.; then 1 ml. taken, converted to dimethylglyoxime, and made up to 100 ml.
[c] Analyzed by the outlined procedure.
[d] Only half the quantities of reagents used in Series A were employed.

For a high concentration of diacetyl, such as was characteristic of the sample whose analysis is reported, a considerable dilution is necessary before the spectrophotometric method may be employed. Any error in the estimation is then multiplied by the dilution factor, even though the relative effect remains the same on the final result. For high concentrations the gravimetric method may be advantageous. The quantities of reagents are not critical since in Series B, which employed only half the amounts of hydroxylamine hydrochloride and sodium acetate used in Series A, the results were essentially the same.

The commercial distillates, as sold to the butter manufacturers, have usually been subjected to oxidation so that the acetoin (3-hydroxy-2-butanone) originally present has been oxidized to diacetyl. In certain food products, one may be concerned with distillate mixtures in which the acetoin is present in quantities many times greater than the diacetyl. The question may then be raised as to the possible interference of the oximation products of these other substances. As an illustration of a monoxime, acetoxime shows high absorption (ϵ_{max} is 5000), but this is at 190 mμ in the far ultraviolet region (*1*). One might predict that acetoin would have similar absorption, although the adjacent hydroxyl group might be expected to affect it to some degree.

To test the possible interference of acetoin, a sample of this material was secured and an aqueous solution prepared. A portion of the solution was added to a sample of starter distillate of known diacetyl content so that the ratio of acetoin to diacetyl was about 25 to 1. The mixture was then subjected to the oximation procedure and subsequent spectrophotometric analysis.

An apparent diacetyl value equivalent to about double the amount known to be present in the distillate was observed. However, an examination of the oximated acetoin alone showed an absorption curve identical with that of dimethylglyoxime. The typical red precipitate was given with a nickel salt, and it was apparent that the acetoin contained about 4% diacetyl. Assuming that the balance of the sample was acetoin, there was no evidence of its presence as a result of the oximation reaction.

Attempts were made to determine diacetyl in butter and butter substitutes without a preliminary distillation. None of these attempts were successful. The determination of acetoin after the usual oxidation with ferric chloride and subsequent distillation constitutes a simple extension of the procedure.

During the progress of the work, an attempt was made to use diacetyl procured from a chemical supply source as a primary standard for the preparation of the dimethylglyoxime and establishment of a working curve. An analysis for diacetyl by the gravimetric method indicated a purity of only 96%. Pure dimethylglyoxime is a much more satisfactory standard.

Some interesting differences in the rate of formation and other factors incidental to the precipitation of the nickel compound were noted, as the original materials were varied. Even though each test represented about the same concentration in terms of diacetyl and all precipitations were made under as nearly identical conditions as possible, the results were slightly different.

For example, the precipitate from pure dimethylglyoxime formed most readily and had a rose-red or slightly bluish color. The precipitate from the "pure" diacetyl formed more readily than did that from the starter distillate. Furthermore, it was a true red, while that from the starter distillate was a brownish red color and more lumpy in character. It is possible that some slight impurity in the starter distillate retarded the formation of the precipitate and may, in some cases, have caused it to be incomplete. The filtrate from the distillate reaction product was of a reddish color, while with the other materials the greenish color of the excess nickel ion predominated.

The most reliable standard curve for spectrophotometric determinations is one prepared from pure dimethylglyoxime. Since concentrations of the order of 1 mg. per 100 ml. (10 p.p.m.) and less can easily be determined with a 1-cm. absorption cell, the sensitivity of the method can be proportionately increased with the use of cells of greater length. In many cases, a total volume of reaction mixture smaller than 100 ml. could be used to attain a similar result. The method compares very favorably with that of Prill and Hammer (*6*) in sensitivity. It is believed the procedure will be advantageous wherever suitable ultraviolet equipment is available.

ACKNOWLEDGMENT

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Polarographic Determination of Diacetyl in Buttermilk

William P. Forrest
Department of Chemistry, Wagner College, Staten Island, New York
and
Norman Shane and Robert Schweigerath
Research Laboratories, Bristol-Myers Corp., Hillside, New Jersey

SUMMARY

A method employing polarography has been developed to determine the presence and amount of diacetyl (2,3-butanedione) in buttermilk. Mixtures of buttermilk and 0.2*N* ammonium chloride (1:1) yielded a polarographic half-wave at 0.76 volts vs SCE (saturated calomel electrode). Changes in diffusion current were related to diacetyl content of buttermilk samples in the parts per million range.

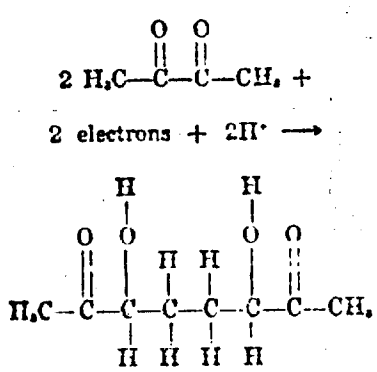
INTRODUCTION

Determination of volatile flavor components in food products often involves a sequence of separatory techniques such as extraction, distillation, selective precipitation and, more recently, gas chromatography. The polarographic method can often be successfully applied to the determination of organic compounds present as flavor components in certain food products. In the present effort, diacetyl was determined in buttermilk in the parts per million range. The determination of diacetyl (and its precursor, acetylmethylcarbinol) is often carried out by the method of van Niel (1927) as modified by Michaelian *et al.* (1933).

Sequence required in this method is as follows: 1) oxidation of product to convert any acetylmethylcarbinol present to diacetyl; 2) steam distillation of product to yield total diacetyl (original diacetyl plus diacetyl formed by oxidation); 3) trapping of distillate in a solution containing hydroxylamine hydrochloride, sodium acetate, and nickel chloride; 4) development of the characteristic red color due to the formation of the nickel salt of dimethylglyoxime (time required for the gravimetric method of Michaelian *et al.* (1933) may be as long as 48 hr); 5) collection of the red nickel salt in a tared gooch or sintered glass crucible; 6) weighing on analytical balance to determine weight of nickel salt; 7) calculation of amount of diacetyl in distillate; 8) conversion of distillate based calculation to determination of diacetyl present in original sample.

Variations on the method have been proposed by Canales and Martinez (1962) and Owades and Jakovac (1963) in the determination of diacetyl in beer. These and other methods described in the literature are essentially all variations on the original Tschugaeff reaction as first used by van Niel (1927). Polarographic determinations of diacetyl *per se* were carried out by Shikata and Tachi (1930), Semerano and Polaczek (1940), Winkel and Proske (1938), Adkins and Cox (1938) and many other workers. The polarographic process utilized in the present method involves the reduction of one of the carbonyl groups of diacetyl to a hydroxyl group at the dropping mercury electrode.

The polarographic process takes place at −0.76 volts relative to the saturated calomel electrode. At a higher voltage (approximately −1.7 volts) the second carbonyl group is reduced to a hydroxyl group; hence any acetoin present will not interfere with the measurement of diacetyl at the first half-wave. Semerano and Polaczek (1940) suggest that the reduction product of the first half-wave may actually be a dimer. The polarographic process may possibly be as follows:



The importance of diacetyl as a natural flavor component resulting from the metabolism of microorganisms such as *Leuconostoc citrovorus* and *Leuconostoc dextranicum* (sometimes called *streptococcus citrovorus* and *streptococcus paracitrovorus*) in numerous food products ranging from butter, beer, honey, fruit, roasted coffee is well established. It seemed desirable to apply the polarographic method to the *direct* determination of diacetyl *(in situ)* in a typical member of this group, i.e., buttermilk. Our objective was to eliminate as many preliminary steps as possible and to evolve a rapid, direct and specific method to be carried out *in the product* and not on a distillate.

EXPERIMENTAL

Numerous microampere vs voltage curves were obtained on buttermilk samples using a variety of polarographic instruments. Instruments used at various times (since 1960) have included: 1) Fisher Electropode; 2) Sargent Model XV Recording Polarograph; 3) Leeds and Northrup Recording Polarograph; 4) Malmstadt-Enke Heath EUW-402M Recording Polarograph. Method evolved requires the following materials and procedures.

Reagents. All compounds used were reagent grade. Distilled water was used throughout. 2,3-butanedione (diacetyl) was obtained from Eastman Organic Chemicals (#1501), as was 3-hydroxyl,2-butanone (acetylmethylcarbinol or acetoin) (#3783). Ammonium chloride (#0660), sodium acetate (#3466), nickelous chloride (#2768), hydroxylamine hydrochloride (#2196), ferric chloride (#1990) from J. T. Baker. All buttermilk used was purchased on the open market.

Stock solutions. There were 10.6 g of ammonium chloride dissolved in distilled water and made to a total volume of one liter to yield 0.2*N* solution. Gravimetric methods used in conjunc-

tion with the present method required the following: A) 40 g of ferric chloride; B) 20 g of hydroxylamine hydrochloride; C) 20 g of sodium acetate; D) 10 g of nickelous chloride; and E) 61.3 mg of diacetyl. These were each dissolved in distilled water to a total volume of 100 ml to yield five stock solutions, i.e., *A*, *B*, *C*, *D*, and *E*.

Analytical procedure. The polarographic method described in this paper may be carried out directly using pure diacetyl as the primary standard. Gravimetric methods used in conjunction with the present method will be described under Results and Discussion.

Treatment of sample. Thoroughly mix equal volumes of buttermilk and 0.2*N* ammonium chloride solution together. A convenient volume is 50 ml of each.

Polarographic analysis. Add 15 ml of the sample solution (now 0.1*N* with respect to ammonium chloride) to the sample side of a polarographic *H* cell (or other suitable sample cell). The SCE (saturated calomel electrode) is employed as the reference electrode. Remove oxygen from the sample system by means of nitrogen bubbling. Current is then measured as a function of voltage in the range 0–2 volts to yield the polarogram of diacetyl having a half-wave of approximately −0.76 volts. Reference to the standard calibration curve relates the limiting (diffusion) current of the polarogram to the diacetyl content of the buttermilk in ppm (room temperature range, 25–28°C).

Standard calibration curve. Into 100 ml volumetric flasks, pipette 1.0, 2.0, 3.0, 4.0, 6.0, 10.0, 20.0, and 30.0 ml of stock diacetyl solution. Pipette 50 ml of 0.2*N* ammonium chloride solution into each flask. Dilute each to a total volume of 100 ml with distilled water. Measure the diffusion current of each system (noting that the half-wave voltage is the same for each system). Plot diffusion current (microamperes) as a function of the diacetyl content in ppm.

RESULTS AND DISCUSSION

Advantages of this method appear to be: 1) elimination of involved pretreatment of buttermilk prior to actual analysis; 2) use of nitrogen purge and blanket prevents conversion of any acetoin present to diacetyl; 3) method is in situ and not on product's distillate.

Correlation with method of Michaelian *et al.* (1933) was deemed desirable in order to relate this polarographic method to the established gravimetric method. Various weights of diacetyl

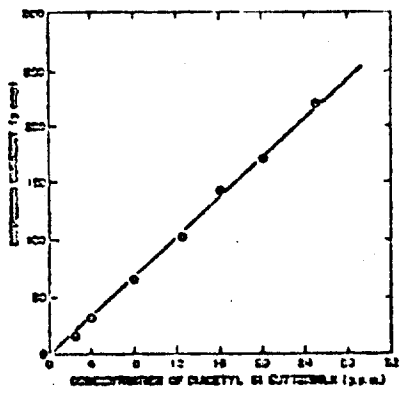


Fig. 1. Diacetyl content of buttermilk (ppm) plotted versus diffusion current.

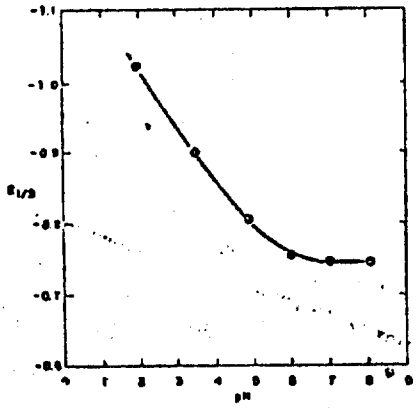


Fig. 2. Effect of varying pH upon $E_{1/2}$ of diacetyl in buttermilk (system is 0.1 *M* in respect to ammonium chloride).

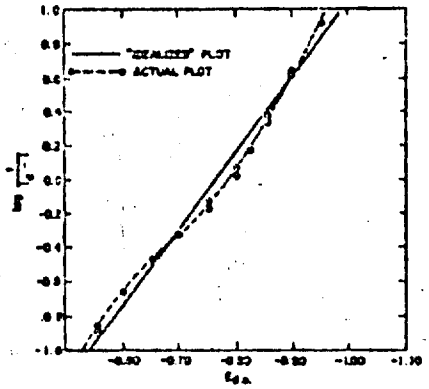


Fig. 3. Plot of $\log \frac{i}{i_d - i}$ versus potential on dropping electrode ($E_{d.e.}$). Term "i" is current at any point in polarographic wave. Term "i_d" is current at polarographic plateau (diffusion current). "i" equals term "$i_d - i$" at $E_{d.e.}$ of −0.76 on "idealized" curve. $E_{1/2}$ equals $E_{d.e.}$ at this point and corresponds to value of half-wave potential of diacetyl in buttermilk (at 0.1 *M* ammonium chloride). System employed in this plot was diacetyl in 0.1 *M* ammonium chloride.

were added to a series of 100 ml volumetric flasks containing 100 ml of buttermilk to yield a series of buttermilk samples containing the original diacetyl present plus a known amount of added diacetyl in the range 0–50 ppm. Each system was thoroughly shaken and a 50 ml portion removed for gravimetric analysis. 50 ml of the 0.2*N* ammonium chloride solution was then added to each 100 ml flask and the contents of each flask were shaken thoroughly. A 15 ml portion was removed from each flask for polarographic analysis as described under Analytical Procedures. The 50 ml portion earmarked for steam distillation was placed in the steam distillation apparatus and 150 ml of distilled water added. The distillate was trapped in a solution containing 2 ml of stock solution *B*, 4 ml of stock solution *C*, and 2 ml of stock solution *D*. Procedures described by Michaelian *et al.* (1933) were followed.

During the course of numerous gravimetric analyses it was found that results obtained were lower than theoretical (due to losses during steam distillation, filtration, etc.) while the results obtained by the polarographic method always corresponded to the amount of diacetyl added. Large amounts of acetoin were deliberately added to the sample systems with no measurable effect upon the determinations. The half-wave potential for acetoin is far removed from that of diacetyl, and hence the method described is specific for diacetyl and excludes acetoin. It was found that the diacetyl content could be determined down to the 3 ppm range with ordinary polarographic equipment and down to 1 ppm when auxiliary units such as the Sargent Micro Range Extender were employed. Ferric chloride stock solution (*A*) was used to oxidize acetoin to diacetyl in experimental steam distillations and yielded expected higher "total" diacetyl values.

The danger of autoxidation during steam distillation is eliminated in the present polarographic method because: 1) no steam distillation is required; 2) the nitrogen blanket used during the polarographic run eliminates oxygen from the system.

pH plays a very important role in the polarographic determination of diacetyl and as shown in Fig. 1, the half-wave is pH-dependent. However, in the system described, pH variations were within a very narrow range and the values of diffusion current obtained as a function of diacetyl content were very reproducible. A plot of $\log i/(i_d - i)$ (Fig. 2) is almost linear

and indicates that the reaction is nearly reversible. This polarographic method for the determination of diacetyl might be extended to other food products by appropriate modifications of the technique. Work is now in progress in our laboratories to apply this method to the determination of diacetyl in fruit products.

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Acetylmethylcarbinol and Diacetyl as Chemical Indexes of Microbial Quality of Apple Juice 443

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M. L. Fields

Food Microbiology and Food Processing Laboratories, Department of Horticulture, University of Missouri, Columbia, Missouri

SUMMARY

Yeasts of sanitary significance in apple juice production and fungi that produce rots in apples were examined to determine whether these organisms produced acetylmethylcarbinol (AMC), diacetyl, or both compounds. Of the yeasts tested that were considered to be significant in apple juice spoilage, all of the pure cultures of *Saccharomyces* spp. produced both compounds. Of the filamentous fungi that produced rots, however, only *Alternaria* sp. produced both AMC and diacetyl in decayed tissue. *Penicillium expansum*, *Mucor* sp., and *Physalospora obtusa* produced only AMC, whereas *Botrytis cinerea* produced no detectable AMC in inoculated apples. Of 80 rots tested from separate apples (both natural and inoculated), only 7 (8.8%) contained diacetyl. Since *Alternaria* is a minor rot producer and since *P. expansum* is the major rot producer in apples, the probability of diacetyl coming from yeast is much greater than from any rot. The presence of diacetyl in apple juice may be considered, therefore, presumptive evidence of poor sanitary practice in the processing plant.

The effects of different processing operations on the retention of AMC and diacetyl were also investigated. Canning had no immediate effect on AMC content; however, there was an average of 26.8% loss of AMC after 5 months of storage at 72-74°F. Analyses of commercial apple juices showed that 17 of 31 samples contained a mixture of AMC and diacetyl whereas the other 14 samples contained only AMC. Studies on fermenting residues on plant equipment indicated that these residues may increase the AMC and diacetyl content of apple juice.

INTRODUCTION

Fields (1962a) showed that the Voges-Proskauer test could be used to detect unfit raw materials and/or the production of apple juice under poor sanitary conditions. No effort was made to separate these undesirable conditions in that study. Also, no differentiation was made between acetyl-

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acetylcarbinol (AMC) and diacetyl. The total value (AMC plus diacetyl) was reported as AMC, although the analyses of fermented apple juices indicated that diacetyl was present. The fact that diacetyl was present in fermenting apple juice and usually was not associated with rots raised the question, "Could the presence of diacetyl be used as an index of poor sanitary conditions in the processing plant?"

After having raised the question, the researcher is faced with the additional problem that little information is available on which yeasts and which filamentous fungi produce diacetyl, AMC, or both compounds. Statements by Ledingham and Neish (1951) suggest that AMC was produced by *Zygosaccharomyces* and *Schizosaccharomyces*. Nothing was said, however, about the genus *Saccharomyces*, which gains the ascendency and dominates in the fermentation of apple juice (Challinor, 1955). Even in a report on studies to determine the mechanism of the formation of acetoin (AMC) by yeast, Juni (1952) made no mention of diacetyl. Beisel *et al.* (1951), in their study on sources of Voges-Proskauer reactants in California Valencia orange juice, showed that yeast as well as bacteria produced Voges-Proskauer reactants in orange juice. Those workers demonstrated that the strains of *Candida*, *Kloeckera*, and *Torulopsis* that were tested were Voges-Proskauer-positive, although no attempt was made to differentiate between AMC and diacetyl. The possibility of using diacetyl as an indicator of sanitation appeared to be feasible when a survey was made of 27 yeast cultures in our laboratory (Fields, 1962d). Nine of the cultures (species from 8 genera) produced diacetyl, based on the rate of color development, when grown on a peptone-glucose medium; however, further information was needed about the production of AMC and diacetyl under actual processing conditions.

Even less information is available about filamentous fungi that produce AMC, diacetyl, or both compounds. Information is also important if the food analyst wishes to distinguish between poor-quality apples and poor processing conditions. In a previous study (Fields, 1962a), both *Penicillium expansum* and *Alternaria* sp. were Voges-Proskauer-positive when inoculated in apples. In pure-culture studies (Fields, 1962b), other filamentous fungi surveyed for Voges-Proskauer reactants indicated that the response was variable; some fungi produced considerable Voges-Proskauer reactants, some small amounts, and some none at all. Other than in the studies in our laboratory, only *Neurospora crassa* has been listed as producing AMC (Strauss, 1955), and this organism does not produce rot in apples. The dominant rot-producing fungus is *Penicillium expansum*. This fungus produces almost all of the rots occurring in storage and the market (Rose *et al.*, 1933; Heald and Ruehle, 1931; Raper and Thom, 1949). Marshall and Walkley (1951a,b,c, 1952) found that the Penicillia possessed the highest incidence as a group of filamentous fungi on the fruit and in the freshly expressed juice, and that *P. expansum* was dominant in the Penicillia group. If diacetyl were to be used as a sanitary index, it should not be present in apple tissue decomposed by *P. expansum*.

To judge the quality of a particular apple juice sample more adequately, the analyst also needs to know the effect of different processing operations on the retention of AMC and diacetyl. As Beyer (1951) has indicated, both diacetyl and AMC are distillable. Since the boiling point of diacetyl is in the temperature range used in pasteurization of apple juice (155–180°F), the question may be asked, "Will diacetyl be lost in processing of apple juice?" Other questions concerning processing may be raised. The following research was instituted in attempts to obtain answers to these questions.

EXPERIMENTAL METHODS

Voges-Proskauer test. The method employed was that of Hill and Wenzel (1957). Three hundred ml of apple juice was distilled in a 1-L Erlenmeyer flask attached to a Liebig condenser with a jacket length of 500 mm. 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-potassium 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 AMC and diacetyl. Beyer (1951) recommended that the diacetyl content could be determined by comparing the value in the first 25-ml fraction of distillate with that in the fourth 25-ml fraction of distillate. To determine what the concentration factor for these compounds would be in apple juice, known amounts were added and recovered. These previous studies (Fields, 1962) indicated that diacetyl was concentrated 9.46 times and AMC 1.39 times in the distillate. Most of the diacetyl comes over in the first 25-ml portion, whereas the distribution of AMC during distillation is constant and independent of the concentration of the solution (Langlykke and Peterson, 1937). This same constant and independent relation was found with apple juice for lower concentrations of AMC than was used by Langlykke and Peterson (1937).

The concentration factor of 9.46 for diacetyl was divided into the value for the first 25-ml fraction of distillate to determine the diacetyl content of the original juice. The value of the fourth fraction was divided by the concentration factor 1.39 for AMC to determine the AMC content of the original solution.

Laboratory studies. Cultures of *Alternaria tenuis* NRRL2169, *P. expansum* strains 973, 976, and 977, and *Saccharomyces* sp. strains Y 129, YB 129, and YB 1188 were obtained from the Northern Utilization Laboratory, Research and Development Division, Peoria, Illinois. *Saccharomyces cerevisiae* var. *ellipsoideus*, *Saccharomyces logos*, and *Cryptococcus laurentii* var. *flavescens* were obtained from Plant Science Department, Purdue University. *Alternaria* sp., *Fusarium* sp., and *Mucor* sp. were isolated from rotting apples in our laboratory. *Botryosphaeria ribis* was obtained from Dr. Ralph Shay, Department of Plant Pathology, Purdue University. *Physalospora obtusa* was obtained from Dr. D. A. Slack, Department of Plant Pathology, University of Arkansas. The culture of *Botrytis cinerea* was obtained from Dr. O. H. Calvert, Department of Field Crops, University of Missouri.

The apples were inoculated with pure cultures of *Alternaria* sp., *Penicillium expansum*, *Physalospora obtusa*, *Mucor* sp., and *Botrytis cinerea* to produce rots in apples by known organisms. A procedure used previously (Fields, 1962a) was also used in this study to determine the AMC and diacetyl content of rots. Thirty g of the decomposed tissue were added to 200 ml of distilled water and blended 1 min in a Waring blender. The blended material was poured into a 1-L Erlenmeyer flask, the blender was rinsed with 100 ml of distilled water, and the rinsings were added to this distillation flask. Both naturally occurring rots were tested in Red

Delicious, Golden Delicious, and Jonathan. For the inoculation studies, however, only Golden Delicious were used. The apples were immersed for 1 min in a 0.5% solution of sodium hypochlorite, removed, and allowed to drain; the apples were inoculated by making a slit in them about 1 inch long and placing mycelium of the test fungus in the slit.

Culture of *Botryosphaeria ribis*, *Fusarium* sp., *Rhizopus stolonifer*, *Alternaria* sp., *Alternaria tenuis* NRRL 2169, strains of *Penicillium expansum* NRRL 977, 973, and 976, *Oidium lactis*, and *Mucor* sp. were used in pure-culture studies in apple juice. These cultures were used to inoculate 300 ml of apple juice. After growth of the fungi was sufficient, the culture and apple juice were distilled and the four fractions were collected and tested.

During the isolation of *Alternaria* sp. from apples, three film yeasts and two bottom yeasts were isolated from the same plates. Bacteria were also isolated. None of these have been identified, but were included in the pure cultures grown in apple juice in 300-ml quantities and distilled.

To determine the effects of aeration on AMC and diacetyl production by yeasts, *Saccharomyces cerevisiae* var. *ellipsoideus* ATCC 4123 was used to inoculate 150 ml of apple juice in 500-ml Erlenmeyer flasks. Half of the flasks were incubated as still cultures and the others were incubated on a shaker at room temperature. As a less vigorous method and a method simulating possible plant conditions, apple juice was dispensed in quantities of 100, 200, 300, 400, and 600 ml in 1 L Erlenmeyer flasks and allowed to undergo natural fermentation for 48 hr at room temperature.

To investigate the effect of pasteurization on the retention of diacetyl, 1 ppm of diacetyl was added to apple juice and the apple juice was run through a laboratory pasteurizer. The pasteurizer consisted of a series of glass coils that gave a retention time of about 1 min. The glass coils were placed in a constant-temperature bath controlled at 153 and 173°F. The pasteurized juice was allowed to flow directly into a glass bottle. After the bottle was filled, it was capped, held for 5 min, cooled, and tested for diacetyl. The same concentration of diacetyl was used in apple juice pasteurized in a small steam-jacketed kettle. In this experiment the juice was heated rapidly to 180°F, and the tin cans were filled, closed, held 5 min, and tested.

Table 1. Acetylmethylcarbinol (AMC) and diacetyl content in naturally occurring and in artificially produced rots in apples (ppm).

Type of inoculum	Name of fungus	Total no. of samples	No. of samples with AMC	AMC Range	AMC Mean[a]	No. of samples with diacetyl	Diacetyl Range	Diacetyl Means
Natural in: Jonathan, Red Delicious, Golden Delicious	Mixed	60	54[b]	0.0–100.9	10.9	4	1.2–7.7	3.5
Inoculated in: Golden Delicious	*Botrytis cinerea*	3	6	0.0	0.0	0	0.0	0.0
	Mucor sp.	1	1	7.6	8.9	0	0.0	0.0
	Penicillium expansum	6	6	11.5–19.8	5.1	0	0.0	0.0
	Phialophora obtusa	3	3	0.5–3.0	2.5	0	0.0	0.0
	Alternaria sp.	4	4	7.6–11.5	9.7	3	2.2–2.7	2.4
Total rots examined		80	71			7		

[a] Mean value for 30 g of decayed tissue.
[b] Two samples contained no AMC.

Pilot-plant studies. A series of apple juices were prepared with commercial equipment and methods. One hundred lb of apples were used in preparing each juice sample. Sound apples were washed, ground, pressed, enzyme-treated, heated to inactivate the enzymes, and filtered. After filtering, the juice was heated to 180°F, poured into 46-oz cans, closed, held for 5 min with can inverted, and water cooled.

To determine the effect of various processing methods on the AMC and diacetyl content, the juice was tested after pressing, after heating to inactivate the enzymes, and after canning and storage. Apple juices containing 3, 6, 9, and 12% rot (based on weight) were prepared and analyzed immediately and again after 5 months at 72–74°F.

To determine the effect of dirty equipment on the AMC and diacetyl content, a pump, a filter, and a vat were contaminated with 1¾ gal of apple juice and allowed to stand at room temperature for 24 hr. Four gallons of an unfermented juice were pumped through the equipment. The juice was sampled before and after the pumping.

Commercial juice studies. Twenty-nine glass-packed and two canned commercial apple juice samples were collected and tested for AMC and diacetyl.

RESULTS AND DISCUSSION

Laboratory studies. Table 1 gives chemical analyses of naturally occurring and artificially produced rots in apples for AMC and diacetyl. The values reported here are in general agreement with those previously reported (Fields, 1962). The values for *Alternaria* are close (a mean of 12.1 ppm calculated as AMC + diacetyl, 9.7 ppm + 2.4 ppm, as shown in Table 1, compared to a mean value of 8.8 ppm in Golden Delicious apples as previously reported, Fields, 1962). The values of AMC produced in Golden Delicious apples by *P. expansum* were lower in this study when compared to the mean value previously reported (8.8 ppm as compared to 47.5 ppm). If the AMC in the tissue were reduced to 2,3-butylene glycol, the Voges-Proskauer reaction would not give a positive reaction until a concentration of 40,000 ppm were reached (Barrett, 1936). Reduction of AMC in the tissue may account for the variation in AMC values as produced by *P. expansum*. The fact that some samples have no detectable AMC in the mixed, natural rots may be due to reduction of AMC or to a species which does not produce AMC.

The data in Table 1 show that diacetyl was found in only 7 out of 80 samples of rots and that these samples were associated with *Alternaria*, a minor rot producer. Also, as shown in Table 1, all samples of pure cultures of *P. expansum* were diacetyl-negative. Of the 60 mixed cultures of naturally occurring rots, 54 contained only AMC, and 2 contained no AMC. These data indicate that rots usually contain AMC, and not diacetyl. The fact that *P. expansum* is the major spoilage organism of apples (Rose *et al.* 1933; Raper and Thom, 1949; Heald and Ruehle, 1931; Marshall and Walkley, 1952) is the basis of using AMC as an indicator of rots. The data, as given in Table 2, also indicate that

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Table 2. Production of acetylmethylcarbinol and diacetyl by pure cultures of filamentous fungi, bacteria, and yeast grown in apple juice.

Type of organism	Name of organism	AMC[a]	Diacetyl[a]
Filamentous fungus	*Mucor* sp.	+[b]	—[b]
Filamentous fungus	*Fusarium* sp.	+	+
Filamentous fungus	*Alternaria* sp.	+	+
Filamentous fungus	*Rhizopus nigricans*	+	—
Filamentous fungus	*Botrytis cinerea*	—	—
Filamentous fungus	*Botryosphaeria ribis*	—	—
Filamentous fungus	*Penicillium expansum*	+	+
Yeast-like fungus	*Oidium lactis*	+	+
Yeast, Y129	*Saccharomyces* sp.	+	+
Yeast, YB29	*Saccharomyces* sp.	+	+
Yeast, YB188	*Saccharomyces* sp.	+	+
Yeast, ATCC4123	*Saccharomyces cerevisiae* var. *ellipsoideus*	+	+
Yeast, PCC253	*Cryptococcus laurentii* var. *flavescens*	+	+
Yeast, PCG	*Saccharomyces lagos*	+	+
Yeast, isolates, 6, 23, 23a, 37, and 9	Unknown, from rotting apple	+	+
Bacteria, isolate 37, 43, 29, 23, 8, and 6	Unknown, from rotting apple	+	+

[a] Varied with age of culture.
[b] + Indicates positive reaction, and — negative, by Voges-Proskauer test.

Alternaria sp. and *P. expansum* both produce diacetyl and AMC in still culture, whereas *P. expansum* did not produce diacetyl in rotting apple tissue (Table 1). *Fusarium* sp. isolated did not produce rot when reinoculated into apples. Therefore, this organism appeared to be a secondary invader and hence would not be a major contributor to the diacetyl content of apple juice. *Botrytis cinerea* did not produce either compound, whereas *Cheralospora obtusa* produced small amounts of AMC.

To separate unfit raw materials from poor sanitary conditions in the processing plant, the dominant spoilage flora must produce a compound which is different from that produced by the spoilage organisms which produce the rots. In fermenting residues in the plant, the dominant flora is *Saccharomyces* sp. (Challinor, 1955). As shown in Table 2, all yeast tested produced AMC and diacetyl. The bacterial and yeast isolates tested, as listed in Table 2, produced both AMC and diacetyl.

As given in Table 3, the amount of aeration, as determined by surface-volume ratio, had an influence upon the amount of AMC and diacetyl in naturally fermenting apple juice. In 1-L flasks containing 100, 200, and 300 ml of apple juice, diacetyl levels were higher than AMC levels, whereas in the 400- and 600-ml quantities the reverse was true. These differences are reflected in the AMC/diacetyl ratio and confirm observations of Ledingham and Neish (1951) that the amount of aeration has an influence on the amount of diacetyl produced.

In another study to determine the effect of aeration on the production of AMC and diacetyl, *Saccharomyces cerevisiae* var. *ellipsoideus* ATCC 4132 was incubated in still and shake cultures. Although the juice in this treatment was given greater aeration than juice in a processing plant, this method was used as another method of checking on the effects of aeration on the production of AMC and diacetyl. Just as residues of various depths can influence the levels of AMC and diacetyl, the shaking magnified the effect of aeration. For example, 0.12 ppm diacetyl and 22 ppm of AMC were found in still cultures of fermenting juice whereas 140 ppm AMC and no diacetyl were found in the shake culture juice after 48 hours. Extrapolating these results to plant operations, it may be expected that operations such as pumping, which may increase the amount of aeration of the juice, would produce conditions more conducive to diacetyl production by yeast.

In the pasteurization studies, there was no significant difference between apple juices with 1 ppm added diacetyl immediately before and immediately after pasteurization at 153 and 173°F. Even with vat pasteurization of 180°F, only 28.6% diacetyl was lost.

Pilot-plant studies. Pilot-plant data of experiment 1 (Table 4) indicate no significant difference between the fresh and canned apple juice immediately after canning. With increasing amounts of rot, AMC increased, which is in agreement with previous studies (Fields, 1962a). A test of the fresh juice containing the 12% rot of experiment 1 and all canned lots of juice in experiment 1 and all juices of experiment 2 indicated that no diacetyl was present. This fact, as well as the previously mentioned pasteurization studies, emphasizes that if diacetyl were present in the apple juice, no heat treatment that is used in apple juice processing would lower its concentration by driving off this compound with steam unless vat pasteurization were used. Another important factor to be considered is that diacetyl is not produced by itself, but is produced in association with AMC. Any heat treatment designed to drive off the diacetyl would not affect the AMC present, and would have only a deleterious effect on flavor of the juice.

The canned apple juices produced in the pilot-plant studies and evaluated after 5 months of storage (Table 5) showed decreased AMC values except for the juice containing 6% rot. This batch, for some unexplained reason, had increased in AMC by 1 ppm.

Table 3. Effect of aeration upon the production of acetylmethylcarbinol (AMC) and diacetyl in apple juice by naturally occurring yeast after 48 hr of incubation at room temperature (ppm).

Amount of juice in 1-L Erlenmeyer flask (ml)	Amount of AMC	Amount of diacetyl	Ratio of AMC/Diacetyl
100	1.9	3.37	0.56
200	0.7	1.58	0.44
300	0.75	1.37	0.55
400	1.7	0.97	1.85
600	2.8	0.65	4.31

Table 4. The relation of acetylmethylcarbinol (AMC) to percent rot in processed apple juice.

Experiment		% rot (by wt.)	AMC (ppm)	Diacetyl + or —
1[a]	Control, sound apples	0	0.95	—
	Before[a]	3	2.50	—
	After[b]	3	2.50	—
	Before	6	6.00	—
	After	6	6.00	—
	Before	9	8.00	—
	After	9	8.00	—
	Before	12	10.00	—
	After	12	10.00	—
2	Control, sound	0	0.4	—
	Before[a]	1	1.7	—
	Before	3	2.5	—
	Before	5	2.7	—

[a] Before canning.
[b] After canning.
+ = Present; — = absent.

Table 5. Loss of AMC in canned apple juice stored 5 months at 72–74°F.

% rot by weight	Time of analysis: At time of canning AMC in ppm[a]	5 months of storage AMC in ppm[b]	% loss
0	0.94	0.6	36.8
3	2.50	2.0	20.0
6	6.00	7.0	—
9	8.00	5.9	26.5
12	10.00	7.6	24.0

[a] Mean of duplicate samples.
[b] Mean of six replicate cans of juice.

Further storage studies involving both bottled and canned apple juice are indicated to determine possible losses of both AMC and diacetyl.

Another pilot plant study, in which apple juice was allowed to contaminate a pump, a filter, and a vat for 24 hr, followed by pumping of unfermenting juice through the systems, illustrates how both the AMC and diacetyl contents of apple juice may be increased by poor sanitary practices in the plant. The diacetyl and AMC level of the juice were respectively 0.24 and 0.72 before pumping through the system, and 0.36 and 1.51 after pumping. In the plant, if the juice is not handled properly, and rapidly, yeast may contaminate the juice in storage vats as well as the production lines.

Evaluation of commercial juices. A total of 31 commercial samples were tested and 17 were found to contain diacetyl. The range of diacetyl varied from 0.27 to 1.01 ppm, with a mean of 0.53 ppm, whereas the AMC in the same samples varied from 0.72 to 5.04 ppm, with a mean value of 2.52 ppm. These data indicate that diacetyl and AMC may be constituents of the Voges-Proskauer reactants in commercial apple juice samples. Diacetyl is present in smaller amounts in the apple juice, but is concentrated more than the AMC during distillation.

Acetylmethylcarbinol and diacetyl as chemical indexes of quality. Both AMC and diacetyl fulfilled the criteria for a useful chemical index. These criteria are: 1) The chemical must be at low levels or absent in sound apples. 2) With increased spoilage, there must be an increase in the chemical index. 3) If possible, the index or indexes should indicate poor-quality apples from poor processing conditions. 4) The index, if a metabolic by-product, should be produced by the dominant spoilage flora.

The fact that diacetyl may be present in commercial apple juice explains how larger Voges-Proskauer values may be obtained. For example, values as high as 10 ppm were previously reported in commercial apple juice (Fields, 1962a). If one assumed that all of the Voges-Proskauer reactants were the result of rot entering the juice, this would mean a rot equivalent of greater than 18% by weight, but the previous work also showed that when apple juice contained 8% rot by weight the rot could be detected by taste. The fact that commercial juice may have a rather high Voges-Proskauer reactant value and still be salable suggests that diacetyl was one of the reactants. Diacetyl is concentrated 9.46 times in the first 25-ml fraction of distillate and it was the first 25 ml fraction of distillate which was used in establishing the proposed standard of quality for apple juice. As found in research as reported herein the diacetyl ranged from .27 to 1.01 ppm which would not have a drastic effect upon the taste of the apple juice like a value of 10 ppm of diacetyl.

As shown in Table 6, the number of samples undergoing fermentation by yeast (94.9%) contained diacetyl while only 8.8% of the samples of rots tested contained diacetyl. All pilot plant juices contained no diacetyl when produced from apples containing rots. Also, two laboratory samples of completely rotten juice contained no detectable diacetyl.

Table 6. The number and percent samples containing diacetyl.

Nature of sample	No. of samples	No. with diacetyl	% containing diacetyl
Commercial apple juice	31	17	54.8
Fermentinar apple juice, yeast; pure and mixed	39	37	94.9
Fermenting apple juice, bacterial	7	7	100.0
Rots in apples natural and pure cultures	80	7	8.8
Pilot plant juices containing rots	7	0	0.0
Laboratory produced apple juice, 100% rotten	2	0	0.0

The data in this study add to the proposed standard in that the presence of diacetyl may be considered as presumptive evidence of poor sanitary conditions because of the association of diacetyl with yeast, the dominant spoilage organisms of apple juice undergoing fermentation and because of the relatively low number of samples of rots which contained diacetyl. The findings in this study do not alter the previously proposed standard but add to its usefulness. The highest-quality juices would contain low levels of AMC and no diacetyl.

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Ms. rec'd 5/28/63.

Contribution from the Missouri Agricultural Experiment Station. Journal Series No. 2599, approved by the Director.

Flavoring Extract Manufacturers' Association of the United States

FROM THE OFFICE OF
FOOD ADDITIVES COMMITTEE
DR. RICHARD L. HALL, Chairman
McCormick & Co., Inc.
BALTIMORE 2, MARYLAND

July 11, 1962

DF

Dr. R. A. Osborn
Division of Food
Food and Drug Administration
Department of Health, Education
and Welfare
Washington 25, D. C.

Dear Doctor Osborn:

Attached is an up-to-date summary sheet for butter starter distillate.

In view of the existing, published information on its manufacture and composition, the expert panel felt that it was, beyond all doubt, generally recognized as safe. Much of the information available on this product was not mentioned on our summary sheet, so will you please destroy the card now in your possession and substitute the attached one in its place.

Sincerely yours,

Dick Hall

R. L. Hall

RLH:JLK

Attachment

179

CLASS II

PRIMARY NAME (A)

BUTTER STARTER DISTILLATE

CHEMICAL FORMULA (B) OR BOTANICAL NAME (IF PLANT OR ESSENTIAL OIL)

COMMERCIAL NAMES

SYNONYMS

NATURAL FOOD SOURCES (AND REFERENCES)

SPECIFICATIONS (C)

Research Bulletin Nos. 203 and 290, Agricultural Experiment Station, Iowa State College, Ames, Iowa; [illegible] Foster et al, Dairy Microbiology, Prentice Hall; Attached papers.

Principal components: Diacetyl, Acetyl methyl carbinol, 2,3-Butylene glycol. Trace components: Acetic acid, Propionic acid, Butyric acid,

FLAVORS IN WHICH USED (D)

Butter

EARLIEST DATE OF USE (E)

GENERAL 1940

EARLIEST 1 [illegible]

IMPORTANCE RATING (F)

A 0

B 1

C 1

Specifications (C) - continued

Ethyl alcohol, Acetaldehyde, [illegible]

PRINCIPAL FOODS IN WHICH USED AND AVERAGE LEVELS OF USE

	BEVERAGES (G)		ICE CREAM, ICES ETC (H) 2		CANDY (I)		BAKED GOODS (J)		Shortening (K)		(L)		(M)	
	USUAL	MAX	USUAL	MAX	USUAL	MAX	USUAL	MAX	USUAL	MAX	USUAL	MAX	USUAL	MAX
AVERAGE	-----	-----	2.00 30.00	20.00 40.00	212.00	417.00	303.00	723.00	[illegible]	[illegible]				
RANGE					75.0- 300.00	20.00- 1200.00	5.0- 1200.0	10.0- 2400.0						

SUMMARY OF TOXICOLOGICAL DATA (N & O)

FDA ACTION

Major Inspection

Extension List - [illegible]

FEMA F.A. COMMITTEE ACTION AND COMMENT

GRAS (10 reports)

[illegible] 4/3/[illegible]

0575

0583

FOOD AND DRUG Research LABORATORIES, INC.

WAVERLY DIVISION
Route 17
P.O. Box 107
Waverly, New York 14892
(607) 565-2931

FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date August 20, 1973

Laboratory No. 1573 p
Contract No. FDA 71-260

Sample: Clear light yellow liquid

Marking: FDA 71-73 (Hansen's; Starter distillate)

Examination Requested: Teratologic evaluation of FDA 71-73 in mice.

Procedure: See Appendix I

Results: See Tables 1 through 4 and Appendix II

Conclusion:

On the basis of the data presented herein, the following conclusion appears to be warranted:

"The administration of up to 1600 mg/kg (body weight) of the test material to pregnant mice for 10 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

FOOD and DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D.
Vice President

This report is submitted for the exclusive use of the person, partnership, or corporation to whom it is addressed, and neither the report nor the name of these Laboratories nor of any members of its staff, may be used in connection with the advertising or sale of any product or process without written authorization.

FOOD AND DRUG RESE . LABORATORIES, INC.

Groups: 291 through 296

Material: FDA 71-73

Date: July 23, 1973

Laboratory No.: 1573 p

Table 1

Fate Summary
(Mice)

Group	Material	Dose** mg/kg	Total		Surviving at Term	
			Mated	Pregnant	Total	Pregnant[1]
291	Sham	0.0	25	21	25	21
292	Aspirin*	150.0	25	23	25	23
293	FDA 71-73	16.0	25	24	25	24
294	FDA 71-73	74.3	25	21	25	21
295	FDA 71-73	345.0	30	21	30	21
296	FDA 71-73	1500.0	25	23	25	23

* Positive Control: 150.0 mg/kg

** Administered as a water solution (See Appendix I); 10 ml per kg of body weight

1) Includes all dams examined at term

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FOOD AND DRUG R[illegible] LABORATORIES, INC.

Group: 291 through 296

Material: FDA 71-73

Table 2
Reproduction Data
(Mice)

Date: July 23, 1973

Laboratory No. 1573 p

Group:	291	292	293	294	295	296
Dose (mg/kg):	Sham	Aspirin**	16.0	74.3	345.0	1600.0
Pregnancies						
Total No.	21	23	24	21	21	23
Died or Aborted (before Day 17)	0	0	0	0	0	0
To term (on Day 17)	21	23	24	21	21	23
Live Litters						
Total No.*	21	23	24	21	21	23
Implant Sites						
Total No.	241	258	284	245	245	261
Average/dam*	11.5	11.2	11.8	11.7	11.7	11.4
Resorptions						
Total No.*	2	10	11	10	9	7
Dams with 1 or more sites resorbed	2	9	6	8	8	6
Dams with all sites resorbed	--	--	--	--	--	--
Per cent partial resorptions	9.52	39.1	25.0	38.1	38.1	26.1
Per cent complete resorptions	--	--	--	--	--	--
Live Fetuses						
Total No.	235	248	269	233	232	253
Average/dam*	11.2	10.8	11.2	11.1	11.1	11.0
Sex ratio (M/F)	0.82	0.76	0.81	0.74	0.97	0.96
Dead Fetuses						
Total*	4	--	4	2	4	1
Dams with 1 or more dead	4	--	3	2	4	1
Dams with all dead	--	--	--	--	--	--
Per cent partial dead	19.1	--	12.5	9.52	19.1	4.35
Per cent all dead	--	--	--	--	--	--
Average Fetus Weight, g	0.92	0.84	0.87	0.91	0.85	0.93

* Includes only those dams examined at term.

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296 Laboratory No. 1573 p

Table 3

Material FDA 71-73 Date July 23, 1973

Summary of Skeletal Findings*
(Mice)

Findings / Group No.: / Dose (mg/kg):	291 Sham	292 Aspirin**	293 16.0	294 74.3	295 345.0	296 1600.0
Live Fetuses Examined (at term)	163/21	175/23	189/24	163/21	162/21	178/23
Sternebrae						
Incomplete oss.	9/5	97/22	27/14	14/9	13/10	23/9
Scrambled						
Bipartite	6/5	3/3	9/6	8/6	8/6	6/6
Fused			1/1			
Extra						
Missing	15/11	45/15	26/12	15/9	26/15	11/7
Other					1/1	
Ribs						
Incomplete oss.			1/1			
Fused/split						
Wavy					1/1	
Less than 12						
More than 13	29/15	44/17	45/21	44/13	40/15	43/20
Other						
Vertebrae						
Incomplete oss.	1/1	3/2	4/4	2/1	5/5	2/2
Scrambled						
Fused						
Extra ctrs. oss.						
Scoliosis						
Tail defects						
Other						
Skull						
Incomplete closure						
Missing						
Craniostosis						
Other						
Extremities						
Incomplete oss.	1/1	7/5	5/4	1/1	6/6	2/2
Missing						
Extra						
Miscellaneous						
Hyoid; missing	52/18	42/17	38/17	25/12	38/16	46/18
Hyoid; reduced	14/8	23/13	25/16	11/8	18/12	10/7
	77.91 127/163					143/175 [illegible]

* Numerator=Number of fetuses affected; Denominator=Number of litters affected.

** Positive control: 150.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296

Date July 23, 1973

Material FDA 71-73

Laboratory No. 1573 p

Table 3-a

Summary of Soft Tissue Abnormalities

(Mice)

Group	Material	Dose Level mg/kg	Dam	Number of Pups	Description

None Observed

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296

Species Mice

Date July 23, 1973

Laboratory No. 1573 p

Table 4

Average Body Weights*

Group	Material	Dose Level mg/kg	Day 0 (g)	6	11	15	17**	
291	Sham	0.0	30.6	33.1	37.3	45.0	51.2 (21)	-20.6
292	Aspirin***	150.0	29.1	31.7	34.7	41.4	47.0 (23)	
293	FDA 71-73	16.0	30.2	32.3	36.1	44.1	50.3 (24)	
294	FDA 71-73	74.3	32.9	34.3	38.4	46.9	53.0 (21)	
295	FDA 71-73	345.0	28.5	32.1	36.1	42.3	49.7 (21)	
296	FDA 71-73	1600.0	29.5	32.7	37.6	45.6	51.3 (23)	-21.8

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1)
*** Positive control: 150.0 mg/kg

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Appendix I

Teratology Study in Mice

Virgin adult female albino CD-1 outbred mice were gang-housed in disposable plastic cages in temperature and humidity-controlled quarters with free access to food and fresh tap water. They were mated with young adult males, and observation of the vaginal sperm plug was considered Day 0 of gestation. Beginning on Day 6 and continuing daily through Day 15 of gestation, the females were dosed with the indicated dosages by oral intubation; the controls were sham treated.

Body weights were recorded on Days 0, 6, 11, 15, and 17 of gestation. All animals were observed daily for appearance and behavior with particular attention to food consumption and weight, in order to rule out any abnormalities which may have occurred as a result of anorexic effects in the pregnant female animal.

On Day 17 all dams were subjected to Caesarean section under surgical anesthesia, and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. The body weights of the live pups were also recorded. The urogenital tract of each dam was examined in detail for anatomical normality.

All fetuses were examined grossly for the presence of external congenital abnormalities. One-third of the fetuses of each litter underwent detailed visceral examinations employing the Wilson technique. The remaining two-thirds were cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 291

Material Sham

Dose 0.0 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date July 23, 1973

Laboratory No. 1573

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
S 3936	NP	0						----	
S 3937	P	11	10		6	4	1	0.93	
S 3938	P	11	11		5	6		0.88	
S 3939	P	10	10		3	7		0.87	
S 3940	P	12	11	1	6	5		0.97	
S 3941	P	9	9		5	4		0.82	
S 3942	P	10	10		4	6		0.92	
S 3943	P	10	9	1	3	6		0.88	
S 3944	P	12	12		4	8		0.83	
S 3945	P	13	13		5	8		0.88	
S 3946	P	15	15		7	8		0.99	
S 3947	P	13	13		6	7		0.81	
S 3948	P	10	10		5	5		0.80	
S 3949	NP	0						----	
S 3950	P	11	11		4	7		0.98	
S 3951	NP	0						----	
S 3952	P	13	13		5	8		0.84	
S 3953	P	15	15		8	7		0.89	
S 3954	P	11	11		4	7		1.23	
S 3955	P	16	15	1	8	7		0.98	
S 3956	P	12	12		4	8		0.97	
S 3957	P	10	9		5	4	1	1.08	
S 3958	P	12	11	1	7	5		0.84	
S 3959	NP	0						----	
S 3960	P	5	5		2	3		0.87	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 292

Material Aspirin

Dose 150.0 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date July 23, 1973

Laboratory No. 1573

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
A 3936	P	12	12		8	4		0.94	
A 3937	P	12	12		3	9		0.75	
A 3938	P	9	8		4	4	1	0.80	
A 3939	P	9	8		7	1	1	0.81	
A 3940	P	10	10		4	6		0.80	
A 3941	P	11	11		5	6		1.20	
A 3942	P	11	10		6	4	1	0.79	
A 3943	P	13	13		5	8		0.74	
A 3944	P	12	11		4	7	1	0.84	
A 3945	P	11	11		3	8		0.85	
A 3946	P	8	8		3	5		0.72	
A 3947	P	4	4		2	2		0.92	
A 3948	P	14	14		6	8		0.91	
A 3949	P	9	9		5	4		0.71	
A 3950	NP	0						----	
A 3951	NP	0						----	
A 3952	P	12	11		6	5	1	0.91	
A 3953	P	15	14		4	10	1	0.79	
A 3954	P	11	11		5	6		0.91	
A 3955	P	12	12		6	6		0.84	
A 3956	P	14	14		4	10		0.98	
A 3957	P	12	11		3	8	1	0.68	
A 3958	P	11	11		5	6		0.73	
A 3959	P	15	14		6	8	1	0.81	
A 3960	P	11	9		3	6	2	0.83	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 293

Material FDA 71-73

Dose 16.0 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date July 23, 1973

Laboratory No. 1573 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 3001	P	17	17		6	11		0.91	
P 3002	P	6	6		2	4		0.95	
P 3003	P	12	12		5	7		0.83	
P 3004	P	10	10		3	7		0.80	
P 3005	P	10	10		7**	2**		0.81	
P 3006	P	15	15		7	8		0.81	
P 3007	P	15	14		6	8	1	0.82	
P 3008	P	11	11		5	6		0.77	
P 3009	P	10	10		5	5		0.84	
P 3010	P	12	12		5	7		0.84	
P 3011	P	12	12		4	8		0.83	
P 3012	P	12	7	2	3	4	3	0.98	
P 3013	P	17	14	1	7	7	2	0.84	
P 3014	P	11	11		6	5		1.20	
P 3015	P	13	13		7	6		0.91	
P 3016	NP	0						----	
P 3017	P	12	12		6	6		0.79	
P 3018	P	7	7		2	5		0.95	
P 3019	P	13	12		4	8	1	0.78	
P 3020	P	12	10	1	7	3	1	0.91	
P 3021	P	12	12		4	8		0.82	
P 3022	P	11	11		8	3		0.98	
P 3023	P	11	8		4	4	3	0.88	
P 3024	P	10	10		4	6		0.81	
P 3025	P	13	13		3	10		0.71	

* P = Pregnant; NP = Not Pregnant

** Not all recorded.

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 294

Material FDA 71-73

Dose 74.3 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date July 23, 1973

Laboratory No. 1573 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 3031	P	12	12		4	8		0.84	
P 3032	P	11	11		4	7		0.98	
P 3033	P	11	10		5	5	1	0.91	
P 3034	P	13	12	1	6	6		0.90	
P 3035	P	11	10		3	7	1	0.90	
P 3036	NP	0						----	
P 3037	P	10	8		3	5	2	0.89	
P 3038	P	10	10		5	5		0.87	
P 3039	P	4	4		1	3		0.86	
P 3040	P	7	7		3	4		0.89	
P 3041	P	12	12		5	7		0.93	
P 3042	P	13	13		5	8		1.24	
P 3043	P	10	9		3	6	1	0.87	
P 3044	NP	0						----	
P 3045	P	17	15		7	8	2	0.85	
P 3046	P	13	13		6	7		1.18	
P 3047	NP	0						----	
P 3048	P	14	13		4	9	1	0.79	
P 3049	P	13	12	1	10	2		1.00	
P 3050	P	15	15		6	9		0.88	
P 3051	NP	0						----	
P 3052	P	13	12		7	5	1	0.81	
P 3053	P	12	12		3	9		0.75	
P 3054	P	9	8		3	5	1	0.92	
P 3055	P	15	15		6	9		0.87	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 295

Material FDA 71-73

Dose 345.0 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date July 23, 1973

Laboratory No. 1573 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 3061	P	12	10		5	5	2	0.87	
P 3062	P	12	12		5	7		0.74	
P 3063	NP	0						----	
P 3064	NP	0						----	
P 3065	P	12	12		7	5		0.85	
P 3066	P	11	11		6	5		0.82	
P 3067	P	9	9		5	4		0.93	
P 3068	P	10	10		4	6		0.89	
P 3069	P	7	6		3	3	1	0.85	
P 3070	NP	0						----	
P 3071	P	13	12		4	8	1	0.80	
P 3072	NP	0						----	
P 3073	NP	0						----	
P 3074	NP	0						----	
P 3075	NP	0						----	
P 3076	NP	0						----	
P 3077	P	11	11		6	5		0.85	
P 3078	P	17	15	1	5	10	1	0.79	
P 3079	P	11	10	1	2	8		0.72	
P 3080	P	13	13		8	5		0.96	
P 3081	P	13	12	1	6	6		0.77	
P 3082	P	15	14		9	5	1	0.84	
P 3083	P	11	10	1	7	3		0.84	
P 3084	P	14	13		9	4	1	0.85	
P 3085	P	7	7		2**	4**		0.98**	
P 3086	P	11	11		5	6		0.85	
P 3087	P	12	12		6	6		0.89	
P 3088	P	12	11		5	6	1	0.88	
P 3089	NP	0						----	
P 3090	P	12	11		5	6	1	0.86	

* P = Pregnant; NP = Not Pregnant

** Not all recorded.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 296

Material FDA 71-73

Dose 1600.0 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date July 23, 1973

Laboratory No. 1573 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 3091	NP	0						----	
P 3092	P	12	12		5	7		0.94	
P 3093	P	12	12		6	6		0.78	
P 3094	P	11	9		5	4	2	0.98	
P 3095	P	12	12		9	3		0.90	
P 3096	P	13	13		6	7		0.82	
P 3097	P	3	3		2	1		0.95	
P 3098	P	12	12		6	6		0.80	
P 3099	P	11	10		5	5	1	0.87	
P 3100	P	13	12		5	7	1	0.82	
P 3101	P	14	14		5	9		0.84	
P 3102	NP	0						----	
P 3103	P	14	14		6	8		1.17	
P 3104	P	12	11	1	5	6		0.87	
P 3105	P	14	13		7	6	1	0.92	
P 3106	P	12	12		7	5		0.93	
P 3107	P	12	12		4	8		0.87	
P 3108	P	10	10		5	5		1.00	
P 3109	P	12	12		5	7		0.98	
P 3110	P	10	10		5	5		0.94	
P 3111	P	10	9		4	5	1	1.19	
P 3112	P	12	12		7	5		0.90	
P 3113	P	10	10		5	5		1.01	
P 3114	P	9	8		4	4	1	0.90	
P 3115	P	11	11		6	5		0.91	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG
Research LABORATORIES, INC.

WAVERLY DIVISION
Route 17
P.O. Box 107
Waverly, New York 14892
(607) 565-2931

FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date August 20, 1973

Laboratory No. 1574 p
Contract No. FDA 71-260

Sample: Clear light yellow liquid

Marking: FDA 71-73 (Hansen's; Starter distillate)

Examination Requested: Teratologic evaluation of FDA 71-73 in rats

Procedure: See Appendix I

Results: See Tables 1 through 4 and Appendix II

Conclusion:

On the basis of the data presented herein, the following conclusion appears to be warranted:

"The administration of up to 1600 mg/kg (body weight) of the test material to pregnant rats for 10 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

FOOD and DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D.
Vice President

This report is submitted for the exclusive use of the person, partnership, or corporation to whom it is addressed, and neither the report nor the name of these Laboratories nor of any members of its staff, may be used in connection with the advertising or sale of any product or process without written authorization.

FOOD AND DRUG RE[illegible] LABORATORIES, INC.

Groups: 291 through 296

Material: FDA 71-73

Date: July 23, 1973

Laboratory No.: 1574 p

Table 1

Fate Summary
(Rats)

Group	Material	Dose** mg/kg	Total		Surviving at Term	
			Mated	Pregnant	Total	Pregnant[1]
291	Sham	0.0	25	22	25	22
292	Aspirin*	250.0	24	21	24	21
293	FDA 71-73	16.0	25	23	25	23
294	FDA 71-73	74.3	25	20	25	20
295	FDA 71-73	345.0	25	22	25	22
296	FDA 71-73	1600.0	25	21	25	21

* Positive Control: 250.0 mg/kg
** Administered as a water solution (See Appendix I)
1) Includes all dams examined at term

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FOOD AND DRUG RE LABORATORIES, INC.

Group: 291 through 296

Material: FDA 71-73

Date: July 23, 1973

Laboratory No. 1574 p

Table 2
Reproduction Data
(Rats)

Group:	291	292	293	294	295	296
Dose (mg/kg):	Sham	Aspirin**	16.0	74.3	345.0	1600.0
Pregnancies						
Total No.	22	21	23	20	22	21
Died or Aborted (before Day 20)	0	0	0	0	0	0
To term (on Day 20)	22	21	23	20	22	21
Live Litters						
Total No.*	22	20	23	20	22	21
Implant Sites						
Total No.	241	237	276	223	263	233
Average/dam*	11.0	11.3	12.0	11.2	12.0	11.1
Resorptions						
Total No.*	6	30	1	2	3	2
Dams with 1 or more sites resorbed	2	5	1	2	3	2
Dams with all sites resorbed	--	1	--	--	--	--
Per cent partial resorptions	9.09	23.8	4.35	10.0	13.6	9.52
Per cent complete resorptions	--	4.76	--	--	--	--
Live Fetuses						
Total No.	235	202	275	221	260	231
Average/dam*	10.7	9.62	12.0	11.1	11.8	11.0
Sex ratio (M/F)	1.03	1.35	0.99	1.01	0.82	1.04
Dead Fetuses						
Total*	--	5	--	--	--	--
Dams with 1 or more dead	--	1	--	--	--	--
Dams with all dead	--	--	--	--	--	--
Per cent partial dead	--	4.76	--	--	--	--
Per cent all dead	--	--	--	--	--	--
Average Fetus Weight, g	3.77	2.53	3.73	3.71	3.76	3.81

* Includes only those dams examined at term.

** Positive Control: 250.0 mg/kg

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296

Laboratory No. 1575 p

Material FDA 71-73

Table 3

Date July 23, 1973

Summary of Skeletal Findings*
(Rats)

Findings / Group No.:	291	292	293	294	295	296
Dose (mg/kg):	Sham	Aspirin**	16.0	74.3	345.0	1600.0
Live Fetuses Examined (at term)	165/22	140/20	191/23	155/20	182/22	162/21
Sternebrae						
Incomplete oss.						
Scrambled	54/20	77/20	62/20	42/17	39/16	43/14
Bipartite	1/1	3/3	1/1	2/2	1/1	
Fused						
Extra			1/1			
Missing	5/3	116/20	9/5	19/9	2/2	14/6
Other						
Ribs						
Incomplete oss.		3/2	1/1			
Fused/split		2/2				
Wavy	18/11	69/17	30/11	19/8	8/5	25/10
Less than 12						
More than 13	4/4	84/16	3/3	4/2	3/2	2/1
Other						
Vertebrae						
Incomplete oss.	23/11	110/20	12/5	30/15	14/10	15/7
Scrambled						
Fused						
Extra ctrs. oss.						
Scoliosis						
Tail defects		2/1				
Other						
Skull						
Incomplete closure	25/14	49/16	25/13	26/14	21/8	35/12
Missing		1/1				
Craniostosis						
Other						
Extremities						
Incomplete oss.		8/5				
Missing						
Extra						
Miscellaneous						
Hyoid; missing	17/10	74/20	21/12	14/8	16/9	27/11
Hyoid; reduced	13/9	13/11	24/10	28/13	14/9	11/8

9696 160/165 162/172

* Numerator=Number of fetuses affected; Denominator=Number of litters affected.

** Positive control: 250.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296 Date July 23, 1973

Material FDA 71-73 Laboratory No. 1574 p

Table 3-a
Summary of Soft Tissue Abnormalities
(Rats)

Group	Material	Dose Level mg/kg	Dam	Number of Pups	Description
292	Aspirin*	250.0	A 4937	1	Umbilical hernia
			A 4940	2	Encephalomyelocele
				1	Anopia
			A 4945	1	Encephalomyelocele
			A 4947	1	Encephalomyelocele
			A 4953	2	Meningoencephalocele
				1	Exophthalmos
			A 4955	3	Encephalomyelocele
				1	Exophthalmos
			A 4960	1	Encephalomyelocele; gastroschisis; renal agenesis
294	FDA 71-73	74.3	P 4052	1	Gastroschisis
295	FDA 71-73	345.0	P 4074	1	Gastroschisis

* Positive Control: 250.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296

Species Rats

Date July 23, 1973

Laboratory No. 1574 p

Table 4

Average Body Weights*

Group	Material	Dose Level	Day 0	Day 6	Day 11	Day 15	Day 20**
		mg/kg	g				
291	Sham	0.0	221	242	257	276	341 (22)
292	Aspirin***	250.0	226	245	256	273	323 (21)
293	FDA 71-73	16.0	228	246	263	286	358 (23)
294	FDA 71-73	74.3	225	242	259	279	344 (20)
295	FDA 71-73	345.0	222	238	255	278	348 (22)
296	FDA 71-73	1600.0	233	249	260	287	350 (21)

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1)
*** Positive control: 250.0 mg/kg

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Appendix I

Teratology Study in Rats

Virgin adult female albino rats (Wistar derived stock) were individually housed in mesh bottom cages in temperature and humidity-controlled quarters with free access to food and fresh tap water. They were mated with young adult males, and observation of the vaginal sperm plug was considered Day 0 of gestation. Beginning on Day 6 and continuing daily through Day 15 of gestation, the females were dosed with the indicated dosages by oral intubations. The controls were sham treated with the vehicle at a level equivalent to the group receiving the highest test dose. The test material was prepared and doses calculated according to the following table:

Dosage (mg/kg)	Dose (ml/kg)	Concentration (mg/ml)
≦ 250	1	≦ 250
251 - 500	2	125 - 250
501 - 750	3	133 - 250
751 - 1000	4	187 - 250
1001 - 1250	5	200 - 250
1251 - 1500	6	208 - 250
1501 - 1600	6.4	235 - 250

Body weights were recorded on Days 0, 6, 11, 15, and 20 of gestation. All animals were observed daily for appearance and behavior with particular attention to food consumption and weight, in order to rule out any abnormalities which may have occurred as a result of anorexic effects in the pregnant female animal.

On Day 20 all dams were subjected to Caesarean section under surgical anesthesia, and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. The body weights of

the live pups were also recorded. The urogenital tract of each dam was examined in detail for anatomical normality.

All fetuses were examined grossly for the presence of external congenital abnormalities. One-third of the fetuses of each litter underwent detailed visceral examinations employing the Wilson technique. The remaining two-thirds were cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 291

Material Sham

Dose 0.0 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date July 23, 1973

Laboratory No. 1574

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
S 4936	P	9	9		6	3		3.76	
S 4937	P	11	11		3	8		3.52	
S 4938	P	11	11		6	5		3.57	
S 4939	NP	0						----	
S 4940	NP	0						----	
S 4941	P	12	12		9	3		3.61	
S 4942	P	14	14		8	6		4.05	
S 4943	P	7	7		4	3		3.96	
S 4944	P	4	4		2	2		4.41	
S 4945	P	7	7		2	5		4.04	
S 4946	P	13	13		5	8		4.10	
S 4947	P	15	15		9	6		4.02	
S 4948	P	9	5		2	3	4	3.09	
S 4949	P	13	13		7	6		3.64	
S 4950	P	14	14		7	7		3.48	
S 4951	P	12	12		6	6		3.81	
S 4952	NP	0						----	
S 4953	P	9	9		3	6		3.71	
S 4954	P	13	13		4	9		3.31	
S 4955	P	13	13		6	7		3.96	
S 4956	P	13	11		3	8	2	3.87	
S 4957	P	14	14		12	2		3.71	
S 4958	P	9	9		5	4		4.11	
S 4959	P	12	12		6	6		3.72	
S 4960	P	7	7		4	3		3.51	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 292

Material Aspirin

Dose 250.0 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date July 23, 1973

Laboratory No. 1574

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
A 4936	P	10	10		6	4		2.72	
A 4937	P	9	9		4	5		2.59	
A 4938	P	12	12		4	8		3.14	
A 4939	P	12	12		4	8		3.03	
A 4940	P	15	15		6	9		2.38	
A 4941	NP	0						----	
A 4942	P	14	14		10	4		3.19	
A 4943	P	11	11		9	2		2.94	
A 4944	P	9	4		3	1	5	1.87	
A 4945	P	10	9		5	4	1	2.20	
A 4946	P	15	15		10	5		2.74	
A 4947	P	11	6		4	2	5	2.12	
A 4948	P	13	8	5	5	3		2.28	
A 4949	P	13	13		7	6		2.67	
A 4950	NP	0						----	
A 4951	P	13	13		11	2		2.83	
A 4952	P	10	10		7	3		2.81	
A 4953	P	7	7		1	6		1.75	
A 4954									Not Assigned
A 4955	P	9	9		6	3		2.27	
A 4956	P	10	10		6	4		2.76	
A 4957	NP	0						----	
A 4958	P	11	11		6	5		2.69	
A 4959	P	12					12	----	
A 4960	P	11	4		2	2	7	1.66	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 293

Material FDA 71-73

Dose 16.0 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date July 23, 1973

Laboratory No. 1574 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 4001	P	11	11		6	5		3.65	
P 4002	P	12	12		8	4		3.32	
P 4003	P	13	13		7	6		4.03	
P 4004	P	9	9		4	5		3.74	
P 4005	NP	0						----	
P 4006	P	14	13		5	8	1	4.22	
P 4007	P	12	12		7	5		4.01	
P 4008	P	13	13		10	3		3.78	
P 4009	P	14	14		7	7		3.65	
P 4010	P	12	12		5	7		3.43	
P 4011	P	11	11		3	8		3.86	
P 4012	P	14	14		5	9		4.18	
P 4013	P	9	9		4	5		3.51	
P 4014	NP	0						----	
P 4015	P	12	12		7	5		3.58	
P 4016	P	13	13		10	3		3.99	
P 4017	P	15	15		9	6		3.28	
P 4018	P	9	9		3	6		3.86	
P 4019	P	12	12		2	10		3.68	
P 4020	P	11	11		4	7		3.84	
P 4021	P	13	13		8	5		3.83	
P 4022	P	11	11		7	4		3.73	
P 4023	P	12	12		5	7		3.79	
P 4024	P	10	10		3	7		3.41	
P 4025	P	14	14		8	6		3.33	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 294

Material FDA 71-73

Dose 74.3 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date July 23, 1973

Laboratory No. 1574 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 4031	P	14	14		11	3		4.06	
P 4032	NP	0						----	
P 4033	P	13	13		5	8		3.60	
P 4034	P	14	14		8	6		3.33	
P 4035	NP	0						----	
P 4036	P	13	13		3	10		3.65	
P 4037	NP	0						----	
P 4038	P	11	11		7	4		3.74	
P 4039	P	12	12		4	8		3.52	
P 4040	P	9	8		5	3	1	3.78	
P 4041	NP	0						----	
P 4042	P	9	9		5	4		3.78	
P 4043	P	13	13		4	9		3.67	
P 4044	NP	0						----	
P 4045	P	8	8		4	4		3.82	
P 4046	P	14	14		5	9		3.73	
P 4047	P	13	12		8	4	1	3.57	
P 4048	P	11	11		5	6		3.82	
P 4049	P	14	14		7	7		3.67	
P 4050	P	10	10		6	4		3.82	
P 4051	P	13	13		10	3		4.05	
P 4052	P	10	10		4	6		3.70	
P 4053	P	12	12		6	6		3.85	
P 4054	P	4	4		1	3		3.31	
P 4055	P	6	6		3	3		3.72	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 295

Material FDA 71-73

Dose 345.0 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date July 23, 1973

Laboratory No. 1574 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 4061	P	13	13		4	9		3.69	
P 4062	P	17	17		9	8		3.79	
P 4063	P	10	9		4	5	1	3.90	
P 4064	P	10	10		6	4		3.72	
P 4065	P	14	14		4	10		4.01	
P 4066	P	14	14		5	9		3.78	
P 4067	P	11	10		4	6	1	3.79	
P 4068	NP	0						----	
P 4069	P	12	12		7	5		3.68	
P 4070	NP	0						----	
P 4071	P	12	12		8	4		3.78	
P 4072	P	11	10		5	5	1	3.91	
P 4073	P	12	12		5	7		3.94	
P 4074	P	11	11		4	7		3.64	
P 4075	P	11	11		6	5		3.64	
P 4076	P	14	14		6	8		3.70	
P 4077	NP	0						----	
P 4078	P	12	12		4	8		3.83	
P 4079	P	13	13		7	6		4.08	
P 4080	P	11	11		5	6		3.90	
P 4081	P	10	10		3	7		3.56	
P 4082	P	12	12		5	7		3.60	
P 4083	P	11	11		7	4		3.71	
P 4084	P	11	11		5	6		3.62	
P 4085	P	11	11		4	7		3.46	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 296

Material FDA 71-73

Dose 1600.0 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date July 23, 1973

Laboratory No. 1574 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 4091	NP	0						----	
P 4092	P	13	13		7	6		3.91	
P 4093	P	10	10		4	6		3.71	
P 4094	P	13	13		7	6		4.08	
P 4095	P	12	12		6	6		3.81	
P 4096	P	12	12		9	3		3.66	
P 4097	P	7	7		3	4		3.85	
P 4098	P	12	12		5	7		3.73	
P 4099	P	12	12		6	6		3.63	
P 4100	P	13	13		12	1		3.96	
P 4101	P	11	11		5	6		3.58	
P 4102	P	8	8		4	4		3.26	
P 4103	P	10	9		3	6	1	3.71	
P 4104	NP	0						----	
P 4105	NP	0						----	
P 4106	P	11	11		8	3		4.00	
P 4107	P	13	13		7	6		5.17	
P 4108	P	14	14		5	9		3.70	
P 4109	NP	0						----	
P 4110	P	14	14		8	6		3.37	
P 4111	P	11	11		4	7		3.77	
P 4112	P	16	16		6	10		3.87	
P 4113	P	5	5		3	2		3.73	
P 4114	P	8	7		3	4	1	3.56	
P 4115	P	8	8		3	5		3.86	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG Research LABORATORIES, INC.

WAVERLY DIVISION
Route 17
P.O. Box 107
Waverly, New York 14892
(607) 565-2931

FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date August 20, 1973

Laboratory No. 1575 p
Contract No. FDA 71-260

Sample: Clear light yellow liquid

Marking: FDA 71-73 (Hansen's, Starter distillate)

Examination Requested: Teratologic evaluation of FDA 71- 73 in hamsters

Procedure: See Appendix I

ults: See Tables 1 through 4 and Appendix II

Conclusion:

On the basis of the data presented herein, the following conclusion appears to be warranted:

"The administration of up to 1600 mg/kg (body weight) of the test material to pregnant hamsters for 5 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

FOOD and DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D.
Vice President

This report is submitted for the exclusive use of the person, partnership, or corporation to whom it is addressed, and neither the report nor the name of these Laboratories nor of any members of its staff, may be used in connection with the advertising or sale of any product or process without written authorization.

FOOD AND DRUG RE[illegible] LABORATORIES, INC.

Groups: 291 through 296

Material: FDA 71-73

Date: July 23, 1973

Laboratory No.: 1575 p

Table 1

Fate Summary
(Hamsters)

Group	Material	Dose** mg/kg	Total		Surviving at Term	
			Mated	Pregnant	Total	Pregnant[1]
291	Sham	0.0	25	21	25	21
292	Aspirin*	250.0	25	24	25	24
293	FDA 71-73	16.0	25	21	25	21
294	FDA 71-73	74.3	25	21	25	21
295	FDA 71-73	345.0	27	25	27	25
296	FDA 71-73	1600.0	26	23	26	23

* Positive Control: 250.0 mg/kg
** Administered as a water solution (See Appendix I)
1) Includes all dams examined at term

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FOOD AND DRUG RES LABORATORIES, INC.

Group: 291 through 296

Material: FDA 71-73

Date: July 23, 1973

Laboratory No. 1575 p

Table 2
Reproduction Data
(Hamsters)

Group:	291	292	293	294	295	296
Dose (mg/kg):	Sham	Aspirin**	16.0	74.3	345.0	1600.0
Pregnancies						
Total No.	21	24	21	21	25	23
Died or Aborted (before Day 14)	0	0	0	0	0	0
To term (on Day 14)	21	24	21	21	25	23
Live Litters						
Total No.*	21	24	21	21	25	23
Implant Sites						
Total No.	316	342	302	303	365	333
Average/dam*	15.1	14.3	14.4	14.4	14.6	14.5
Resorptions						
Total No.*	4	2	--	6	4	2
Dams with 1 or more sites resorbed	3	2	--	3	4	2
Dams with all sites resorbed	--	--	--	--	--	--
Per cent partial resorptions	14.3	8.33	--	14.3	16.0	8.70
Per cent complete resorptions	--	--	--	--	--	--
Live Fetuses						
Total No.	311	339	301	297	361	329
Average/dam*	14.8	14.1	14.3	14.1	14.4	14.3
Sex ratio (M/F)	0.70	0.78	0.72	0.68	0.64	0.80
Dead Fetuses						
Total*	1	1	1	--	--	2
Dams with 1 or more dead	1	1	1	--	--	2
Dams with all dead	--	--	--	--	--	--
Per cent partial dead	4.76	4.17	4.76	--	--	8.70
Per cent all dead	--	--	--	--	--	--
Average Fetus Weight, g	1.73	1.75	1.76	1.75	1.80	1.76

* Includes only those dams examined at term.

** Positive Control: 250.0 mg/kg

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296 — Laboratory No. 1575 p

Table 3

Material FDA 71-73 — Date July 23, 1973

Summary of Skeletal Findings*
(Hamsters)

Findings / Group No.:	291	292	293	294	295	296
Dose (mg/kg):	Sham	Aspirin**	16.0	74.3	345.0	1600.0
Live Fetuses Examined (at term)	216/21	235/24	208/21	206/21	251/25	227/23
Sternebrae						
Incomplete oss.	68/20	56/18	46/17	68/20	48/14	97/22
Scrambled						
Bipartite	25/13	30/15	18/13	20/13	27/17	15/9
Fused						
Extra		2/1	1/1	5/3		
Missing	40/14	30/12	38/13	38/15	37/11	52/17
Other						
Ribs						
Incomplete oss.						1/1
Fused/split	1/1			1/1		
Wavy						
Less than 12						
More than 13	34/11	70/20	42/17	45/18	51/18	56/17
Other						
Vertebrae						
Incomplete oss.	6/4	10/2		5/1	3/2	6/5
Scrambled						
Fused						
Extra ctrs. oss.						
Scoliosis				1/1		
Tail defects						
Other						
Skull						
Incomplete closure	1/1			1/1		1/1
Missing						
Craniostosis						
Other						
Extremities						
Incomplete oss.	7/4	9/1		10/4	5/2	11/6
Missing						
Extra						
Miscellaneous						
Hyoid; missing	5/5	10/2	3/3	5/3	2/1	4/2
Hyoid; reduced	14/6	1/1	10/6	7/6	5/2	10/4

* Numerator=Number of fetuses affected; Denominator=Number of litters affected.

** Positive control: 250.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296 Date July 23, 1973

Material FDA 71-73 Laboratory No. 1575 p

Table 3-a
Summary of Soft Tissue Abnormalities
(Hamsters)

Group	Material	Dose Level mg/kg	Dam	Number of Pups	Description
292	Aspirin*	250.0	A 5945	1	Gastroschisis
293	FDA 71-73	16.0	P 5009	1	Gastroschisis
294	FDA 71-73	74.3	P 5031	1	Hydrocephalus
			P 5043	1	Hydrocephalus
295	FDA 71-73	345.0	P 5080	1	Gastroschisis

*Positive control: 250.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296

Date July 23, 1973

Table 4

Species Hamsters

Laboratory No. 1575 p

Average Body Weights*

Group	Material	Dose Level	Day 0	6	8	10	14**
		mg/kg	g				
291	Sham	0.0	129.1	130.9	136.4	147.8	173.5 (21)
292	Aspirin***	250.0	125.0	130.0	134.3	145.5	169.8 (24)
293	FDA 71-73	16.0	125.6	128.6	133.0	144.6	170.9 (21)
294	FDA 71-73	74.3	121.2	123.9	128.8	139.9	164.1 (21)
295	FDA 71-73	345.0	123.1	125.8	132.6	142.3	165.6 (25)
296	FDA 71-73	1600.0	125.2	128.8	132.8	145.0	166.8 (23)

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1)
*** Positive control: 250.0 mg/kg

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Appendix I

Teratology Study in Hamsters

Virgin adult female golden hamsters from an outbred strain were individually housed in mesh bottom cages in temperature and humidity controlled quarters with free access to food and fresh tap water at all times. They were mated (1 to 1) with mature males and the appearance of motile sperm in the vaginal smear was considered as Day 0 of gestation. Beginning on Day 6 and continuing daily through Day 10 of gestation, the indicated dose levels of the test material were administered by oral intubation. The controls were sham treated with the vehicle at a level equivalent to the group receiving the highest test dose. The test material was prepared and doses calculated according to the following table:

Dosage (mg/kg)	Dose (ml/kg)	Concentration (mg/ml)
≦ 250	1	≦ 250
251 - 500	2	125 - 250
501 - 750	3	133 - 250
751 - 1000	4	187 - 250
1001 - 1250	5	200 - 250
1251 - 1500	6	208 - 250
1501 - 1600	6.4	235 - 250

Body weights were recorded on Days 0, 6, 8, 10 and 14 of the gestation period. All animals were observed daily for appearance and behavior with particular attention to food consumption in order to better recognize any abnormalities resulting from anorexic effects in the pregnant animal.

On Day 14, all animals were subjected to Caesarian section under deep anesthesia and the numbers of implantation sites, resorption

sites, live and dead fetuses were recorded. All live pups were weighed and the genital tract of each dam was examined for any anatomical abnormalities.

All fetuses were examined grossly for the presence of external congenital defects, and one-third of each litter underwent detailed visceral examination employing the Wilson technique. The remaining two-thirds of the pups were cleared in potassium hydroxide, stained with alizarin red S dye, and examined for the presence of skeletal abnormalities.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 291

Material Sham

Dose 0.0 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date July 23, 1973

Laboratory No. 1575

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
S 5936	NP	0						----	
S 5937	P	15	14		5	9	1	1.83	
S 5938	NP	0						----	
S 5939	P	14	14		8	6		1.68	
S 5940	NP	0						----	
S 5941	P	15	15		7	8		1.92	
S 5942	P	14	13		5	8	1	1.50	
S 5943	P	13	13		6	7		1.96	
S 5944	P	15	15		7	8		1.89	
S 5945	P	16	16		7	9		1.68	
S 5946	P	14	14		6	8		1.63	
S 5947	P	14	14		7	7		1.59	
S 5948	P	14	14		6	8		1.62	
S 5949	P	18	18		7	11		1.69	
S 5950	NP	0						----	
S 5951	P	20	20		7	13		1.57	
S 5952	P	11	11		6	5		1.54	
S 5953	P	16	16		7	9		1.98	
S 5954	P	13	13		4	9		1.81	
S 5955	P	15	14	1	5	9		1.69	
S 5956	P	15	15		4	11		1.75	
S 5957	P	16	16		6	10		1.71	
S 5958	P	17	17		5	12		1.75	
S 5959	P	15	15		8	7		1.83	
S 5960	P	16	14		5	9	2	1.71	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 292

Material Aspirin

Dose 250.0 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date July 23, 1973

Laboratory No. 1575

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
A 5936	P	12	12		5	7		1.96	
A 5937	P	17	17		8	9		1.73	
A 5938	P	10	10		4	6		1.73	
A 5939	P	14	14		7	7		1.90	
A 5940	P	14	14		6	8		2.01	
A 5941	P	12	12		7	5		1.74	
A 5942	P	13	13		6	7		1.84	
A 5943	P	8	8		4	4		1.98	
A 5944	P	14	14		5	9		1.79	
A 5945	P	15	15		7	8		1.75	
A 5946	P	17	17		7	10		1.81	
A 5947	P	15	14		5	9	1	1.10	
A 5948	P	17	17		6	11		1.69	
A 5949	P	13	13		7	6		1.55	
A 5950	P	12	12		4	8		1.67	
A 5951	P	16	16		7	9		1.70	
A 5952	P	19	19		7	12		1.68	
A 5953	P	17	17		6	11		1.92	
A 5954	P	13	13		7	6		1.62	
A 5955	P	17	17		8	9		1.70	
A 5956	P	14	14		8	6		1.74	
A 5957	P	14	14		7	7		1.83	
A 5958	P	13	12		3	9	1	1.73	
A 5959	P	16	15	1	8	7		1.71	
A 5960	NP	0						----	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 293

Material FDA 71-73

Dose 16.0 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date July 23, 1973

Laboratory No. 1575 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 5001	NP	0						----	
P 5002	P	14	14		6	8		1.77	
P 5003	P	13	13		6	7		1.85	
P 5004	P	10	10		5	5		1.70	
P 5005	P	14	14		6	8		1.90	
P 5006	NP	0						----	
P 5007	P	14	14		6	8		1.75	
P 5008	P	16	16		8	8		1.64	
P 5009	P	9	9		5	4		1.70	
P 5010	NP	0						----	
P 5011	P	15	15		5	10		1.84	
P 5012	P	11	11		4	7		1.93	
P 5013	P	14	14		5	9		1.61	
P 5014	P	16	16		7	9		1.73	
P 5015	NP	0						----	
P 5016	P	17	17		7	10		1.62	
P 5017	P	14	14		6	8		1.69	
P 5018	P	12	12		6	6		1.67	
P 5019	P	15	15		4	11		1.88	
P 5020	P	17	17		4	13		1.75	
P 5021	P	15	15		7	8		1.66	
P 5022	P	15	14	1	5	9		1.91	
P 5023	P	18	18		9	9		1.72	
P 5024	P	15	15		6	9		1.94	
P 5025	P	18	18		9	9		1.68	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 294

Material FDA 71-73

Dose 74.3 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date July 23, 1973

Laboratory No. 1575 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 5031	P	18	15		5	10	3	1.29	
P 5032	P	14	14		5	9		1.88	
P 5033	NP	0						----	
P 5034	P	13	13		5	8		1.65	
P 5035	NP	0						----	
P 5036	P	14	14		5	9		1.75	
P 5037	P	11	11		3	8		1.76	
P 5038	P	11	11		5	6		1.91	
P 5039	P	13	13		5	8		1.97	
P 5040	P	14	14		7	7		1.81	
P 5041	NP	0						----	
P 5042	P	14	14		5	9		1.56	
P 5043	P	11	10		4	6	1	1.86	
P 5044	P	17	17		8	9		1.49	
P 5045	P	11	11		5	6		1.89	
P 5046	P	16	16		7	9		1.72	
P 5047	P	15	15		8	7		1.81	
P 5048	P	12	12		5	7		1.64	
P 5049	P	17	15		1	14	2	1.79	
P 5050	NP	0						----	
P 5051	P	17	17		6	11		1.46	
P 5052	P	18	18		5	13		1.74	
P 5053	P	17	17		9	8		1.91	
P 5054	P	15	15		9	6		1.87	
P 5055	P	15	15		8	7		1.91	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 295

Material FDA 71-73

Dose 345.0 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date July 23, 1973

Laboratory No. 1575 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 5061	P	20	20		8	12		1.71	
P 5062	P	14	14		4	10		1.63	
P 5063	P	12	12		4	8		1.78	
P 5064	P	16	16		7	9		1.75	
P 5065	P	18	18		7	11		1.59	
P 5066	P	11	11		5	6		1.64	
P 5067	P	12	12		4	8		1.59	
P 5068	P	16	15		6	9	1	1.82	
P 5069	P	14	14		5	9		1.94	
P 5070	P	10	9		2	7	1	1.91	
P 5071	P	12	12		3	9		1.88	
P 5072	P	14	14		6	8		1.46	
P 5073	P	17	16		6	10	1	1.53	
P 5074	NP	0						----	
P 5075	P	14	14		4	10		1.91	
P 5076	P	14	13		5	8	1	1.83	
P 5077	P	19	19		11	8		1.90	
P 5078	P	20	20		10	10		1.93	
P 5079	P	16	16		3	13		1.74	
P 5080	P	16	16		4	12		1.63	
P 5081	P	14	14		7	7		1.94	
P 5082	P	14	14		3	11		2.41	
P 5083	NP	0						----	
P 5084	P	13	13		7	6		1.89	
P 5085	P	13	13		8	5		1.95	
P 5086	P	12	12		5	7		1.78	
P 5087	P	14	14		7	7		1.88	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 296

Material FDA 71-73

Dose 1600.0 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date July 23, 1973

Laboratory No. 1575 p

Dam No.	Fate*	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 5091	P	7	7		4	3		1.60	
P 5092	P	10	10		5	5		1.97	
P 5093	NP	0						----	
P 5094	P	14	13		5	8	1	1.75	
P 5095	NP	0						----	
P 5096	P	11	11		3	8		1.72	
P 5097	P	13	13		7	6		1.88	
P 5098	P	14	14		7	7		1.77	
P 5099	P	15	15		6	9		2.01	
P 5100	P	15	15		6	9		1.91	
P 5101	P	12	12		5	7		1.83	
P 5102	P	10	10		4	6		1.55	
P 5103	P	16	15	1	5	10		1.56	
P 5104	P	19	19		9	10		1.57	
P 5105	P	13	13		11	2		1.90	
P 5106	P	16	16		6	10		1.62	
P 5107	P	20	19	1	10	9		1.83	
P 5108	P	16	15		7	8	1	1.77	
P 5109	P	18	18		3	15		1.80	
P 5110	NP	0						----	
P 5111	P	17	17		6	11		1.70	
P 5112	P	16	16		7	9		1.63	
P 5113	P	18	18		7	11		1.70	
P 5114									Not Assigned
P 5115	P	16	16		7	9		1.68	
P 5116	P	13	13		7	6		1.85	
P 5117	P	14	14		9	5		1.82	

* P = Pregnant; NP = Not Pregnant

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FOOD AND DRUG Research LABORATORIES, INC.

WAVERLY DIVISION
Route 17
P.O. Box 107
Waverly, New York 14892
(607) 565-2931

FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date October 26, 1973

Laboratory No. 1576 p
Contract No. FDA 71-260

Sample: Clear light yellow liquid

Marking: FDA 71-73 (Hansen's; Starter distillate)

Examination Requested: Teratologic evaluation of FDA 71-73 in rabbits

Procedure: (See Appendix I)

lts: See Tables 1 through 4 and Appendix II

Conclusion:

On the basis of the data presented herein, the following conclusion appears to be warranted:

"The administration of up to 1600 mg/kg (body weight) of the test material to pregnant rabbits for 13 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

FOOD and DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D
Vice President

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FOOD AND DRUG R[illegible] R[illegible] LABORATORIES, INC.

Groups: 291 through 296

Material: FDA 71-73

Date: September 26, 1973

Laboratory No.: 1576 p

Table 1

Fate Summary
(Rabbits)

Group	Material	Dose ** mg/kg	Total		Surviving at Term	
			Mated	Pregnant	Total	Pregnant[1]
291	Sham	0.0	23	13	20	11
292	6-AN*	2.5	24	10	24	10
293	FDA 71-73	16.0	26	11	25	10
294	FDA 71-73	74.3	22	11	22	11
295	FDA 71-73	345.0	14	10	13	10
296	FDA 71-73	1600.0	23	11	23	11

* Positive Control: 2.5 mg/kg of 6-aminonicotinamide dosed on Day 9

** Administered as a water solution (See Appendix I)

1) Includes all dams examined at term

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

P: 291 through 296

Material: FDA 71-73

Table 2

Reproduction Data

(Rabbits)

Date: September 26, 1

Laboratory No.: 1576 p

Group:	291	292	293	294	295	296
Dose (mg/kg):	Sham	6-AN**	16.0	74.3	345.0	1600.0
Pregnancies						
Total No.	13	10	11	11	10	11
Died or Aborted (before Day 29)	2	0	1	0	0	0
To term (on Day 29)	11	10	10	11	10	11
Corpora Lutea						
Total No.	153	142	224	142	139	225
Average/dam mated	9.56	7.89	14.9	11.8	11.6	14.1
Live Litters						
Total No.*	10	9	10	10	10	11
Implant Sites						
Total No.	53	46	69	74	42	52
Average/dam*	4.82	4.60	6.90	6.73	4.20	4.73
Resorptions						
Total No.*	13	11	1	4	7	8
Dams with 1 or more sites resorbed	4	4	1	1	3	4
Dams with all sites resorbed	1	--	--	--	--	--
Per cent partial resorptions	36.4	40.0	10.0	9.09	30.0	36.4
Per cent complete resorptions	9.09	--	--	--	--	--
Live Fetuses						
Total No.	40	34	68	60	35	44
Average/dam*	3.64	3.40	6.80	5.45	3.50	4.00
Sex ratio (M/F)	0.82	1.43	1.27	1.46	1.50	1.67
Dead Fetuses						
Total No.*	--	1	--	10	--	--
Dams with 1 or more dead	--	1	--	1	--	--
Dams with all dead	--	--	--	--	--	--
Per cent partial dead	--	10.0	--	9.09	--	--
Per cent all dead	--	--	--	--	--	--
Average Fetus Weight, g	42.7	36.8	37.7	39.5	43.0	42.4

* Includes only those dams examined at term.

** Positive Control: 2.5 mg/kg of 6-aminonicotinamide dosed on Day 9

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296 | Laboratory No. 1576 p

Table 3

Material FDA 71-73 | Date September 26, 1973

Summary of Skeletal Findings*
(Rabbits)

Findings / Group No.:	291	292	293	294	295	296
Dose (mg/kg):	Sham	6-AN**	16.0	74.3	345.0	1600.0
Live Fetuses Examined (at term)	40/10	33/8[a]	68/10	60/10	35/10	44/11
Sternebrae						
Incomplete oss.	1/1	5/1	2/1	2/2	4/2	4/3
Scrambled						
Bipartite	2/1	1/1		1/1	1/1	
Fused					2/2	1/1
Extra	1/1	1/1	2/2	1/1	2/1	
Missing	1/1			2/2		
Other						
Ribs						
Incomplete oss.						
Fused/split		8/5				
Wavy						
Less than 12		1/1				
More than 13						
Other						
Vertebrae						
Incomplete oss.						
Scrambled		5/4				
Fused						
Extra ctrs. oss.						
Scoliosis						
Tail defects		9/4	1/1	1/1		1/1
Other						
Skull						
Incomplete closure						1/1
Missing						
Craniostosis						
Other						
Extremities						
Incomplete oss.						
Missing						
Extra						
Miscellaneous						

* Numerator=Number of fetuses affected; Denominator=Number of litters affected.

** Positive control: 2.5 mg/kg of 6-aminonicotinamide dosed on Day 9.

[a] One pup lost in processing

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296 Date September 26, 1973

Material FDA 71-73 Laboratory No. 1576 p

Table 3-a
Summary of Soft Tissue Abnormalities
(Rabbits)

Group	Material	Dose Level mg/kg	Dam	Number of Pups	Description
291	Sham	0.0	S 6515	1	Short tail
292	6-AN*	2.5	Z 6498	2	Medial rotation of hind limbs
			Z 6504	2	Anopia; medial rotation of hind limbs
				3	Anopia
			Z 6512	1	Anopia; medial rotation of hind limbs; short tail; anteroposterior facial dysplasia
296	FDA 71-73	1600.0	P 6089	1	Meningoencephalocele

*Positive Control: 2.5 mg/kg of 6-aminonicotinamide dosed on Day 9

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 291 through 296

Species Rabbits

Date September 26, 1973

Laboratory No. 1576 p

Table 4

Average Body Weights*

Group	Material	Dose Level	Day 0	Day 6	Day 12	Day 18	Day 29**
		mg/kg	kg	kg	kg	kg	kg
291	Sham	0.0	2.19	2.38	2.41	2.45	2.42 (11)
292	6-AN***	2.5	2.31	2.43	2.37	2.38	2.55 (10)
293	FDA 71-73	16.0	2.41	2.55	2.48	2.64	2.62 (10)
294	FDA 71-73	74.3	1.91	2.02	2.06	2.13	2.22 (11)
295	FDA 71-73	345.0	2.34	2.46	2.49	2.56	2.70 (10)
296	FDA 71-73	1600.0	2.29	2.39	2.45	2.59	2.63 (11)

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1)
*** Positive control: 2.5 mg/kg of 6-amoninicotinamide dosed On day 9

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Appendix I

Teratology Study in Rabbits

Virgin, adult, Dutch-belted female rabbits were individually housed in mesh bottom cages in temperature and humidity-controlled quarters with free access to food and fresh tap water. On Day 0, each doe was given an injection of 0.4 ml of human chorionic gonadotropin (400 IU) via the marginal ear vein. Three hours later, each doe was inseminated artificially with 0.3 ml of diluted semen from a proven donor buck using approximately 20×10^6 motile sperm according to the procedure described by Vogin et al (Pharmacologist 11, 282 (1969)). Beginning on Day 6 and continuing daily through Day 18 the females were dosed with the indicated dosages by oral intubation. The controls were sham treated with the vehicle at a level equivalent to the group receiving the highest test dose. The test material was prepared and doses calculated according to the following table:

Dosage (mg/kg)	Dose (ml/kg)	Concentration (mg/ml)
≦ 250	1	≦ 250
251 - 500	2	125 - 250
501 - 750	3	133 - 250
751 - 1000	4	187 - 250
1001 - 1250	5	200 - 250
1251 - 1500	6	208 - 250
1501 - 1600	6.4	235 - 250

Body weights were recorded on Days 0, 6, 12, 18, and 29 of gestation. All animals were observed daily for appearance and behavior, with particular attention to food consumption and body weight in order to rule out any abnormalities which may have occurred as a result of anorexic effects in the pregnant female animal.

On Day 29 all does were subjected to Caesarean section under surgical anesthesia, and the numbers of corpora lutea, implantation sites, resorption sites and live and dead fetuses were recorded. Body weights of the live pups were also recorded. The urogenital tract of each animal was examined in detail for normality. In addition all fetuses underwent a detailed gross examination for the presence of external congenital abnormalities. The live fetuses of each litter were then placed in an incubator for 24 hours for the evaluation of neonatal survival. All surviving pups were sacrificed, and all pups examined for visceral abnormalities (by dissection). All fetuses were then cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 291

Material Sham

Dose 0.0 mg/kg

Appendix II

Reproduction Data in Rabbits (Individual)

Date September 26, 1973

Laboratory No. 1576

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
S 6496	NP	--**	0						----	
S 6497	NP	--**	0						----	
S 6498	P	9	5					5	----	Died Day 22
S 6499	NP	0	0						----	Died Day 28
S 6500	NP	--**	0						----	
S 6501	NP	7	0						----	
S 6502	NP	--**	0						----	
S 6503	NP	13	0						----	
S 6504	NP	--**	0						----	
S 6505	P	12	5	5		2	3		42.3	
S 6506	P	5	4	4		1	3		37.2	
S 6507	P	13	4					4	----	
S 6508	P	--	--	--		--	--		----	Aborted Day 14
S 6509	P	13	9	9		4	5		36.1	
S 6510	NP	9	0						----	
S 6511	P	8	3	3		2	1		34.5	Two Neonatal Deat
S 6512	P	9	6	6		3	3		42.3	
S 6513	P	8	8	3		2	1	5	39.1	
S 6514	P	13	4	4		2	2		42.0	
S 6515	P	11	5	3		1	2	2	47.4	
S 6516	P	14	2	2		0	2		49.0	
S 6517	P	9	3	1		1	0	2	56.6	
S 6518	NP	--**	0						----	

* P = Pregnant; NP = Not Pregnant

** Not Recorded

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 292

Material 6-AN

Dose 2.5 mg/kg

Appendix II

Reproduction Data in Rabbits (Individual)

Date September 26, 1973

Laboratory No. 1576

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Z 6496	NP	--**	0						----	
Z 6497	P	12	6		1			5	----	
Z 6498	P	11	5	5		3	2		40.0	Four Neonatal Dea
Z 6499	NP	--**	0						----	
Z 6500	P	6	4	4		2	2		41.5	
Z 6501	NP	--**	0						----	
Z 6502	P	8	5	3		1	2	2	35.2	One Neonatal Deat
Z 6503	NP	7	0						----	
Z 6504	P	10	5	5		3	2		35.8	Five Neonatal Dea
Z 6505	NP	--**	0						----	
Z 6506	NP	--**	0						----	
Z 6507	P	11	1	1		1	0		37.7	One Neonatal Deat
Z 6508	NP	6	0						----	
Z 6509	NP	3	0						----	
Z 6510	NP	9	0						----	
Z 6511	NP	8	0						----	
Z 6512	P	12	4	1		1	0	3	32.2	One Neonatal Deat
Z 6513	NP	2	0						----	
Z 6514	NP	9	0						----	
Z 6515	NP	6	0						----	
Z 6516	P	2***	5	5		4	1		36.2	
Z 6517	P	8	4	3		2	1	1	49.4	
Z 6518	P	12	7	7		3	4		23.4	Four Neonatal Dea
Z 6519	NP	--**	0						----	

* P = Pregnant; NP = Not Pregnant
** Not Recorded
*** One ovary missing

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 293

Material FDA 71-73

Dose 16.0 mg/kg

Appendix II

Reproduction Data in Rabbits (Individual)

Date September 26, 1973

Laboratory No. 1576 p

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 6001	NP	--**	0						----	
P 6002	P	14	8	8		5	3		32.3	
P 6003	NP	0	0						----	
P 6004	NP	--**	0						----	
P 6005	P	32	5	4		2	2	1	35.6	
P 6006	NP	--**	0						----	
P 6007	NP	--**	0						----	
P 6008	P	14	7	--		--	--		----	Died Day 10
P 6009	P	11	8	8		5	3		39.6	
P 6010	P	12	7	7		4	3		44.6	
P 6011	NP	35	0						----	
P 6012	P	12	3	3		2	1		47.5	
P 6013	NP	--**	0						----	
P 6014	P	18	11	11		6	5		29.7	
P 6015	NP	--**	0						----	
P 6016	NP	13	0						----	
P 6017	NP	--**	0						----	
P 6018	NP	14	0						----	
P 6019	NP	--**	0						----	
P 6020	NP	7	0						----	
P 6081	NP	--**	0						----	
P 6082	P	11	3	3		2	1		39.6	
P 6085	P	19	11	11		5	6		34.1	
P 6086	P	12	5	5		3	2		31.6	
P 6087	NP	--**	0						----	
P 6088	P	--**	8	8		4	4		42.0	

* P = Pregnant; NP = Not Pregnant

** Not Recorded

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 294

Material FDA 71-73

Dose 74.3 mg/kg

Appendix II

Reproduction Data in Rabbits (Individual)

Date September 26, 1973

Laboratory No. 1576 p

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 6021	P	14	5	5		3	2		45.9	
P 6022	P	10	4	4		2	2		43.1	
P 6023	NP	--**	0						----	
P 6024	NP	--**	0						----	
P 6025	NP	--**	0						----	
P 6026	NP	--**	0						----	
P 6027	P	7	5	5		2	3		40.0	
P 6028	P	11	7	7		4	3		40.5	
P 6029	NP	--**	0						----	
P 6030	P	10	7	7		4	3		35.4	
P 6031	P	33	14		10			4	----	
P 6032	NP	4	0						----	
P 6033	P	13	4	4		3	1		41.5	
P 6034	P	17	7	7		5	2		32.5	One Neonatal De
P 6035	NP	0	0						----	
P 6036	NP	--**	0						----	
P 6037	NP	--**	0						----	
P 6038	NP	--**	0						----	
P 6039	P	13	7	7		4**	2**		30.9	
P 6040	P	10	6	6		3	3		45.1	
P 6083	P	--**	8	8		5	3		40.2	
P 6084	NP	--**	0						----	

* P = Pregnant; NP = Not Pregnant
** Not Recorded

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Rabbits (Individual)

Group 295

Material FDA 71-73

Dose 345.0 mg/kg

Date September 26, 1973

Laboratory No. 1576 p

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 6041	P	22	4	4		2	2		34.2	
P 6042	P	13	3	1		1	0	2	43.0	
P 6043	P	12	4	1		0	1	3	50.7	
P 6044	P	13	1	1		0	1		52.3	
P 6045	P	8	4	4		3	1		33.8	
P 6046	NP	5	0						----	Died Day 13
P 6047	P	11	5	3		3	0	2	46.6	
P 6048	P	12	6	6		3	3		44.9	
P 6049	P	17	3	3		2	1		45.0	One Neonatal Dea
P 6050	NP	4	0						----	
P 6051	NP	--**	0						----	
P 6052	NP	--**	0						----	
P 6053	P	12	5	5		3	2		44.3	
P 6054	P	10	7	7		4	3		34.7	

* P = Pregnant; NP = Not Pregnant
** Not Recorded

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Rabbits (Individual)

Group 296

Material FDA 71-73

Dose 1600.0 mg/kg

Date September 26, 1973

Laboratory No. 1576 p

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
P 6061	NP	--**	0						----	
P 6062	NP	--**	0						----	
P 6063	NP	3	0						----	
P 6064	P	15	6	6		4	2		38.5	
P 6065	NP	--**	0						----	
P 6066	NP	--**	0						----	
P 6067	P	22	2	1		1	0	1	49.3	
P 6068	P	15	7	5		3	2	2	46.1	
P 6069	P	21	4	4		--**	--**		35.3	
P 6070	NP	--**	0						----	
P 6071	P	24	1	1		1	0		52.6	
P 6072	P	9	3	3		1	2		43.2	
P 6073	P	10	5	5		3	2		43.0	
P 6074	P	17	4	4		3	1		46.2	
P 6075	NP	--**	0						----	
P 6076	NP	6	0						----	
P 6077	NP	20	0						----	
P 6078	P	29	8	4		2	2	4	35.0	
P 6079	NP	3	0						----	
P 6080	NP	4	0						----	
P 6081	P	14	6	6		4	2		41.0	One Neonatal De
P 6090	P	13	6	5		3	2	1	35.8	
P 6091	NP	--**	0						----	

P = Pregnant; NP = Not Pregnant

Not Recorded

0584

0585

Food Protection Committee, Food and Nutrition Board. 1965
Chemicals Used in Food Processing
National Academy of Sciences, National Research Council, Washington, D.C. Publication 1274

J. Dairy Res. 34, 131

(0589)

Volatile compounds in butter oil

I. Lower boiling compounds

By D. A. FORSS, W. STARK and GERDA URBACH
Division of Dairy Research, C.S.I.R.O., Melbourne, Australia

(*Received* 28 *December* 1966)

SUMMARY. The following compounds were isolated from best-quality Australian butter oil by high vacuum degassing, and identified by the combination of gas chromatography and mass spectrometry: the $C_{3,5,7,9}$ alkan-2-ones, the $C_{2,4,6,8,10,12}$ n-alkanoic acids, the $C_{6,8,10}$ 5-lactones (or their hydroxyacids), dimethyl sulphone, all isolated in amounts above 1 pt in 10^8 of butter oil, and butanone, undecan-2-one, 5-dodecalactone, diacetyl, n-nonanoic acid, a decenoic acid, methyl n-decanoate, toluene, o-methoxyphenol, a cresol, phenol, benzaldehyde, methyl benzoate and benzothiazole, all isolated in amounts between 1 pt in 10^{11} and 1 pt in 10^8 of butter oil.

No compound was isolated in an amount greater than flavour threshold, in fact most compounds were below one-tenth threshold level. However, the distillate obtained by degassing produced a very attractive creamy flavour when added to bland non-cultured butter.

In Australia, butter for export is made only from sweet or neutralized farm-ripened cream. Its flavour is therefore not a predominantly diacetyl-type derived from fermentation, but is due to the flavours inherent in the cream or derived from processing. Butter is graded according to flavour and aroma (maximum 50 points), texture (maximum 30 points) and condition (maximum 20 points). Most butters are awarded the full 50 points for texture and condition, the main variation being in the flavour score. Butter graded as 93 points and over is called choicest quality. A small proportion may receive 94 points and such terms as 'a good 93', '93+' are used to indicate that the butter has some desirable characteristic over and above the bland 93-point butter which merely lacks undesirable flavours.

The Dairy Produce Inspectors of the Commonwealth Department of Primary Industry grade butter for export and complete reliance is placed on their judgement. The aim of the present series of investigations was to relate grade scores on butters to qualitative and quantitative chemical data on volatile compounds isolated from butter oil.

Monerieff (1965) reviewed the literature on butter flavour up to the end of 1963. Since then Parliment, Nawar & Fagerson (1965) have identified 5-hexalactone in heated milk fat and Jurriens & Oele (1965) have reported the presence of 4- and 5-lactones from C_{10} to C_{16} inclusive in butter. Haverkamp Begemann & Koster (1964) claimed that hept-*cis*-4-enal is responsible for a creamy flavour in milk fat and occurs

in fresh milk fat at concentrations of 1·5 pts in 10^9. Badings' (1965) observations were similar; hept-*cis*-4-enal added to high vacuum degassed milk fat at a concentration of 1 pt in 10^9 conferred a creamy flavour as judged by experienced Dutch butter graders. Kawanishi & Saito (1965) isolated from Japanese sweet cream butter a series of normal ($C_{3-6,8}$) and iso ($C_{4,5,8}$) fatty acids, and aliphatic carbonyl compounds having 3–8 carbon atoms and they claimed that this mixture reproduced the flavour of sweet cream butter.

It became obvious in our investigation of butter flavour that neither reduced pressure steam distillates nor high vacuum distillates (pressure below 10^{-3} torr) of butter or butter oil held at 45 °C had typical buttery flavours. However, cold finger molecular distillates of highest quality butter oil had a strong butter odour.

This paper describes the identification of the compounds collected during the degassing stage prior to molecular distillation. Subsequent papers will describe the identification of the higher boiling compounds which collected on the cold finger and which are mainly responsible for the characteristic flavour of butter. The presence of 4-dodecalactone and other lactones deposited on the cold finger has already been reported (Forss, Urbach & Stark, 1966).

EXPERIMENTAL

The butter oil was prepared commercially from a mixture of farm and factory separated cream (35% fat) by the Alfa-Laval process (1963). After vacreation the cream was concentrated to 45% fat and fed into a 'Clarifixator' where it was homogenized and concentrated to 80% fat. After further centrifugal concentration to 97–98% fat, the product was passed through a plate heat exchanger (94 °C; 9 s) into a vacuum vessel where the water content was reduced to below 0·1%. In the manufacture of butter oil more than in the manufacture of butter, a mild-flavoured product free of taints is sought so that due to the more severe processing some of the most volatile compounds would be lost. All butter oil investigated came from the same factory and was graded highest quality. Distillations were performed on autumn and spring samples less than 3 weeks old which had been stored at −12 °C.

The distillation apparatus is shown in Plate 1. Butter oil (3·0 kg) was allowed to flow downwards through 2 Davies condensers (the first filled with Fenske helices) at 55 °C into a reservoir at such a rate that the pressure was less than 5×10^{-2} torr. Most of the water (1–2 ml) collected in trap 1. Very little water or flavour passed into trap 2. Partially degassed butter oil was allowed to flow into the 10 l. flask at such a rate that the pressure measured on a Penning gauge after trap 4 did not exceed 2×10^{-3} torr. The flask was immersed in water at 50 °C and the butter oil was magnetically stirred. The complete degassing took 4–5 h. Trap 3 was removed and replaced by a similar trap (no. 5). All stopcocks were closed overnight. On the following day the butter oil was degassed to a pressure of less than 5×10^{-5} torr on the Penning gauge (2 h) after which liquid nitrogen was added to the cold finger. After 5 h the cold finger was removed, the liquid nitrogen replaced by water at 60 °C and material on the cold finger (1–2 g) was allowed to flow into a flask or was washed with chloroform. Control experiments on the distillation apparatus established the technique did not yield any contaminants apart from benzothiazole which

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from the milking machine rubber tubing initially used to connect the Edwards diffusion pump to the first stage of the degassing section. This rubber tubing was replaced by a glass tube.

The volatile compounds isolated during the degassing (traps 1, 3 and 5) are reported in this paper. The contents of traps 1, 3 and 5 were combined to give the total distillate.

The distillate was separated into 'acid' and non-acid fractions. The distillate, which was acid to litmus, was made alkaline with 50 μl of N-NaOH and the volatiles removed by vacuum sublimation of the frozen distillate followed by distillation at 50°C and 1×10^{-5} torr. Under these conditions synthetic C_6 and C_8 5-lactones stayed behind with the 'acid' fraction.

'Free acids' were liberated from the 'acid' residue by treatment with water and Dowex 50W–H^+ form (Bio-Rad). The methyl esters were prepared by suspending the 'acid' residue in methanol and adding Dowex 50W–H^+ form resin previously washed with methanol. The reaction was carried out in a sealed glass tube heated at 70°C for 10 min.

The total distillate and the 'acid' fraction (free and esterified) were analysed by combined gas chromatography and mass spectrometry (Stark, Smith & Forss, 1967) on a glass column 8 ft × $\frac{1}{8}$ in. O.D. packed with 5% Carbowax 20M on 60–70 mesh, acid-washed 'Chromosorb G', held at 60°C for 10 min and then programmed to 220°C at 2°C/min. Mass spectra were recorded in 4–15 s.

The Carbowax 20M column was suitable for separating most of the isolated compounds but acids were strongly adsorbed, and desorbed very slowly. This was first shown by the mass spectra which contained peaks at m/e = 60, 73, 87 even when there was no gas chromatographic peak. No indication of the C_{12} acid was given from the unesterified sample but this was shown to be a major component after esterification.

Authentic compounds were treated in the same way to obtain retention data and mass spectra, and compounds were regarded as identified if retention times and mass spectra of known and isolated compounds agreed.

Trap 1 was also analysed by 2-dimensional gas chromatography/thin-layer chromatography (Janák, 1964) with manual movement of the thin-layer chromatographic plate. This confirmed the presence of the $C_{6,8,10}$ 5-lactones.

Bland, unsalted 93-point butter was used as the vehicle for flavour evaluations by the Dairy Produce Inspectors (Graders). In this way the total distillate and the 'acid' and non-acid fractions were examined. The following synthetic compounds were also examined for flavour: gas chromatographically purified $C_{6,8,10}$ 5-lactones (the decalactone from Price's (Bromborough) Ltd., Cheshire, England), $C_{2,6,8}$ n-alkanoic acids and 5-hydroxyhexanoic acid. The hydroxyacid was not gas chromatographically purified.

RESULTS

Identifications

The following compounds were isolated from all samples of butter oil in amounts above 1 pt in 10^8: the $C_{3,5,7,9}$ alkan-2-ones, the $C_{2,4,6,8,10,12}$ n-alkanoic acids, the $C_{6,8,10}$ 5-lactones (or their hydroxyacids) and dimethyl sulphone. Butanone, undecan-

2-one, 5-dodecalactone, methyl n-decanoate, diacetyl, n-nonanoic acid, a decenoic acid, toluene, o-methoxyphenol, a cresol, phenol, benzaldehyde, methyl benzoate and benzothiazole were isolated in amounts between 1 pt in 10^{11} and 1 in 10^4 of butter oil. In different experiments these compounds were differently distributed in traps 1, 3 and 5 both qualitatively and quantitatively. Most of the compounds were found to some extent in all the traps and there is indication that they were also collected on the cold finger.

Thin-layer chromatography and mass spectrometry established that the compounds reported as 5-lactones were substituted in the 5-position.

The total distillate consisted of about 80% n-alkanoic acids and about 10% 5-lactones.

Flavour evaluations

The Graders' comments clearly indicated that the distillate contained some very desirable elements described by them as 'sweet' and 'creamy'. These desirable elements were associated with the 'acid' fraction. Experiments showed that the flavour of this fraction was not appreciably affected by gas chromatography. The non-acid fraction contained undesirable flavours. Compounds identified in the 'acid' fraction were the $C_{2,4,6,8,9,10,12}$ n-alkanoic acids, a decenoic acid, the $C_{6,8,10,12}$ 5-lactones, phenol, o-methoxyphenol, and a cresol. It is not clear whether the lactones were originally present as such or as their hydroxyacids because in the separation of 'acid' and non-acid fractions the lactones remain with the 'acid' fraction and during gas chromatography on a polar phase (Ansell & Palmer, 1963) 4- and 5-hydroxy-acids are converted to the corresponding lactones.

Table 1. *Flavour of synthetic compounds added to 93-point butter*

Compound	Added concentration in butter pt/pts butter	Comments
5-Lactone C_6	1 in 10^4	Scarcely perceptible, undesirable
5-Lactone C_8	1 in 10^6	Strong coconut oil, undesirable
5-Lactone C_8	1 in 10^7	Just perceptible, undesirable
5-Lactone C_{10}	1 in 10^5	Coconut, rancid, anty, undesirable
n-Alkanoic acid C_2	2·5 in 10^4	Fragrant, desirable
n-Alkanoic acid C_6	1 in 10^6	Desirable
n-Alkanoic acid C_8	1 in 10^6	Desirable
5-Hydroxyhexanoic acid	1 in 10^4	Astringent, flat

No compound was isolated in amount greater than flavour threshold, in fact most compounds were below one-tenth flavour threshold level. Yet examination by the Graders clearly showed that a creamy element was contained in this lower boiling fraction and that some components in it improved 93-point to 94+ butter.

Table 1 lists the comments of the Graders on various synthetics added to 93-point butter. The concentration given is additional to the amount naturally present in 93-point butter. None of the $C_{6,8,10}$ 5-lactones, $C_{2,6,8}$ n-alkanoic acids or 5-hydroxyhexanoic acid produced a creamy flavour.

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DISCUSSION

In Australia, the very top quality butters are those completely free of off-flavours and containing one or more desirable flavour elements such as those usually described as creamy or nutty.

In the present investigation we have shown that 93-point butter can be changed to 94+ butter by the addition of compounds trapped during the degassing of butter oil. The elements responsible for this improvement are in the 'acid' fraction but we have not been able to determine as yet whether any of the compounds identified are actually responsible for the creamy flavour or whether the creamy flavour is due to a single compound or a mixture. We were not able to detect any hept-*cis*-4-enal at a concentration of 1 pt in 10^{11} in butter oil (Haverkamp Begemann & Koster, 1964).

The presence of dimethyl sulphone is interesting in relation to the presence of dimethyl sulphide in butter (Day, Lindsay & Forss, 1964) although the sulphone itself is odourless. Dimethyl sulphone was recently isolated from pasteurized milk (Williams, Burstein & Layne, 1966*a*) but no dimethyl sulphoxide was detected. We also did not detect any dimethyl sulphoxide in butter oil. Williams *et al.* (1966*b*) observed that in humans and rabbits, dimethyl sulphide is oxidized to the sulphone and eliminated in the urine. The sulphone might therefore be a normal metabolite of the cow.

Since we showed that benzothiazole is a volatile constituent of some rubber, it is possible that the benzothiazole detected in the butter oil may have been absorbed during milking. Alternatively, since Arnold, Libbey & Day (1966) observed benzothiazole in stale but not in fresh sterilized concentrated milk, it may be a product of ageing. Several other aromatic compounds from butter oil were identified, but as no anti-oxidants were used in the preparation of this butter oil, the aromatic compounds could not have been derived from them.

The Firmenich group (Winter, Stoll, Warnhoff, Greuter & Büchi, 1963), Japanese investigators (Kawanishi & Saito, 1965) and the Unilever group (Boldingh & Taylor, 1962) claim that the compounds which they have isolated together give a complete butter flavour. In view of the difference between their findings—carbonyl compounds by the Firmenich group, a mixture of fatty acids and carbonyl compounds by the Japanese workers and $C_{8,10,12}$ 5-lactones by the Unilever group, one wonders whether the term 'butter flavour' means very different things to people in different countries. Certainly, the 'degassing fraction' reported in this paper, although highly odorous and on the whole pleasant, does not represent 'butter flavour' but only a part of it. Examination of the cold finger distillate showed that a fraction containing esters, acids and indole-type compounds conferred a very desirable nutty flavour when added to butter.

From the point of view of the dairy industry, the most important finding emerging from the present investigation is the fact that 93-point butter can be changed to 94+-point butter by compounds trapped during the degassing of butter oil. It will, therefore, be necessary to direct future work towards the origin and mode of formation of these compounds.

The authors' thanks are due to their colleague, Mr J. F. Smith, for help with the mass spectrometry, and to Mr M. E. McShane and Dairy Produce Inspectors of the Department of Primary Industry, Melbourne, for grading butters.

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EXPLANATION OF PLATE

Plate 1

Apparatus for the degassing and cold-finger distillation of butter oil.

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Handbook of Food Additives
Chemical Rubber Company, Cleveland,
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Production of Acetoin and Diacetyl by the Genus *Salmonella*

J. A. GARIBALDI AND H. G. BAYNE

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Albany, California 94710

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Members (all 42 tested) of the genus *Salmonella*, although Voges-Proskauer-negative when grown on MR-VP Medium, produce abundant quantities of acetoin or diacetyl, or both, when cultured on a minimal medium containing 1% glucose. The designation that the genus *Salmonella* does not produce acetoin should be re-examined.

One of the characteristics used to define the genus *Salmonella* is its inability to produce acetylmethylcarbinol [acetoin (*Bergey's Manual*, 7th ed.; 3, 4)]. The members of the genus do not produce acetoin under the carefully controlled conditions of the Voges-Proskauer test (3). However, we found that under somewhat different nutritional conditions salmonella produce abundant quantities of acetoin or diacetyl, or both.

MATERIALS AND METHODS

Bacteria. The following species of *Salmonella* were tested: *S. typhimurium* LT-2, TM-1, 6087; *S. cerro* 4837, 6268; *S. senftenberg* 6101, 4361, S8, 3434-63, 775W; *S. milwaukee; S. macallen; S. montevideo; S. urbana; S. memphis; S. albuquerque; S. mission; S. newport; S. barielly; S. heidelberg; S. choleraesuis* var. *kunzendorf; S. oranienburg; S. worthington; S. bredon; S. meleagridis; S. derby; S. anatum; S. austin; S. tennessee; S. blockley; S. thompson; S. gallinarum; S. pullorum; S. sendai; S. dublin;* S1982, S1917, S547; S729, S280, S2, S1, 3417.

Media. MR-VP Medium (Difco) was prepared as recommended by the manufacturer and was also modified by increasing the glucose concentration to 1% or adjusting the initial *p*H to 6.0, or both. The minimal medium was composed of K_2HPO_4, 11 g; $(NH_4)_2SO_4$, 0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4$ to give 8 μg of Fe/ml; $MnCl_2$ to give 8 μg of Mn/ml; and $ZnSO_4$ to give 8 μg of Zn/ml; water, 1.0 liter (*p*H 7.6). Glucose was sterilized separately and added after autoclaving to give a final concentration of 1%. In some tests, this medium was supplemented with yeast extract at levels of 0.3, 0.1, or 0.03%. In others, it was supplemented with the amino acids and vitamins necessary for the growth of *S. gallinarum* and *S. pullorum* (10).

The media were inoculated from 15-hr slant cultures, incubated at 30 C on a rotary shaker, and tested after 24 and 48 hr.

Detection and analysis of acetoin or diacetyl or both. Two milliliter samples of the cultures were tested with the reagents 5% α-naphthol in 95% ethyl alcohol and 0.3% creatine in 40% KOH (3).

Analyses for acetoin and diacetyl were conducted as described by Krampitz (5) on cell-free culture supernatants.

Gas chromatography of diacetyl in culture supernatant. The volatiles from 5 ml of cell-free supernatant of *S. typhimurium* TM-1, grown on the minimal medium at 30 C for 48 hr, were isolated, trapped, and injected into the gas chromatograph as previously described (7). Helium carrier gas (30 ml/min) was used in the stainless-steel column [outer diameter, 24 ft by 1/8 inch (7.3 m by 0.32 cm)], packed with 5% FFAP on 60/80 mesh chromosorb G. The column was operated isothermally at 75 C, the flame ionization detector at 250 C, and the injector at 225 C. Toluene was used as an internal standard for the calculation of relative retention times.

RESULTS

MR-VP Medium. The bacteria tested did not give a positive reaction for acetoin or diacetyl, or both, either on the unmodified MR-VP Medium (in which all of the glucose was metabolized) or on the medium modified to a glucose concentration of 1% or an initial *p*H of 6.0, or both (in which glucose was in excess). The final *p*H of the cultures after 48 hr of incubation in all cases was approximately 5.0.

Minimal medium. Of the 42 strains grown on minimal medium, 33 gave a positive reaction for acetoin after either 24 or 48 hr of incubation at 30 C. The nine strains giving a negative reaction (strains of *S. dublin, S. sendai, S. pullorum*, and *S. gallinarum*) grew sparsely on this minimal medium and are known to have specific growth requirements for vitamins or amino acids, or both (1, 10). In a subsequent experiment when the growth was increased by supplementing this minimal medium with either 0.3, 0.1, or 0.03% yeast extract, seven of these nine strains of *Salmonella* converted glucose to acetoin or diacetyl, or both, to give a positive reaction with the Voges-Proskauer reagents described above. The two *salmonellae* which still did not produce acetoin in these media were strains of *S. pullorum* amd *S. gallinarum*.

A positive reaction for acetoin or diacetyl, or both, resulted when these two organisms were cultured on the minimal medium supplemented with the vitamins and amino acids necessary for growth of *S. pullorum* and *S. gallinarum* (10).

A gas chromatogram of the volatiles from the cell-free supernatant of a 48-hr culture of *S. typhimurium* TM-1 showed four well-defined fractions. The fourth fraction had a definite diacetyl-like odor as it emerged from the column, and its relative retention time (0.587) agreed well with that observed for authentic diacetyl (0.585). The level of acetoin-diacetyl in cell-free supernatants of this strain was 28 μg/ml.

DISCUSSION

It is apparent that members of the genus *Salmonella* have the capability of producing acetoin. It is also obvious that they do not produce amounts detectable with the Voges-Proskauer test under the nutritional conditions of the MR-VP Medium. As discussed by several investigators (6, 8, 11), the initial glucose concentration and the initial *p*H of the MR-VP Medium greatly affect the production of acetoin by the genus *Aerobacter*. Our exploratory experiments with these two variables indicate that they are not the factors which prevent the accumulation of acetoin by *Salmonella*.

The detrimental effect of phosphate on the production of acetoin by sporeformers was described (9). It was recommended that the phosphate be replaced with sodium chloride when the VP test is conducted on anaerobic sporeformers (9). However, in the present study, the minimal medium contains more than twice the concentration of phosphate as the MR-VP Medium without restricting the formation of acetoin by *Salmonella*.

The nutritional or environmental factors, or both, which prevent the accumulation of acetoin by the genus *Salmonella* under the conditions of the Voges-Proskauer test, are still to be determined.

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The Volatile Flavor Substances of Celery

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HARVEY J. GOLD AND CHARLES W. WILSON, III

U. S. Fruit and Vegetable Products Laboratory, Winter Haven, Florida*

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SUMMARY

The isolation and identification of the volatile compounds of celery are described. Non-linear temperature-programmed gas chromatography was found to give resolution superior to that of either isothermal operation or linear temperature programming. The identification of 24 compounds from celery is reported. Of the 38 compounds thus far identified from celery distillates, the following six compounds are primarily responsible for the characteristic flavor and aroma of celery: 3-isobutylidene-3a,4-dihydrophthalide; 3-isovalidene-3a,4-dihydrophthalide; 3-isobutylidene phthalide; 3-isovalidene phthalide; cis-3-hexen-l-yl pyruvate; and diacetyl. The phthalide derivatives are also implicated in the occurrence of certain celery off-flavors reported in the literature.

INTRODUCTION

The composition of the flavor of celery has not previously received intensive investigation, although celery is used in cooking and in processing primarily for its unique and characteristic flavor. Guenther (1950) listed what was known of the composition of the essential oil of celery seed. He cited sedanonic anhydride (3-butylidene-3a,4,5,6-tetrahydrophthalide) and sedanolide (3-butylidene 5,6,7,7a-tetrahydrophthalide) as the primary flavor constituents. The character of the flavor and aroma of the stem portion of the celery plant is different, however, from that of the seed. In a preliminary report, the present authors (Gold and Wilson, 1961) described collection of the volatile substances from celery. These volatile materials were divided, by differential cold-trapping, into relatively high-boiling and relatively low-boiling fractions, and the analysis of the acidic portion of the high-boiling fraction was discussed. A separate paper (Gold and Wilson, 1963) described the structural elucidation of four new phthalide derivatives from the neutral portion. The present paper describes the completion of the analysis of the high-boiling fraction and the analysis of the relatively low-boiling materials. The characteristic flavor of celery has been found to be due to six of the compounds identified.

EXPERIMENTAL

Celery juice was prepared and distilled in pilot-plant equipment, and the distillate fractions were taken to the laboratory for examination by chemical and physical means.

Preparation and distillation of juice. Details of preparation and distillation of the celery juice have been reported (Gold and Wilson, 1961). About 5,000 kg of fresh celery was used, yielding 4130 kg of juice, which was distilled in a modified continuous pilot-plant essence recovery unit (Morgan *et al.*, 1953). In this unit, the juice was flash vaporized under vacuum, the condensate given a second flash vaporization, and the liquid remaining after this second stage was fractionally distilled. Fractions were collected in the following portions of the apparatus: 1) column-bottom (material continuously withdrawn as it accumulated in the fractional distillation boiling pot); 2) chilled-water trap; 3) ice trap; 4) salt and ice trap; 5) dry-ice trap; 6) liquid nitrogen trap. All compounds found in traps 2, 3, and 4 were also found in either the column-bottom fraction or the dry-ice trap.

Gas chromatography. The analytical gas chromatographic equipment consisted of a Perkin-Elmer 154-D Vapor Fractometer combined with a Beckman Thermotrac temperature programmer.

* A laboratory of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

Mention of brand names is for identification only and does not imply recommendation by the U. S. Department of Agriculture.

The Perkin-Elmer exhaust was modified to come straight out of the bottom to prevent condensation of high-boiling materials in the line. The following columns were used: A) 0.01-inch by 150-foot capillary column, coated with polypropylene glycol; B) ⅛-inch by 16-foot column, containing 0.25% Silicone 200 on glass microbeads; C) 3/16-inch by 10-foot column containing 0.25% Silicone 200 on glass microbeads; D) ¼-inch by 16-foot column, containing 0.25% Silicone 200 on glass microbeads. Columns A, B, and C were used with a flame ionization detector, and Column D with a thermistor detector.

The preparative gas chromatographic equipment, constructed in the laboratory, consisted of eight ⅝-inch by 2-foot columns, connected in series and encased in a 4-inch-diameter galvanized sheet-metal tube. The tube was wrapped with asbestos-insulated heating wire, and placed inside a 6-inch-diameter tube. Air was circulated up through the column compartment and down through the outer concentric chamber, thereby reducing temperature gradients. Insulation was provided by 1-inch-thick pipe lagging, and temperature was controlled by a Wheelco model 292 controlling pyrometer. A four-filament diffusion-type detector was used, provided with a separate, independently heated compartment. The ⅝-inch by 16-foot column was filled with 25% silicone 200 on Gas-Chrom P.

Retention times were corrected for column hold-up before log retention-time plots were constructed. When the flame ionization detector was used, column hold-up time was estimated by the procedure of Gold (1962). The use of retention time to substantiate identifications was considered valid only when the retention time of the unknown matched with that of known material within the readability of the chart (0.03 min.).

Infrared measurements. Infrared spectra were taken with either a Perkin-Elmer 137 Infracord or a Beckman IR-7 Infrared Spectrophotometer.

Analysis of the liquid nitrogen trap fraction. The liquid nitrogen trap constituted a relatively simple system, comprising four compounds. These were separated by gas-liquid chromatography (GLC), using the preparative column at 110°C. Infrared spectra were taken on the collected compounds.

Analysis of the dry-ice trap fraction. Contents of the dry-ice trap consisted of 25 compounds, as determined by temperature-programmed GLC (column B). No acids, aldehydes, or phenolic compounds were found. Preliminary information was obtained by application of the log retention time plots of Evans *et al.* (1962). Information was obtained on alcohols and esters by the techniques of "subtractive" gas chromatography, as follows:

Alcohols. A 10-μl portion was treated with *p*-phenylazobenzoyl chloride (Woolfolk, *et al.*, 1955) to remove alcohols. Chromatograms of the alcohol-free mixture were compared with those of the original material (columns A and C).

The mixture of *p*-phenylazobenzoates was saponified (Van Etten, 1951), the reaction mixture acidified with hydrochloric acid, and the alcohols extracted with approximately 50 μl of carbon disulfide. The azo linkage of the reagent split during saponification, giving analine and *p*-amino benzoic acid. The use of hydrochloric acid as the acidifying agent caused the precipitation of these compounds as the hydrochlorides, and prevented their carry-over into the carbon disulfide. The carbon disulfide extract was examined by infrared spectrometry and by GLC.

Esters. A 10-μl portion was saponified by the microsaponification technique cited above. Chromatograms were compared with those of the original material, as described above.

The acids formed were removed with aq. sodium bicarbonate, and the bicarbonate solution acidified, extracted with 35 μl of carbon disulfide, and examined by GLC.

In order to reduce the complexity of the gas-chromatograms obtained from this fraction, thereby simplifying their interpretation and facilitating the collection of individual compounds, the remaining fraction was chromatographed on silica-gel before attempting fractionation by GLC. Maximum separation on the silica-gel column was achieved by grading the eluent polarity. The sequence of solvents used was: hexane-methylene chloride; methylene chloride; methylene chloride-acetonitrile; acetonitrile. The eluted fractions were separated by GLC on the preparative column, and infrared spectra taken on the individual compounds.

Analysis of the neutral fraction from the column-bottoms. Analysis of the acidic fractions of the column-bottoms has been reported (Gold and Wilson, 1961).

The neutral fraction, which was in ether solution, was concentrated *in vacuo* to 30 ml and treated with Girard-T reagent (Girard and Sandelusco, 1936; Hunter and Struck, 1962; Stanley *et al.*, 1961; Teitelbaum, 1958). Aldehydes were regenerated by the method of formaldehyde exchange. After separation of the aldehydes from the reaction mixture, ketones were recovered by acid regeneration. Compounds in each case were purified by GLC (column D). Fractions were collected directly in Connecticut Instrument Co. type "D" cavity cells, and infrared spectra taken. The ketone- and aldehyde-free neutral fraction

was taken up in ether, concentrated to 30 ml, and chromatographed on silica-gel, as described for the dry-ice trap. The eluted fractions were submitted to GLC (column D), and infrared spectra were taken on the collected compounds.

Flavor evaluations. The contributions of individual fractions and compounds to the overall flavor of celery were evaluated by a group of five to seven members of the laboratory staff.

RESULTS AND DISCUSSION

When it was necessary to examine complicated mixtures directly by GLC, the technique of programming the temperature non-linearly was found to give resolution superior to that of isothermal operation or of linear temperature programming. This is demonstrated by a comparison of the isothermal, linear temperature-programmed and non-linear temperature-programmed chromatography of a mixture of normal alcohols (Fig. 1).

Compounds identified from celery are listed in Table 1. In most cases, identification is based upon comparison of both GLC retention times and infrared spectra with those of commercially available compounds.

Exceptions are as follows: Cis-3-hexen-1-yl pyruvate was identified by comparing its infrared spectrum with that of the synthetically prepared ester (Weisberger and Kibler, 1955), comparison of the infrared spectrum of the saponified mixture with that of a mixture of cis-3-hexen-1-ol and pyruvic acid, and by retention times of the parent compound and the saponification products. Ozonolysis, followed by reduction of the ozonide with Raney nickel, gave propionaldehyde, thus confirming that the double bond is in the 3 position.

The identifications of ethyl isovalerate, carvyl acetate, and neryl acetate were based upon the retention times of the parent compounds and of the saponification products.

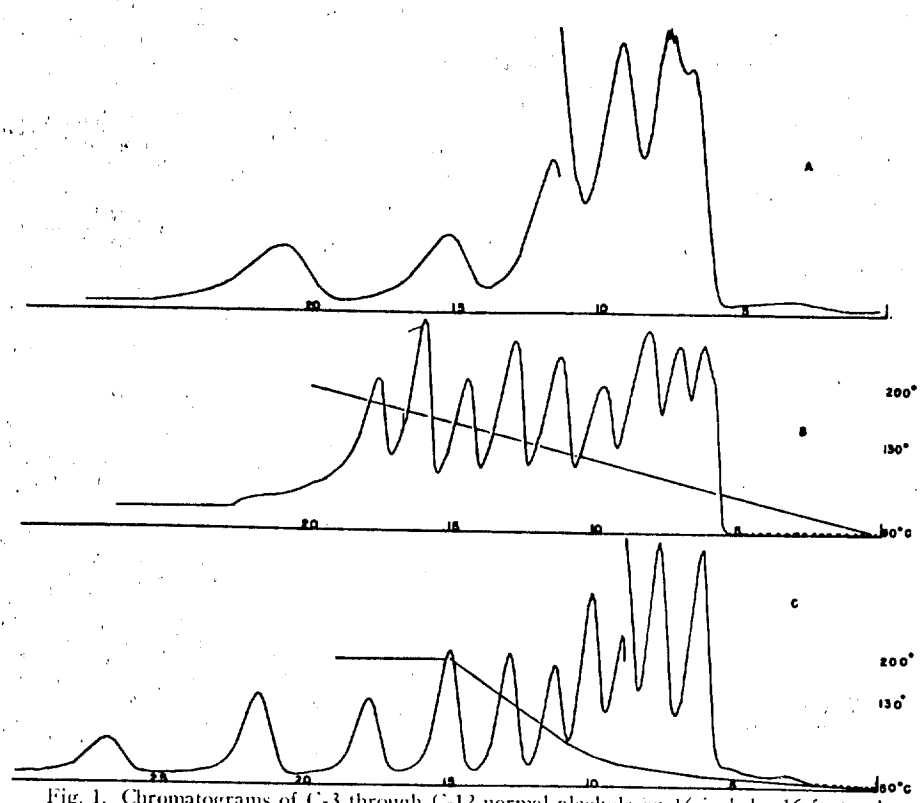

Fig. 1. Chromatograms of C-3 through C-12 normal alcohols on ⅛-inch by 16-foot column containing 0.25% silicone 200 on glass microbeads. A) isothermal operation at 150°C; B) temperature linearly programmed, according to the superimposed line; C) temperature non-linearly programmed, according to the superimposed curve.

Table 1. Volatile substances from celery.

Aldehydes	*Esters*	*Alcohols*
formaldehyde (6)	ethyl isovalerate (5)	iso-amyl alcohol (5)
acetaldehyde (6)	cis-3-hexen-1-yl pyruvate (5)	hexanol (2,3,5)
propionaldehyde (6)	decyl acetate (5)	heptanol (1,2,3,5)[a]
hexanal (1)	linalyl acetate (5)	
heptanal (1,4)	terpinyl acetate (5)	*Acids*
octanal (1,4)[a]	geranyl acetate (5)	n-valeric (1)[a]
undecanal (1)	citronellyl acetate (5)	iso-butyric (1)[a]
dodecanal (1,4)	neryl acetate (5)	pyruvic (1)[a]
neral (1)[a]	carvyl acetate (5)	
citronellal (1)	terpinyl propionate (4,5)	*Phenol*
	geranyl butyrate (4,5)	guiacol (1)[a]
	benzoyl benzoate (1)	
Hydrocarbons	*Phthalides*	
d-limonene (5)[a]	3-isobutylidene-3a,4-dihydrophthalide (1,2,3)[b]	
myrcene (5)[a]	3-isovalidene-3a,4-dihydrophthalide (1,2,3)[b]	
Ketones	3-isobutylidene phthalide (1,2,3)[b]	
carvone (5)	3-isovalidene phthalide (1,2,3)[b]	
diacetyl (6)	sedanonic anhydride (1)[a]	

(1) column-bottoms; (2) chilled-water trap; (3) ice trap; (4) salt-ice trap; (5) dry-ice trap; (6) liquid nitrogen trap: a) Gold and Wilson, 1961; b) Gold and Wilson, 1963.

In addition to the compounds listed in Table 1, valeraldehyde, nonanal, geranial, and the C-3, C-4, C-8, C-9, C-10, C-11, and C-12 normal alcohols were indicated by functional group determination and by GLC retention time. Sufficient quantities of these materials were not available for detailed study. An additional compound gave, upon saponification, acetic acid and an unidentified branched-chain alcohol. The alcohol retention time coincided with that predicted for 2-methylhexanol by a log retention-time plot for 2-methyl alkanols, and the ester retention time approximately coincided with that predicted for 2-methylhexanyl acetate by a log retention-time plot for acetates of the 2-methyl alkanols (predicted, 12.0 min; observed, 12.4 min).

Contribution of compounds to flavor and aroma. Many of the compounds listed in Table 1 are normally considered rather malodorous. Their odors, however, become less objectionable, and even pleasant, when dilution is sufficiently high (Gold and Wilson, 1961). While most of the compounds listed probably make some contribution to the over-all flavor and aroma of celery, six are of primary importance: the 3-isobutylidene-3a,4- and 3-isovalidene-3a,4-dihydrophthalides, the 3-isobutylidene and 3-isovalidene phthalides, cis-3-hexen-1-yl pyruvate, and diacetyl.

Early in this work, it was noted that the column-bottoms exhibited a strong characteristic celery odor. The principal odor constituents of this fraction were the four phthalide derivatives listed above. They were isolated in the ratio of 6:3:1:1. It has been found (Berlingozzi, 1927; Kariyone and Shimizu, 1953) that a celery-like odor is generally characteristic of synthetically prepared 3-alkylidene phthalides and hydrophthalides. The presently reported compounds exhibit a readily detectable celery aroma in concentrations as low as 0.1 ppm in water. Such solutions are characterized by a mild bitterness, and an occasionally noted burning sensation. When tasted in neat form, the dihydrophthalides exhibited the intense burning-numbing sensation described by Pan (1960), the isobutylidene compound being more intense than the isovalidene. Examination of the infrared spectrum presented by Pan for a burning-numbing fraction from celery, showed it to be similar to the spectrum presented by the present authors (Gold and Wilson 1963) for the isoalkylidene dihydrophthalides.

Notwithstanding the characteristic celery odor of the column-bottoms fraction, addi-

tion of this material to tomato juice did not reproduce the flavor of tomato-celery juice blends unless material from the dry-ice and liquid-nitrogen traps was included. As indicated above, the principal odor constituent of the dry-ice trap was cis-3-hexen-1-yl pyruvate, and that of the liquid-nitrogen trap was diacetyl. When 2 μl of the following mixture was added to 100 ml of tomato juice, a strong celery flavor and aroma were imparted: 3-isobutylidene-3a,4-dihydrophthalide (26 parts); 3-isovalidene-3a,4-dihydrophthalide (13 parts); cis-3-hexen-1-yl pyruvate (4 parts); diacetyl (1 part). The contribution of the aromatic phthalides could not be assessed, because of insufficient material.

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VOLATILE COMPONENTS OF RAW CHICKEN BREAST MUSCLE

T. C. GREY[1] AND D. H. SHRIMPTON[2]

Low Temperature Research Station, Downing Street, Cambridge

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SYNOPSIS

The volatile components of raw chicken breast muscle have been analysed. Twenty-two components have been separated and twenty have been tentatively identified. Circumstantial evidence is presented to support the hypothesis that these volatile components may be divided into two classes according to their origin: namely those that are endemic, mainly carbonyls, and those that are adventitious, mainly thiols, sulphides and alcohols. From analyses of the caeca of both living and dead birds it is further proposed that the substantial microflora present in this organ may be a major metabolic source of adventitious muscle components.

INTRODUCTION

Comparative analyses by Watt and Merrill (1963) of non-volatile and by von Sydow and Westberg (1962) of volatile components of different foods demonstrate a surprising degree of similarity in qualitative composition and the prospect of any one component being solely responsible for the characteristic stimulus evoked by any particular food appears to be remote. More attractive as a working hypothesis is the permutation within foods of a limited number of components to give the variety of flavour stimuli. Hence, analytically, the problem is both to identify and to quantify the components of characteristic flavour profiles.

Raw chicken muscle has been selected for study for two main reasons. First, the system is more simple than that resulting from cooking where the techniques are difficult to standardise, and chemical reactions, particularly autoxidations, are difficult to follow. Secondly, it has been assumed that provided no substances are added, the range of chemical change during cooking will be determined by the chemical composition of the raw meat before cooking begins. Hence, if real differences exist in the flavour of chickens reared and processed in the packing station in different ways their origin should be apparent in differences of composition in the raw muscle. Unfortunately such differences may be quantitative and not qualitative and consequently be more difficult to identify. In these experiments one aspect only of the problem has been studied: namely to determine the analytical nature and the origin of what in England is termed "gamey" flavour and sometimes described in the U.S.A. as "visceral taint". Only the volatile components of raw muscle have been analysed, although it is recognised that some of the volatiles of cooked muscle may have their origin in interaction between compounds with high boiling points.

The raw breast muscle of birds has been analysed immediately after slaughter and

[1] Present address: A.R.C. Food Research Institute, Earlham Laboratory, Recreation Road, Norwich, Norfolk.

[2] Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford.

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after the carcass containing the viscera had been hung at room temperature. This procedure has not been chosen because it simulates commercial practice in the United Kingdom, where evisceration immediately after death is the rule rather than the exception, but because it is a readily controlled experimental procedure that exaggerates the older traditional practice in the United Kingdom when uneviscerated birds were retailed and which, it is asserted, are more desirable than the present-day product.

The second objective of the experiments has been to determine the origin of volatile components found in the muscle. Because Barnes and Shrimpton (1957) showed that the hydrogen sulphide found in the muscle of hung birds had its origin in the chemical activities of the microflora of the caecum, this organ has been studied here. The experiments have been extended to study the caeca of live birds to determine whether there is any possibility of the production of some of the compounds associated with the hung bird whilst the bird is alive.

Although it would have been desirable to compare the results obtained from analysing the muscle of hung uneviscerated birds with those from eviscerated birds, the rapid spoilage of the latter by micro-organisms at room temperature makes such a control experiment impossible.

MATERIALS AND METHODS

Origin of birds

White Leghorn cockerels and Light Sussex hens aged 11-15 months were housed in individual battery cages and fed on a standard ration containing approximately 18 per cent of crude protein and 5·4 per cent of crude fibre (Beattie and Shrimpton, 1958).

The caeca were exteriorised in some birds, following the procedure of Beattie and Shrimpton (1958) but modifying this to allow the insertion of a permanent cannula designed primarily for convenience of sampling for subsequent analysis by gas-liquid chromatography.

Dissection of muscle

The major pectoral muscles were used for all experiments. Preliminary results were obtained from muscles removed from birds killed in a packing station, sealed into a polythene bag and subsequently held at −20° C. until analysed.

Because of the possibility of contamination of the samples during dissection, storage and sampling, a final experiment was carried out with one bird under specially controlled conditions. A year-old Light Sussex hen was killed (by severing the jugular vein) on a nearby experimental poultry farm and the right pectoral muscle dissected immediately. It was placed in a polythene-foil bag, vacuum sealed in an annexe to the laboratory and held at −20° C. until analysed. The carcass with the left pectoral muscle *in situ* was transferred to a room, but still away from the laboratory, and held for 4 days at 10°-15° C. before it was dissected. After dissection the left pectoral muscle was treated in the same way as the right one.

Sampling of volatiles

Muscle. The basic design of the sampling apparatus is shown in Figure 1. Three grams of minced frozen breast muscle were placed in the sampler on top of the glass ballotini. The complete sampler, containing the mince, was connected to the gas line and immersed in a water bath at 50° C. It was found necessary to remove some of the water from the system during collection and a glass cold trap was placed in the collecting line between the sample and collecting trap. The cold trap was kept at −4·5° C. throughout the collection of volatiles; this allowed most of the volatiles to be collected and increased the collection time from less than ½ hr to 1 hr. The collecting trap was placed

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in liquid oxygen. The apparatus was allowed to equilibrate for 15 min. before gassing. A "concentric tube trap" developed by Swoboda (personal communication) was used to collect all the volatiles in this series of experiments. The rate of flow of the argon carrier gas was controlled by a needle valve at 40 ml. per min. and measured after the collecting trap by a soap bubble flowmeter.

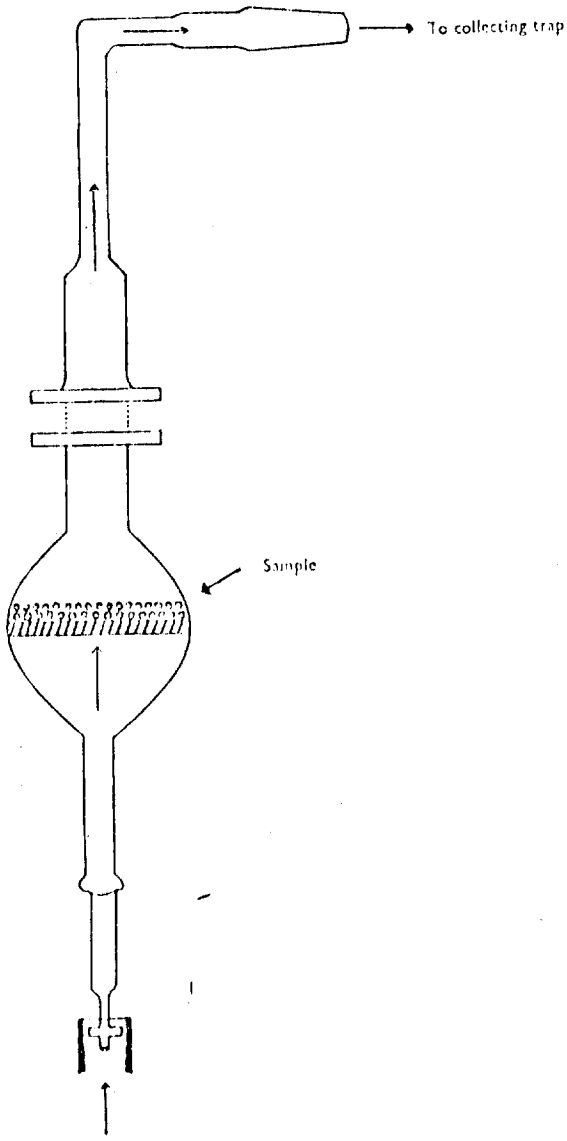

FIG. 1.—Apparatus for sampling volatile components of minced muscle.

Caecal. The design of the cannula and sampler is shown in Figure 2. The volatiles

were displaced from the caecum by passing argon, saturated with water vapour to reduce dessication, through the cannula and trapped from the returning stream. The trap consisted of a 3 ft length of no. 11 gauge stainless steel tubing bent into four loops dipping into liquid oxygen (Swoboda, personal communication). "Fogging" was prevented by heating the exposed portions of the loops, about one-third, in a stream of hot air. The gas flow through the system was measured at the other end of the trap using a soap bubble flowmeter and was controlled by a needle valve. Gas was passed through the apparatus until the trap blocked from water vapour frozen out of the gas stream. However, the minimum collecting period of 30 min. still enabled a representative sample, suitable for analysis, to be obtained. Samples were collected consecutively before and after death so that a complete picture of the pattern of volatiles could be obtained.

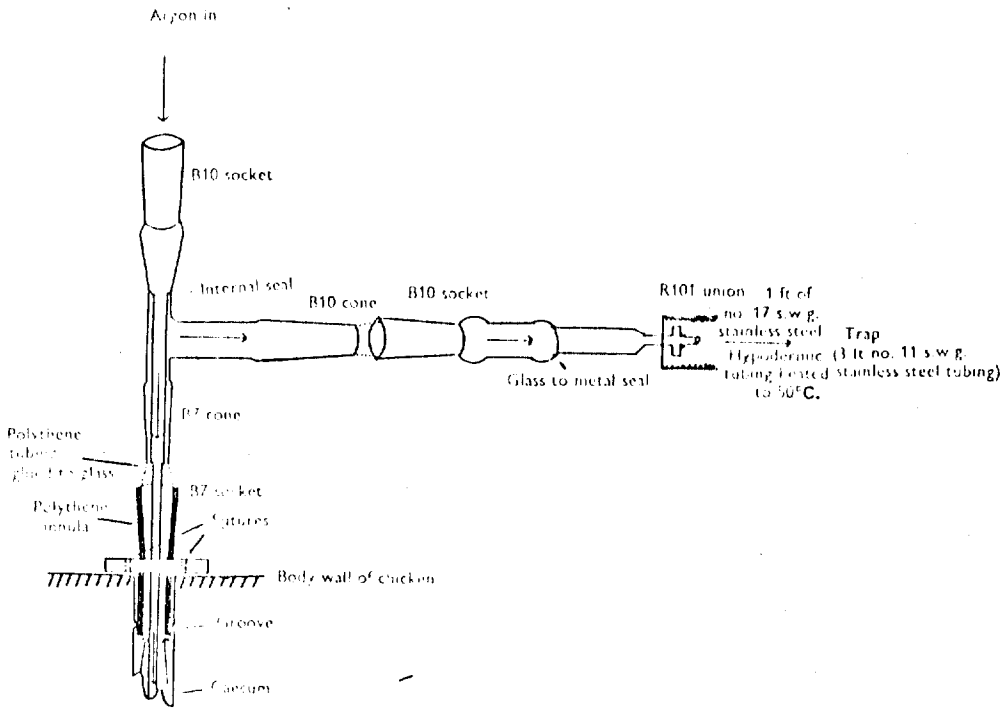

FIG. 2.—Apparatus including cannula, for sampling volatile components of caecal metabolism *in situ*.

Gas-liquid chromatography

A β-ionisation chromatograph (W. G. Pye Ltd, Cambridge) with a 20 mc strontium 90 detector, was operated isothermally at 50° C. The volatiles were fractionated in a stainless steel column, 6 m. long and 4 mm. internal bore, packed with 15 per cent (w/w) Triton X 305 (Rohm and Haas Co.) on 100-120 mesh acid/alkali washed celite. Argon was used as carrier gas at a rate of 40 ml./min. measured at the outlet from the detector. This fractionation procedure is based upon the experience of Adams and Koppe (1959).

Precolumn. Water was prevented from entering the main column by the use of a remote stainless steel precolumn (Swoboda,

1960; personal communication) 17 cm. long and 4 mm. internal bore and fitted with a back-flushing mechanism to cleanse it after use. The precolumn was packed with 20 per cent diglycerol (May and Baker Ltd) on 85-100 mesh siliconised celite. The temperature was maintained at 60° C. using resistance wire coupled up to a low voltage A.C. supply and insulated by covering with layers of glass fibre tape, 0·009 in. × 1½ in. (Fibre Glass Ltd), which was treated when in position with surface coating "Araldite". The complete column was finally covered tightly with a ½ in. layer of felt and the temperature calibrated with argon flowing through using a thermocouple, and then packed with the support.

Injection. The injection system is shown diagrammatically in Figure 3. It is basically similar to Swoboda's (personal communication), using stainless steel no. 17 s.w.g. hypodermic tubing and unions (Edwards R101) throughout but modified at the inlet to withstand the higher pressure necessary to achieve a satisfactory flow through the 6 m. column. The connection between the back-flush valve (W. G. Pye Ltd) and the union with the column is maintained above 50° C. by ohmic heating.

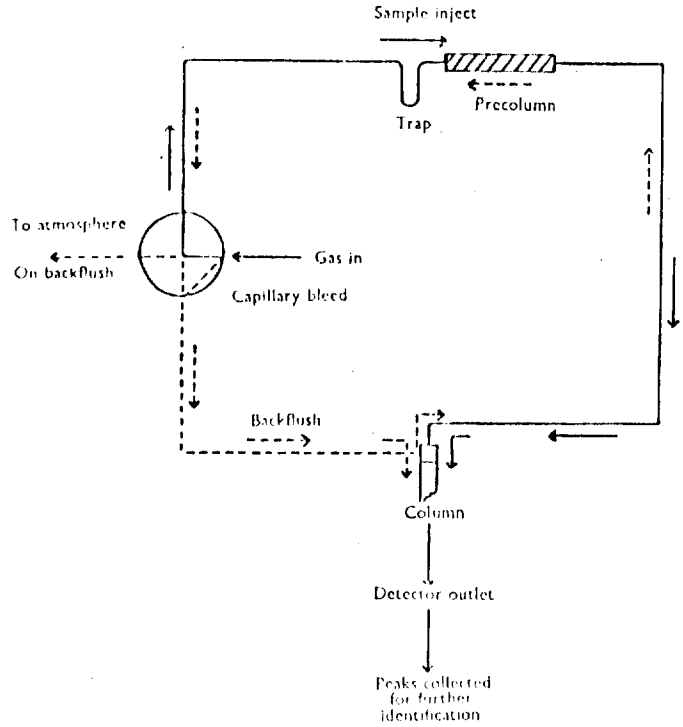

FIG. 3.—Injection system for introducing sample to gas chromatograph.

Injection from the trap into the head of the precolumn was accomplished by ohmic heating, the current being passed 30 to 45 sec. before the start of the gas flow and maintained for 2 to 3 min. Water vapour was prevented from entering the analytical column by use of the back-flush valve after an interval predetermined by calibration of the apparatus for water retention by the precolumn.

Identification. Peaks were identified by using the retention index system of Kováts (1958; 1961) and reviewed by Ettre (1964).

The retention indices were determined with and without the precolumn and it was found that the differences were no greater than would be accounted for by experimental error in the method. To help to confirm particular peaks from the Triton X 305 phase, samples were collected at the detector outlet and retention data obtained on one of two other phases. Ten per cent polyethylene glycol adipate and β, β^1-oxydipropionitrile (May and Baker) were used for the analysis of the higher and lower boiling fractions respectively.

RESULTS

The results of the analysis for volatile components in muscle have been examined primarily for two characteristics: the qualitative and quantitative relationships of the components and their identity. In further experiments, also reported here, the hypothesis has been tested that bacterial metabolism within the caecum has a significant effect on the profile of volatile components found in the muscle.

All the results quoted have been obtained by the techniques of gas-liquid chromatography and the identification of components, without corroboration from other and independent physical or chemical methods, is necessarily tentative. Nevertheless, for the purpose of constructive discussion and because of the absence of conflict between the interpretation of analyses from different stationary phases, these tentative identifications are quoted hereafter without further qualification. The profile, too, is in part a consequence of the analytical techniques used and particularly of the method of sampling prior to chromatography.

With muscle, for example, both the temperature selected (50° C.) and the duration of sampling established readily repeatable experimental conditions in which the relatively large sample obtained was similar in profile to that of samples taken at a range of temperatures (17°-55° C.); at 70° C. the profile changed. The components of the profiles established by these techniques are thus regarded as primary constituents of breast muscle, some of which may have orgnaoleptic significance.

Volatile components of breast muscle

Experiments have been made on muscles excised from 6 birds and in each case sampling was continued until reproducible results were obtained. The results, shown in the form of a histogram in Figure 4 are taken from one complete experiment on one bird and are typical of the results obtained from many replicates on each of the 6 birds.

Immediately after death. Of the 14 identified components, hydrogen sulphide, propanal, 2-methylpentanal and 5-methyl hexanal and 2-hexanone were absent or present in only trace amounts (Figure 4*a*). Moreover, the amounts of the other components were small compared with those in samples obtained after storage (*cf.* Figure 4*b*).

After storage for 4 days at room temperature. Twenty-one components were recognised, compared with 14 in the fresh sample, and 18 were tentatively identified (Figure 4*b*). By comparison with the fresh sample, the relative amounts of most components common to both were increased 2 to 5 times. Three components, however, differed from this general pattern; ethanol was increased 100 times, 2-butanone 20 times and butanal was absent. Of the new components, methanol dominated the profile, being of comparable concentration with ethanol. The other 6 components were present in relatively smaller amounts and included 2,3-butanedione, hexanal, 4-methyl-pentanal and 2-hexanone.

Volatile components of caecal contents

The results, shown in the form of a histogram in Figure 5, are the mean values from 2 complete experiments with 2 birds

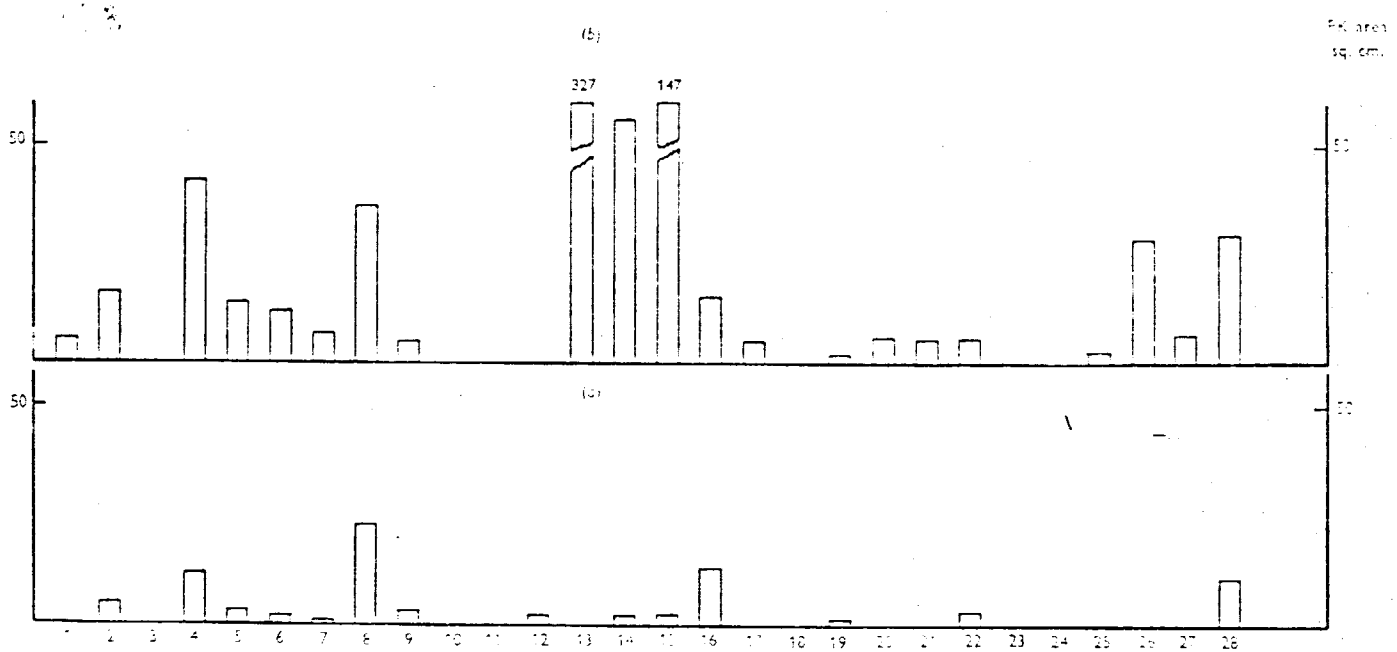

FIG. 4.—Volatile components of raw chicken breast muscle.
(*a*) immediately after death. (*b*) after hanging for 4 days at 10 to 15° C.

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and are typical of those obtained in 11 complete experiments, involving 11 birds. The 3 parts of the figure show, in (*a*) the pattern of volatiles in caecal gas, excluding low hydrocarbons and the permanent gases, from a live but anaesthetised bird and in (*b*) and (*c*) changes in the pattern following the death of the bird from intravenous injection of barbiturate.

In all there were 22 separate components. Of the 19 which were tentatively identified (Table 1) 14 were also observed in the pectoral muscle after storage.

From a living bird. A steady state was reached between 1 and 2 hr after the start of passing the carrier gas and the profile obtained at this time is shown in Figure 5*a*. Nine of the 10 components have been tentatively identified including ether which, like the others, was present in similar proportions in successive experiments. Unlike the other components though, there may be some doubt about its origin because it was always necessary to use ether as a respiratory anaesthetic when deep anaesthesia was required. Nevertheless, it should be

TABLE 1

Tentative identification of volatile components from breast muscle and caecum

Stationary phase:—Triton X 305 50° C.

Peak no.	Retention index (experimental)	Retention index (standard)	Tentative identification	Source muscle or caecum
1	503	500	Hydrogen sulphide	Both
2[1]	609	609	Ethoxyethane (ether)	Both
3[1]	638	637	Methanethiol	Caecum
4[1]	666	665	Ethanal (acetaldehyde)	Muscle
5[1]	705	704	Ethanethiol	Both
6[1]	718	717	Methylthiomethane (dimethyl sulphide)	Both
7[1, 2]	754	754	Propanal	Both
8[1, 2]	776	775	2-Propanone (acetone)	Both
9	800	800	Propenal (acrolein)	Muscle
10[1]	805	805	Propanethiol	Caecum
11	820	—	—	Caecum
12[2]	842	842	Butanal	Both
13[1]	852	851	Methanol	Both
14[2]	864	863	2-Butanone	Both
15[1, 2]	886	884	Ethanol	Both
16[2]	897	897	3-Methyl-2-butanone	Both
17[1]	924	923	2,3-Butanedione	Muscle
18[2]	940	938	3-Pentanone	Caecum
19[2]	971	972	2-Methyl-1-pentanal	Both
20	979	—	—	Muscle
21[2]	984	984	4-Methyl-1-pentanal	Both
22	990	—	Unidentified carbonyl	Both
23[1]	1011	1010	Methyldithiomethane (dimethyl disulphide)	Caecum
24	1020	—	—	Caecum
25[2]	1035	1035	2-Hexanone	Muscle
26[2]	1045	1045	Hexanal	Both
27	1065	—	—	Muscle
28	1084	1084	5-Methyl-hexanal[3]	Both

[1] Confirmed on ββ-oxydipropionitrile.
[2] Confirmed on 10 per cent polyethylene glycol adipate.
[3] Obtained by extrapolation from a homologous series of isoalkanals.

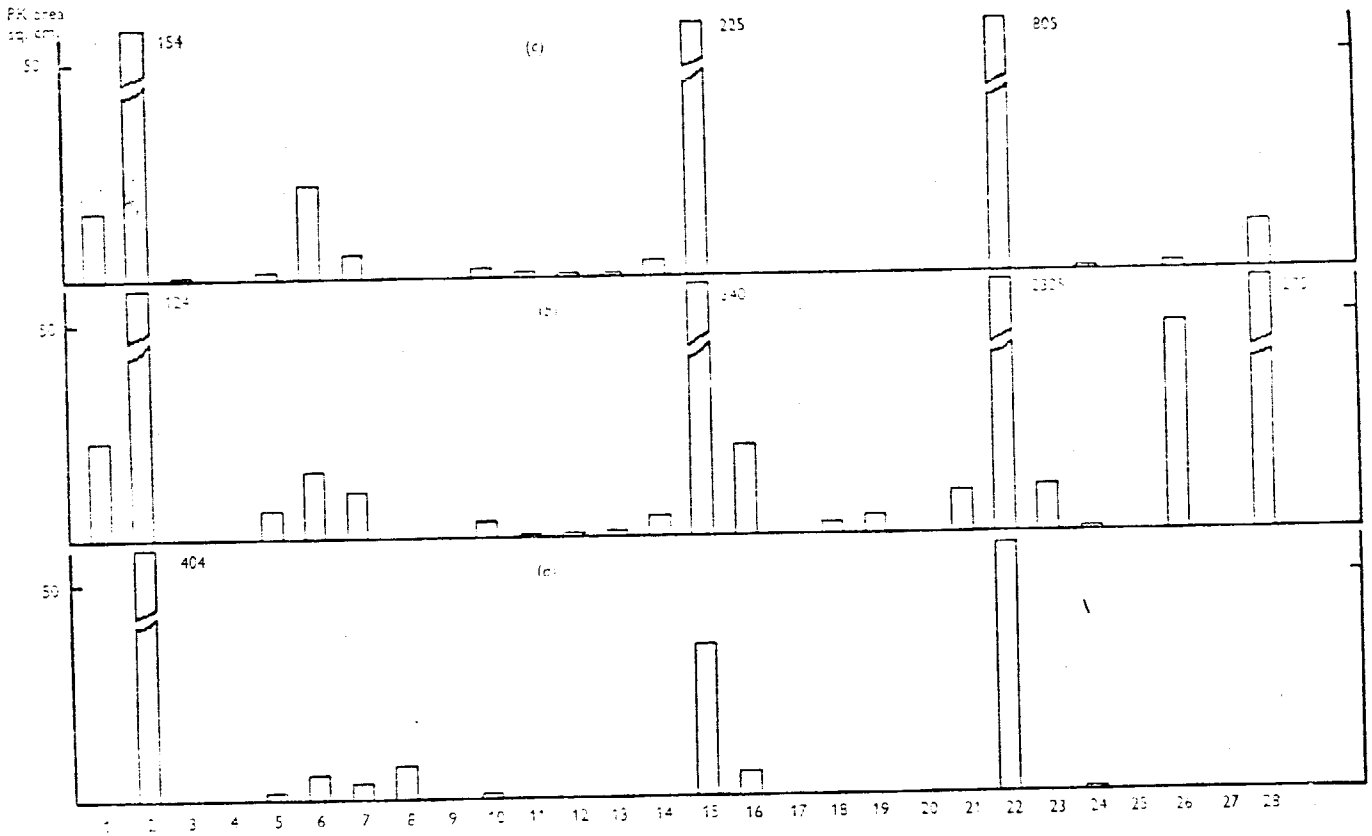

FIG. 5.—Volatile components of caecal contents.

(*a*) from a living (anaesthetised) bird. (*b*) 18 hr after death (held at 10 to 15° C.).
(*c*) 24 hr after death (held at 10 to 15° C.).

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remembered that a closed sampling system was used and that it would be expected that the caecal sphincter was closed. Ethanol and an unidentified carbonyl were the other two major components.

18 hr after death. Twenty components were characterised. The unidentified carbonyl was now by far the largest, the other major components being ethanol, 5-methyl-hexanal and n-hexanal. Acetone, which was present in samples from the live bird, disappeared. Of the 11 components which were additional to the pattern from the live bird, 5-methyl-hexanal was dominant and n-hexanal was present in large amounts compared with the remainder. Four of the components common to both samples all increased 3 to 4 fold, 1-propane thiol 8 fold, ethanol 10 fold and the unidentified carbonyl 40 fold. Ether decreased 3 to 4 times and acetone was not found.

24 hr after death. Sixteen components were separated. The unidentified carbonyl remained the largest component and ethanol and ether were the other major components. The only component to increase, apart from ether, was dimethyl sulphide and methanethiol appeared in trace amounts. Hexanal and 5-methyl-hexanal decreased markedly but the proportionate decrease of the remainder, relative to the profile of the sample 18 hr after death, was only of the order of $\frac{3}{4}$ to $\frac{1}{4}$. 3-Methyl-1-butanone 2-methyl-pentanal, 4-methylpentanal, 3-hexanone and one of the unknown components previously found in the 18 hr samples were not present.

DISCUSSION

The analyses reported here of the volatile components of freshly excised raw chicken breast muscle substantially extend the results reported by Pippen and Nonaka (1963) of analyses of raw frozen muscle and discussed in relation to other meats by Hornstein and Crowe (1964). Also the experimental conditions were chosen to provide some evidence on which to speculate on the biological origin of these components and it is these aspects which will be considered now. For this purpose it was necessary to have one common analytical procedure for the fractionation of substances differing widely in both polarity and boiling point, *e.g.* H_2S, b.p. −61·8° C. and hexanal, b.p. 131° C., and also sampling procedures which might be expected to cause the least disturbance to the profiles being studied.

It is well recognised that sampling procedures have the tendency either to change components or, more often, to lose trace components during fractionation and concentration. The procedure used was selected as a compromise between head space and vacuum distillation techniques in the case of muscle and as an alternative to individual gas samples from the caeca. Many of the pitfalls encountered in sampling small amounts of biological material were thereby overcome to give reproducible samples which were large enough to be analysed at relatively low sensitivity in the gas chromatograph. For the subsequent fractionation, to obtain a single profile of the volatile components up to C_7, only Triton X 305 was satisfactory under isothermal conditions and for confirmation, two separate phases had to be used to cover the same range.

Changes in the profile of the muscle during hanging appear to reflect some of the changes taking place concurrently in the caecum. Thus, ten components of muscle which increased during hanging were found also in the caecal samples of live birds and increased there, too, after death. By contrast acetaldehyde, acetone, diacetyl, acrolein and 2-hexanone were found in the muscle only. Butanal, which disappeared from the muscle during ageing, was present in trace amounts only in the caecum even after ageing.

It is possible, therefore, although the evidence at present is circumstantial, that the volatile components of chicken breast muscle may fall into two classes according

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Formation Dynamics of some Compounds in Starter Cultures Originating from Various Countries

B. HABAJ, K. HABAJ, T. RAPCZYNSKI and M. RUSIECKI

Dairy Research Institute — Dairy Pure Culture Laboratory Olsztyn, Kortowo 35, Poland

It was frequently observed that desirable quantitative characteristics of particular single-strain cultures disappeared in mixed cultures. The properties encountered in single-strain cultures may also be reduced in the mixture and no reliable method is available to catalogue the properties of mixed strain cultures. This is due to the fact that single-strains may not always be able to mix well, in respect to their cultural, physiological and biochemical properties.

It can be demonstrated by mathematical calculation that even some minute differences in the growth of a generation — due, for example, to the temperature being more or less suitable for a given strain — may result in the domination or disappearance of that strain in the following transfer. Jespersen (1) found that after a year mixed strain cultures might show a quite different composition as compared with the original one. Deryabina (2) reported that she could obtain as much as 15 mg/l of diacetyl with associated strains of Str. lactis and Str. diacetilactis, while certain strain associations failed to give diacetyl at all. Under such circumstances, composition and properties of mixed strain cultures may be considered as characteristics resulting from given biological systems and definite cultural conditions. The characteristics of mixed strain cultures may be reflected in butter or cheese but will not necessarily occur because fermentation processes must be considered as strictly connected with technology, physical and chemical processes, chemical composition of raw material etc. That is pointed out by Pijanowski (3) Doleżalek (4) and others. It was frequently observed, that various strain compositions showed considerable differences in the content of diacetyl and other components. The attempt was made to investigate the change dynamics of some metabolites produced in the milk acidification process when using stabilized mixed strain cultures of varied composition prepared both in Poland and supplied from other countries. The studies concerned such fermentation products as diacetyl and acetoin (C_4-compounds), volatile fatty acids as well as their mutual relationship and acidification dynamics.

MATERIAL AND METHODS

a) Skim milk of cows under constant veterinary survey, pasteurized under the same conditions (90 °C, 30 min.) was used as culture medium. Metabolite determination was made after 6, 12, 18 and 24 hrs. Three cultures were tested on one day, and the three other cultures on another one, with an interval of one day. All tests were carried out in duplicate.

b) Mixed-strain cultures which had been composed in Poland had a definite strain composition. That was not the case with one Soviet and one Danish culture, the composition of which was unknown. 0.5 % inoculum of an 18 hrs. milk culture was used. Incubation took place at 23 °C. The percentages of individual strains contained in the Polish mixed strain cultures are shown in Table 1.

TABLE 1

Origin of cultures and their symbol	*Percentage of individual strains in the moment of composition*				*Total number of strains in the mixture*
	Str. lactis	*Str. diacetilactis*	*Str. cremoris*	*Str. paracitrovorus*	
Polish T+R	33	67	-	—	6
Polish B_4	40	7	-	53	15
Polish RZ	90	10			10
Polish N	72	4	12	12	25
Danish C. Hansen	Composition unknown				
Soviet					

c) Diacetyl and acetoin were determined by using the method originally devised by Pien (5, 6) for butter examination, which was adapted for starters.

d) Volatile acids were quantitatively determined by steam distillation of 100 ml of starter with addition of 3 ml 1 N H_2SO_4 and a drop of oleic acid. 500 ml of distillate were titrated with 0.1 N NaOH against phenolphthalein. Qualitative determinations of volatile acids were made by paper chromatography as described by Lindquist (7).

e) CO_2 content was determined by distillation to 0.1 N $Ba(OH)_2$. A special closed-circuit distillation set excluded analytical errors due to the excessive or changeable CO_2 content in laboratory air. The excess of free $Ba(OH)_2$ was titrated with 0.1 N HCl using 2 % phenolphthalein solution. 1 ml of combined 0.1 N $Ba(OH)_2$ corresponds to 4.4 mg CO_2.

f) Citric acid was determined as described by Mariet and Boulet (8).

g) Titrable acidity in starters was determined after 6 and 24 hrs of incubation at 20 and 30 °C in two parallel samples. Results were expressed as percentage of lactic acid.

RESULTS

a) Acidifying capacities of starters

Table 2 represents the level of titrable acidity which was reached by cultures after 24 hrs of incubation at 20° and 30°C. The figures show that individual cultures reacted to the incubation temperature in a different way. These data may provide some information about which mixture would be more viable in the cheese vat during scalding or during drainage and salting, which is of considerable importance for lactose fermentation rate of cheese.

TABLE 2

Titrable acidity of mixed strain cultures in %, after 24 hrs incubation at 20 and 30° C and the acidity product

	Percentage of titrable acidity in mixed strain cultures after 24 hr incubation					Product of titrable acidity at 20 and 30°C after 24 hr incubation × 1000		
	at 20 C			at 30 C				
No.	1	%	No.	2	%	No.	3	
1	Polish (R+1)	0.92	1	Polish B_1	1.01	1	Polish (1+R)	920
2	Polish N	0.90	2	Polish (1+R)	1.00	2	Polish B_1	888
3	Danish	0.88	3	Polish N	0.98	3	Polish N	882
4	Polish B_1	0.88	4	Soviet	0.97	4	Danish	827
5	Soviet	0.83	5	Polish RZ	0.95	5	Soviet	869
6	Polish RZ	0.81	6	Danish	0.94	6	Polish RZ	705

The Polish starter (R+1) which contained ⅔ of Str. diacetilactis strains and ⅓ of Str. lactis ones, showed a good activity at both temperatures, while the culture RZ, which contained only one Str. diacetilactis strain (10%) appeared to be more susceptible to the reduction of temperature down to 20°C. The Danish starter was slower at 30°C and moderately good at 20°C. The susceptibility of cultures to the temperature may be expressed as a product of acidity (in % of lactic acid) as it was reached after a definite incubation time, multiplied by 1000. This product decreases, if a culture is more susceptible to a fall in temperature. The less the acidifying rate is influenced by temperature, the more the acidification product is close to 1000 after 24 hrs. Column 3 in the Table 2 illustrates the susceptibility of mixed-strain cultures to the temperature reduction to 20°C in descending order.

The probable activity of starter in cheese manufacture may be expressed by the increase of titrable acidity after 6 hrs of incubation at 30°C. The aptitude of a culture to continue fermentation during drainage of cheese was generally determined after 6 hrs at 20°C. The results for individual cultures are given in Table 3.

The higher is the product of acidity after 6 hrs incubation and the closer it approaches to 100 (Column 3 in Table 3) the more rapid may be the acid development in the cheese vat. If such a starter is active, too, at 20°C, the fermentation should continue during drainage and salting of the cheese. The high activity of starters at 20°C is a good reason believing that a rapid lactose fermentation will possibly be assured in cheese by this starter, or that it appears to be suitable for cream acidification assuring that its flavour and aroma producing abilities are adequate and a rapid acid development in cream is required.

TABLE 3

Titrable acidity of mixed strain cultures in %, after 6 hrs of incubation at 20 and 30°C and the acidity product

	Percentage of titrable acidity in mixed strain cultures after 24 hr incubation					Product of titrable acidity at 20 and 30°C after 24 hr incubation × 1000		
	at 20°C			at 30°C				
No.	1	%	No.	2	%	No.	3	
1	Soviet	0.235	1	Danish	0.38	1	Danish	83
2	Polish RZ	0.23	2	Polish (R+1)	0.36	2	Polish (R+1)	81
3	Polish (1+R)	0.225	3	Polish N	0.34	3	Polish N	71
4	Danish	0.22	4	Polish B_1	0.31	4	Soviet	70
5	Polish N	0.21	5	Soviet	0.30	5	Polish RZ	67
6	Polish B_1	0.205	6	Polish RZ	0.29	6	Polish B_1	63

b) Capacity of producing aroma, CO_2, volatile and utilization of citric acid

The diagram 2 and 3 indicate that the particular starters differed extremely in their diacetyl and acetoin content. These differences were both quantitative and qualitative ones. They concerned also the phase of their occurrence after 6, 12, 18 and 24 hrs. The Danish starter and the Polish ones, N and B_1, yielded a very similar amount of diacetyl after 12 hrs, i. e. 5.16, 5.12 and 4.04 mg/l respectively. After 18 hours the content of this compound was reduced to 0.6, 2.25 and 2.8 mg/l in the Danish starter, B_1 and N respectively. After 24 hrs the diacetyl content was established at a level of 0.3 mg/l for all three cultures. The Soviet starter reached its maximum diacetyl content (7.8 mg/l) most rapidly after 18 hrs. After 24 hrs this value dropped to the level of 5.6 mg/l which was still higher than that reached with any other starter examined. It may be thought that the Soviet starter had low reducing capacities and its high diacetyl content could be related to the slow decrease of citric acid, as is shown in Fig. 1. However, that relationship did not appear in the Polish starter RZ with low aroma producing capacity. This culture showed a similar reduction in citric acid content to the Soviet one, but simultaneously it had the lowest diacetyl level (1.08 mg/l).

On the other hand, the culture RZ being the slowest Polish mixture for citric acid fermentation, this fact may be connected with its low aroma producing capacity. This characteristic distinguished it from the Soviet starter which showed the similar slow decrease of citric acid but notwithstanding the highest diacetyl content.

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The Diagram 1 indicates that all starters, except for the Soviet one, showed the highest diacetyl content after 12 hrs from the moment of inoculation followed by a decrease in all cultures when the incubation continued. The high aroma producing Soviet starter showed simultaneously a slow increase of acetoin content which was 64 mg/l in a period of maximum diacetyl content (7.8 mg/l) after 18 hrs and then the increase continued till 24 hrs, although the diacetyl content decreased at the same time.

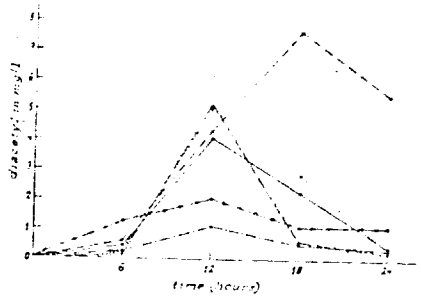

Fig. 1. Changes of diacetyl content in starters prepared with dairy cultures of different origin.

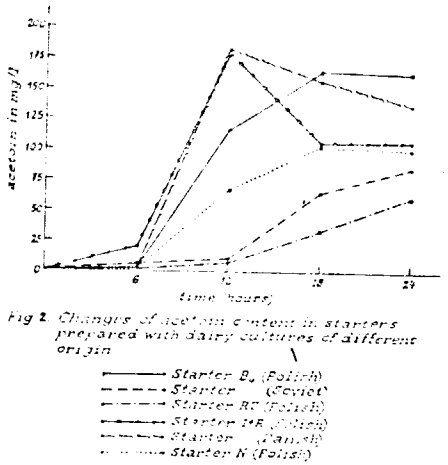

Fig 2. Changes of acetoin content in starters prepared with dairy cultures of different origin

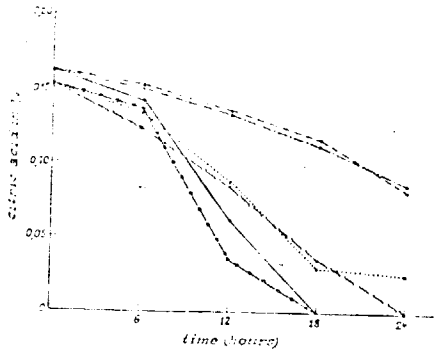

Fig 3 Decrease of citric acid content in starters prepared with dairy cultures of different origin

Citric acid content (Diagram 3) fell to zero after 18 hrs in starters (1 + R) and B_4, and after 24 hrs in the Danish one which corresponds with the low diacetyl content at that time. Notwithstanding, the rapid citric acid utilization correlated neither with aroma producing capacities nor with the rate of diacetyl decrease when

incubation continued up to 24 hrs. Nevertheless generally it may be said that no great amount of diacetyl could be expected, if a culture was found to contain only small or no amounts of citric acid. That view was supported by results obtained with 5 of 6 cultures analysed.

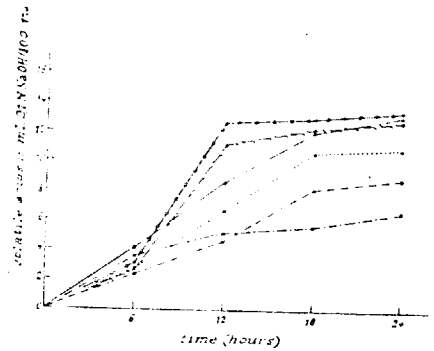

Fig 4 Changes in volatile acid content in starters prepared with dairy cultures of different origin.

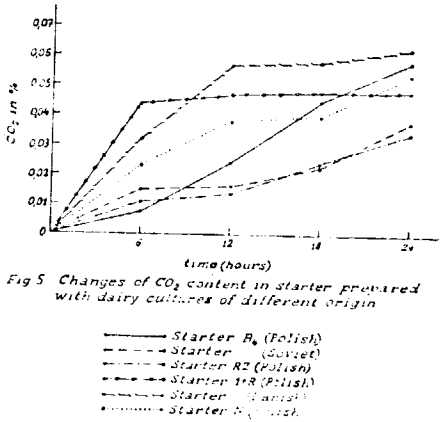

Fig 5 Changes of CO_2 content in starter prepared with dairy cultures of different origin

The content of volatile acids and that of CO_2 (Diagram 5) in the mixed strain cultures appeared to be on a remarkably different level for particular stages of investigation. The Soviet starter generally showed a low content of volatile acids (up to 6.3 ml 0.1 N NaOH/100 ml of starter) and CO_2 (up to 0.34‰). The Danish starter and the Polish (1 + R) gave the highest volatile acid contents and the relatively highest (up to 0.65‰) CO_2 content, both generally in particular stages of investigations. The intensive production of volatile acids and CO_2 by these starters corresponds with the highest acetoin content after 12 hrs.

The content of volatile acids, acetoin and diacetyl appeared to be considerably differentiated in particular starter cultures after 12 hrs and the same is true of CO_2 after 6 hrs which indicated that CO_2 was the first metabolite to be analysed which was found to be present in starters before all others were detected. The low capacity to produce volatile acids and CO_2, that was found in the Soviet starter and the Polish RZ (Fig. 4 and 5) may be correlated only with slow decrease of citric acid content (Fig. 3). The ratio between diacetyl and acetoin showed various levels. There were also differences in their mutual proportion during acidification, as is seen in Table 4.

Table 4 indicates that the highest percentage of diacetyl in relation to acetoin was still observed after 6 hrs of incubation in all starters but one studied. Only trace amounts of diacetyl were found after 24 hrs in all starters except the Soviet one.

TABLE 4

Per cent ration of diacetyl to acetoin in course of acidification of mixed strain starters

Acidification time after inoculation	Symbol of starter tested and the per cent ratio of diacetyl to acetoin					
	RZ Polish	N Polish	B_1 Polish	Danish	Soviet	A—E Polish
After 6 hrs	35.0	18.2	16.6	15.0	11.0	6.0
.. 12 ..	13.5	7.5	3.5	2.8	42.0	1.1
.. 18 ..	1.5	2.7	1.1	0.4	11.5	1.1
.. 24 ..	0.5	0.2	0.2	0.2	6.4	1.1

DISCUSSION

Considerable amounts of acetic acid and possibly those of formic acid (similar Rf) were found on chromatograms of all cultures studied. Almost imperceptible trace quantities of propionic acid could be observed in Polish N and Soviet starters. The organoleptic estimation (in skim milk) showed a stronger flavour and effervescent properties of starters with high content of volatile acids and CO_2, while those with low content of these compounds possessed a slight acid and mild flavour. Such mild and less strong flavour could be also correlated with a little higher pH values after 12 hrs (4.5—4.6) as compared with pH 4.2—4.4 of starters which revealed a stronger flavour at the same time.

The differences in produced metabolites which were found in the starter mixtures studied indicate that different characteristics are present in these cultures. These differences could not be explained by such factors as milk properties, pasteurizing conditions, oxidation degree, the ratio of the surface of starter to the total volume etc., because all these conditions were in all cases the same. The main source of these differences must be involved in the properties of strains which were contained in the mixtures, as well as in their interaction. The differentiated characteristics of starters studied seem to be due in the first place to the enzyme system of strains which were responsible for the metabolism of milk components.

Harvey and Collins (9) emphasized the particular role played by citrase in the break-down of citrate to acetate, oxalate, pyruvate and CO_2. The same workers reported that lactose and citrates were necessary for C_1-compounds to be synthetized. That was recently confirmed by Andersen (10) and by Mizuno and Jezeski (11) who applied the labelled atom procedure in their studies.

The signs of diacetyl reduction in Betacoccus type cultures were observed by Sobeck-Skal (12) when the cell counts were the highest, while the Streptococcus diacetilactis strains showed the same reduction only when they were in the death phase, and even then at a different level. Therefore it is probable that the cell counts of the cultures studied varied in the particular incubation phases and that

the decreases rate of diacetyl could be more or less influenced by these changes after 12 hrs of incubation when the cell tends to die.

There seems to be no doubt that it would be desirable to stop the further acidification after 10—12 hrs or after coagulation (according to the propagation temperature) in order to prevent the ageing of bacterial population. It is difficult to give any precise and unquestionable advice on which the cultures studied are better and in what respect. The only reliable answer can be given by the manufactured product i. e. cheese of various types or butter which would be made under comparable conditions.

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food technology

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III. GRAS Substances

0734

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CHEMICALS/See Cover

Recent Progress in the Consideration of Flavoring Ingredients Under the Food Additives Amendment

III. GRAS Substances

RICHARD L. HALL and BERNARD L. OSER

■ THE Flavoring Extract Manufacturers' Association has conducted a program since 1958 to determine the status of flavoring substances under the Food Additives Amendment of 1958. The key portion of this program has involved the creation of a panel of expert pharmacologists and toxicologists to determine, on the basis of all available data, including experience based on common use in food, what substances are "generally recognized as safe" (GRAS). This article, in a series describing the program, lists all substances which are GRAS and the average maximum use levels at which each has been reported to be used in different categories of food. Inasmuch as all food ingredients, including those that are GRAS, must be used only in accord with good manufacturing practice, certain general guidelines to good manufacturing practice may be drawn from the available data. Such guidelines are presented and discussed.

Previously published articles (Anon., 1961a–f, 1962; Hall, 1959, 1960; Hall and Oser, 1961) and Committee reports (Food Additives Committee, 1958–1964) have given in detail the chronology of the steps taken by the flavoring industry in compliance with the Food Additives Amendment of 1958. The key portion of the program has been the organization of a panel of highly qualified experts who are equipped by both experience and current knowledge to determine general recognition of safety. The background leading to the present article will be found in the list of references, and the article represents the next logical step in the series mentioned above. The data on levels of use which the panel employed in making its judgments are presented in detail for each substance generally recognized as safe. Also stated and discussed are certain general interpretive principles by which these data may be used as guidelines to certain aspects of good manufacturing practice.

COMMON USE FACTOR

The Food Additives Amendment of 1958 states that the term "food additive" means "any substance the intended use of which results . . . directly or indirectly, in its becoming a component . . . of any food . . ., if such substance is not generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or *experience based on common use in food*) to be safe under the *conditions of its intended use; . . .*" The phrases from the Food Additives Amendment inextricably couple general recognition of safety with the conditions of intended use. Any adverse indications regarding safety derived from experience in common use may militate against general recognition of safety. Almost all of the flavoring ingredients contained herein were in use prior to January 1, 1958. In almost every case, therefore, common use was a factor, sometimes a major factor, in the panel's final judgment.

The principal source of information on "common use" was a survey of flavoring and representative food manufacturers, conducted by the Flavoring Extract Manufacturers' Association (FEMA). Included in addition to the information derived from this survey are data on chewing gum flavors obtained through the National Association of Chewing Gum Manufacturers, and a limited amount of additional data on candy flavors obtained from several leading manufacturers. While this report does not include all actual flavor uses, it does provide widely representative data from which "common use" can be judged.

It must be emphasized that there can be no general recognition of safety without knowledge of the conditions of intended use and reasonable assurance that actual use conforms appropriately to the intended conditions. This statement applies as fully to those substances which are on the Food and Drug Administration's "White Lists" as to the flavoring ingredients which are on the FEMA GRAS list. This report, therefore, includes both. Those substances which appear only on an FDA White List are indicated in the accompanying survey tabulation with an asterisk.

The enormous variety of flavoring ingredients and the uses to which they may be put make it completely impossible to establish hard-and-fast rules defining their use. As an illustration, oil of clove may be used as a trace ingredient at less than a part per million in the finished food at which level it is not recognizable as such. Or it may be used at a few parts per million as one of the principal flavor notes in a product. Finally, it may reasonably be used at more than 1,000 parts per million when it is the dominant flavor note, for example, in hard candy. Thus in some cases, the use of a substance to impart a dominant note may be at a level more than 1,000 times that of the same substance used as a trace ingredient. In general, the upper limit of use is governed by the general acceptability of the product. Those who have worked with flavors are familiar with this self-limiting characteristic.

A complication which should be noted arises from multiple introductions of a particular chemical entity into a single food product. This occurs because a substance may be added

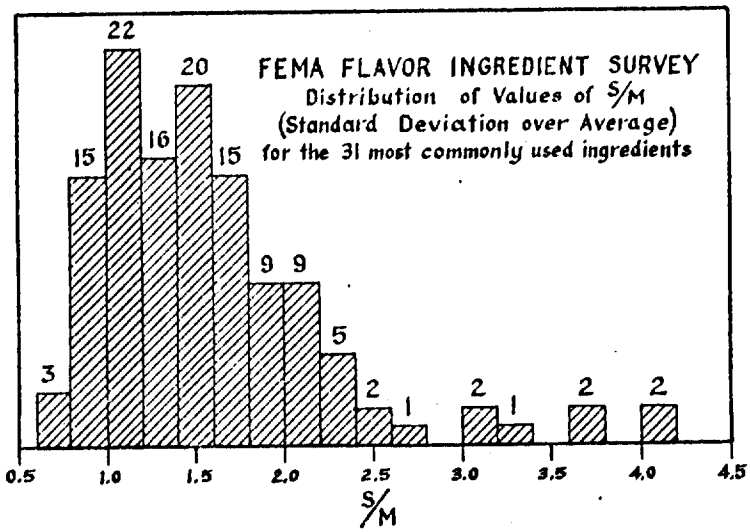

Fig. 1

both as such, and as a normal constituent of one or more natural flavoring materials. For example, the uses reported here of benzaldehyde do not include its use as almond oil added to the same food product, and the uses of eugenol do not reflect that which is separately listed under oil of clove.

GENERAL INTERPRETATIONS

This report reproduces for each major food category on which information was obtained the average of the maximum use levels reported by those firms participating in the survey which appears at the end of this article. The heading "Candy" is taken in the broadest sense, to include chocolate and hard candies such as sour balls, pressed sugar tablets, lozenges, etc. The category "Condiments" includes salad dressings, mustard, relishes, sauces, and comparable highly seasoned food products which are not ordinarily eaten as such but are consumed in conjunction with other foods. The category "Beverages" includes primarily soft drinks, but also some flavored wines and liqueurs which are not specifically listed separately. It also includes beverage tablets and powders, in which cases the use levels involved are computed on the basis of an 8-ounce serving of finished beverage.

In instances where there was only one report, the figure "1" in parentheses appears with the reported maximum use level. Two replies are shown individually rather than as the average.

It is obvious that most averages are derived from sets of figures, some of which may individually depart widely from the mean. Usually, though not necessarily, the average is close to the median, i.e., approximately half of the individual responses will exceed the average, and half be less. One or a few very low or high figures may greatly influence the average. Whether or not this influence by extreme figures is misleading depends on two other factors, neither of which was possible to control. One of these factors is the reliability of the apparently aberrant figure, and the other is the weight which it should be given in the total picture.

A maximum use level that appears to be unreasonably low may show either that the reporting firm used the substance only as a trace ingredient, or that the value may actually have resulted from an error in calculation (e.g., in converting ounces per 100 gallons to parts per million). An extremely high value may represent a reasonable but unusual use, or it, too, could have been erroneous. While an effort was made to recheck extremely high figures, it clearly was not possible, in many cases, to be sure whether the figure represented an error in calculation or an unusual, but nevertheless actual, use.

The second factor concerns the weight that should be given each report. In theory, the averages would be most meaningful if each reply were weighted in proportion to the percentage of total consumption within each food category which that reply covered. In practice, this was impossible to determine, and the only feasible alternative was to use a simple average. This meant, however, that an extreme value could influence the average out of all proportion to its actual importance. This is particularly true of high figures. As every food manufacturer knows, progressively higher flavoring levels (above the most generally preferred or acceptable levels) appeal to progressively smaller segments of the population. Thus, an extremely high use level, even though actual, would ordinarily represent a very limited total consumption. All factors considered, these use figures are reasonably accurate, even though we must remain constantly aware of the qualifications which affect their interpretation.

It should also be noted that the use levels obtained in the survey and reported here are those *introduced* into the food. They do not reflect the losses which may occur in later processing—through volatilization, leaching, or other means.

THE BASIC PROBLEM

We come, then, to the basic problem of how to employ an average maximum use level (bearing in mind the qualifications just stated) as a guide to that "good manufacturing practice" which should govern its "intended use" and is required by both common sense and government regulation. In preparing for this, two steps have been taken:

1) In the calculation of average maximum use levels reported here, responses from the individual manu-

facturer have been omitted which exceed four times the average calculated from data submitted by other firms, provided that these high levels appeared to be separate and distinct from the common levels of use, rather than at the end of a continuous spectrum of values. This was done to eliminate patently erroneous values. In such cases, this obviously resulted in figures lower than the actual arithmetic averages, because (a) unusually low figures were not eliminated, and (b) some isolated high values were undoubtedly real, even though unusual.

2) *All* reports for four principal food categories (beverages, frozen desserts, candy, and baked goods) on the 31 ingredients on which 50 or more replies were received were analyzed statistically. In the analysis of these 124 cases, the elimination procedure described in (1) above was not followed, except for one clearly erroneous value. The average (M) and the standard deviation (S) of the average maximum use levels for each food category were calculated. The data for a possible relationship between M and S were then examined and it was found that one did, indeed, exist, as shown in Fig. 1.

In the most frequent (modal) case, the ratio of the standard deviation to the mean (S/M) ranged between 1.0 and 1.19. In 100 out of 124, S/M was less than 2.00. Careful analysis of those cases where the ratio was 2.0 or greater revealed that either (a) the reported value was obviously in error, or (b) an exceptionally high single flavor note was employed.

Clearly, the great majority of maximum use levels are less than five times the average (M). An even higher proportion of *all* uses (including those less than the maxima) fall below $5M$.

Finally, as mentioned above, extremely high uses strongly influenced S, and to a lesser extent M, in this unweighted average, out of all proportion to the necessarily limited volume of food covered by such high use.

Thus, it is reasonable to conclude that in actual manufacturing practice, a level of five times the average maximum use will include nearly all normal applications of the ingredient.

RATIONALE

This multiple of five is subject to both upward and downward variation in a number of special cases. To define it more usefully and specifically required consideration of a number of determinants, which may operate singly or in combination. The numbered points which follow summarize the effect of these determinants upon use levels, and upon the factor which relates the average maximum level to the general range of good manufacturing practice. A brief discussion of the rationale is included where appropriate.

1) If a flavoring substance is used to provide the principal, or a single, flavoring note, the level of use may be more than five times the average maximum use level.

2) While there are many exceptions, natural flavors are, in general, used at higher levels than synthetic components. In the 124 cases analyzed, only 15 concern an average maximum use in excess of 500 ppm in any single food category. Thirteen of these cases involve natural oils or extracts (Table 1).

Table 1. Cases in which the average maximum use level is greater than 500 ppm.

Substance	Recalculated av. max. use level (ppm)*	S/M	Food category
Anise, oil	570.	1.82	Candy
Cassia bark, oil	750.	.78	Candy
Cinnamaldehyde	670.	1.72	Candy
Grapefruit, oil	570.	1.18	Candy
Lemon, oil	1,000.	1.04	Candy
	540.	.90	Baked goods
Lime, oil	700.	1.00	Candy
Methyl salicylate	930.	2.29	Candy
Orange peel, sweet, oil	1,200.	1.22	Candy
	530.	.92	Baked goods
Peppermint, oil	1,200.	1.17	Candy
Vanilla, extract	1,300.	1.48	Beverages
	3,900.	1.16	Ice cream, ices, etc.
	3,700.	1.09	Candy
	4,100.	1.68	Baked goods

*These are recalculated, including *all* data (see text).

3) Substances used at very high levels usually entail a smaller multiple of the average maximum than those used at lower levels. In the 15 cases (Table 1) where the levels of use are more than 500 ppm, only one, methyl salicylate, involves an S/M value higher than 1.82. The use of methyl salicylate in hard candy and chewing gum presents some unusual aspects, of which this is one. Ten of the fifteen cases are below 1.23. Thus, most would be covered by a factor of $3M$, rather than $5M$. On the other hand, all of the 22 cases in Table 2 in which S/M is greater than two, and thus not covered by $5M$, are concerned with uses under 200 ppm, again except for methyl salicylate. In two instances, S/M is exactly 2.0. All but six of these 24 cases are below 120 ppm.

Table 2. Cases in which S/M is 2.0 or greater.

Substance	Recalculated av. max. use level (ppm)*	S/M	Food category
Acetic acid	110.	2.16	Ice cream, ices, etc.
Anise, oil	50.	4.19	Beverages
Benzaldehyde	96.	3.1	Beverages
	120.	3.1	Ice cream, ices, etc.
Butyric acid	26.	2.02	Beverages
	84.	2.1	Baked goods
Cassia bark, oil	49.	3.32	Beverages
	100.	2.35	Ice cream, ices, etc.
Clove bud, oil	14.	2.00	Beverages
Coriander, oil	78.	3.6	Candy
	67.	3.6	Baked goods
	66.	4.0	Ice cream, ices, etc.
Diacetyl	3.7	2.00	Beverages
Ethyl butyrate	40.	2.15	Beverages
	38.	2.23	Ice cream, ices, etc.
Ethyl methyl phenylglycidate	18.	2.23	Ice cream, ices, etc.
Ethyl vanillin	170.	2.30	Candy
	170.	2.60	Baked goods
Lemon oil, terpeneless	39.	2.47	Beverages
	93.	2.23	Ice cream, ices, etc.
	100.	2.17	Candy
	120.	2.56	Baked goods
Methyl salicylate	930.	2.29	Candy
Nutmeg, oil	200.	2.04	Baked goods

*These are recalculated, including *all* data (see text).

4) Products such as hard candies and some baked or fried goods involve processing with resultant high flavor losses. This requires the flavor to be introduced at high levels, and may involve a wider range than in other foods, such as beverages, in which no processing losses occur. Of the 15 high-level cases (average maximum use level greater than 500 ppm) in Table 1, 13 deal with baked goods and candies. The other two are vanilla extract in beverages and ice cream. Vanilla extract is ordinarily used at a relatively high level in any food product.

5) Of the 22 cases involving S/M ratios greater than 2.0, most are

explainable by the foregoing causes. In addition, it must be pointed out that foods consumed in small portions tend to be flavored at high levels. Obvious examples are chewing gum, flavored wines, liqueurs, and, hard candies. The special considerations applicable to chewing gum are discussed in considerable detail by Heggie *et al.* (1965). The FEMA survey reflects primarily soft-candy uses, since hard candies (including lozenges and pressed mints) are only approximately 12.5 percent of total U. S. candy production (Steinberg, 1963). It was evident from the data that the "average maxima" of hard-candy uses ran from 2 to 10 times the average maximum use levels reported for all candy in the survey reported here, and on rare occasions were higher.

One may estimate the confidence which can be placed in the reported average maximum-use levels from the total number of replies on which these figures are based. This is given in the first column to the right of the name of the substance. Obviously, for substances on which a large number of responses were received, the average figure is more reliable than in cases where only a handful of reports were received.

These considerations, applied to the average maximum-use levels, are consistent with good manufacturing practice, and were taken into account by the panel in arriving at its judgments that these substances are generally recognized as safe. The panel's conclusions were based on over-all conditions of use of each substance in relation to its total intake.

GUIDELINES, NOT TOLERANCES

The Food and Drug Administration has constantly emphasized the necessity of observing "good manufacturing practice." In Paragraph 121.101, it defines the term to include the following restrictions:

"(1) The quantity of the substance added to food does not exceed the amount reasonably required to accomplish its intended physical, nutritive, or other technical effect in food.

"(2) Any substance intended for use in or on food is of appropriate food grade and is prepared and handled as a food ingredient."

The figures presented and discussed in this report are not tolerances. The word "tolerance" means a level within the safe range established by scientific procedures, but no greater than necessary to achieve the desired effect, and hence above which the substance may not legally be used. In contrast, the figures cited here are averages to which certain flexible principles, stated above, must be applied. It is the opinion of the expert panel that, except where specifically noted, it is neither necessary nor practical to establish tolerances or rigid use limits for the flavoring substances covered by this report.

It is clear that the fact that a flavor ingredient may have been reported as used only in certain food categories does not necessarily preclude its use, as a substance generally recognized as safe, in other categories within the principles stated above. However, in special cases where flavoring substances are used in a manner, or at levels, substantially different from those embraced by current good manufacturing practice, justification for such uses, in terms of safety and necessity, may have to be accomplished independently by the user concerned.

It is again emphasized that these are guidelines to "good manufacturing practice" or to "intended use" or to "common use" in which these substances are generally recognized as safe. They are not tolerances in any sense of the word.

ACKNOWLEDGMENTS

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THE AUTHORS are respectively director of research and development, McCormick & Co., and president, Food and Drug Research Laboratories.

SURVEY OF FLAVORING INGREDIENT USAGE LEVELS

Flavoring Extract Manufacturers' Association average maximum use levels (in ppm) on which the expert panel based its judgments that the substances are generally recognized as safe. Those substances which appear only on an FDA "White List" are indicated with an asterisk.

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
2001 *ACACIA, GUM [*Acacia senegal* (L.) Willd.]--Arabic, gum	102	330.	430.	410.	460.	840.	740 2,800.	*Meringue* 750. 7,000.	*Syrups* 210. 240.	
2002 ACETAL--Acetaldehyde diethyl acetal	7	7.3	52.	39.	6.0 120					
2003 ACETALDEHYDE--Ethanal	33	3.9	25.	22.	12.	6.8	20. 270.			
2004 ACETALDEHYDE PHENETHYL PROPYL ACETAL--Acetal R, Pepital	1	-	-	(1)2.5	(1)2.5					
2005 ACETANISOLE--4'-Methoxyacetophenone; p-Acetyl anisole, p-Methoxyacetophenone; Novatone	12	2.3	2.5	4.6	5.8		(1)810			
2006 ACETIC ACID--Ethanoic acid	60	39.	32.	52.	38.	(1)15.	60. 60.	*Condiments* 5,900.		
2007 (tri-)ACETIN--Glyceryl triacetate; Enzactin, Vanex	9	190.	60. 2,000	560.	1,000.		(1)4,100.			
2008 ACETOIN--3-Hydroxy-2-butanone, Acetyl methyl carbinol, 2,3-Butanolone, γ-Hydroxy-β-oxobutane, Dimethylketol	34	7.4	3.3	18.	32.	0.60 21.		*Cottage Cheese* (1)7.0	*Margarine* 0.80 50.	*Shortening* (1)8.0
2009 ACETOPHENONE--Methyl phenyl ketone; Acetyl benzene, Hypnone	24	0.98	2.8	3.6	5.6	(1)7.0	0.60 20.			
2010 ACONITIC ACID--1-Propene-1,2,3-tricarboxylic acid, Achilleic acid, Citridic acid, Equisetic acid	4	0.20 2.0	(1)0.60	0.60 30.	0.60 15.		(1)28.	*Alcoholic Beverages* (1)20.		
2011 ADIPIC ACID--1,4-Butanedicarboxylic acid, Hexanedioic acid	2	(1)40.	-	-	-	(1)5,000.				
2012 *AGAR--*Gelidium cartilagineum* (L.) Gaillon and *Gracilaria confervoides* (L.) Greville and related red algae	10	420. 1,000.	130. 1,000.	-	490.			*Icings* 300. 30,000.	*Meringue* (1)2,000.	
2013 *ALFALFA, EXTRACT--*Medicago sativa* L.	2	(1)10.	-	-	-			*Alcoholic Beverages* (1)200.		
2014 *ALGIN--*Laminaria* spp. and other kelps	9	100. 240.	2,000. 2,400.	-	-	(1)1,000.		*Emulsions* (1)100.		
2015 ALGINATES, SODIUM, CALCIUM, and AMMONIUM	25	340.	1,000.	-	70. 200.			*Condiments* 5,200.	*Meats* (1)1,000.	*Toppings* 4,500.
2016 ALKANET ROOT, EXTRACT [*Alkanna tinctoria* Tausch]--Alcannin, extract, Alkannin, extract, Anchusin, extract	6	(1)1.0	(1)3.0	(1)10.	(1)10.			*Icings* (1)70.	*Meats* (1)0.20	
2017 *ALLSPICE--*Pimenta officinalis* Lindl.	36	120.	1.5 2.0	(1)2.0	1,400.		(1)40.	*Condiments* 1,000.	*Meats* 670.	
2018 *ALLSPICE, OIL--*Pimenta officinalis* Lindl.	56	18.	15.	66.	48.		40. 1,700.	*Alcoholic Beverages* (1)5.0 *Meats* 110.	*Condiments* 70. *Pickles* 29.	*Soups* (1)55.
2019 *ALLSPICE, OLEORESIN--*Pimenta officinalis* Lindl.	8	-	-	-	(1)600.			*Condiments* 25. 130.	*Meats* 69.	
2020 ALLYL ANTHRANILATE	5	1.1	0.67	2.0	0.02 1.0	(1)2.0				
2021 ALLYL BUTYRATE	6	1.2	0.50 1.0	1.3	0.50 3.0	(1)1.0				
2022 ALLYL CINNAMATE	8	1.0	1.4	1.8	2.6					
2023 ALLYL CYCLOHEXANEACETATE	5	1.1	1.6	3.5	4.0					

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
2024 ALLYL CYCLOHEXANEBUTYRATE	6	1.0	1.4	3.3	3.8					
2025 ALLYL CYCLOHEXANEHEXANOATE	3	(1)1.4	(1)3.3	8.0	8.5					
2026 ALLYL CYCLOHEXANEPROPIONATE--Allyl 3-cyclohexylpropionate, Allyl β-cyclohexylpropionate	30	3.7	3.1	13.	7.1	7.7	(1)30.	*Icings* (1)0.20		
2027 ALLYL CYCLOHEXANEVALERATE	5	1.2	2.3	4.4	4.8					
2028 ALLYL DISULFIDE--Diallyl disulfide	5	-	-	-	-			*Condiments* 6.5	*Meats* 7.0	
2029 ALLYL 2-ETHYLBUTYRATE	2	0.50 1.0	-	(1)2.0	-	(1)1.0				
2030 ALLYL 2-FUROATE	5	0.53	0.05 2.0	1.6	0.75 2.0	(1)1.0				
2031 ALLYL HEPTANOATE--Allyl enanthate; Allyl heptoate; Allyl heptylate	15	1.3	2.7	6.4	6.4	2.9	(1)86.			
2032 ALLYL HEXANOATE--2-Propenyl hexanoate	52	7.0	11.	32.	25.	22.	210			
2033 ALLYL α-IONONE--1-(2,6,6-Trimethyl-2-cyclohexene-1-yl)-1,6-heptadien-3-one. Cetone V	6	0.50	1.4	2.6	3.1	(1)1.0		*Toppings* (1)2.0		
2034 ALLYL ISOTHIOCYANATE--Mustard oil	23	0.02 0.50	(1)0.50	(1)0.50	5.2			*Condiments* 52.	*Meats* 87.	*Pickles* 10. 88.
2035 ALLYL MERCAPTAN--2-Propene-1-thiol; Allylthiol, Allyl sulfhydrate	4	(1)0.25	0.50 2.0	(1)0.50	0.50 2.0			*Condiments* 2.0 3.0	*Meats* (1)0.50	
2036 ALLYL NONANOATE	4	0.70	0.50 3.0	5.0	3.0 5.0				*Meats* (1)1.0	
2037 ALLYL OCTANOATE	15	1.7	3.3	5.1	4.0	(1)0.10				
2038 ALLYL PHENOXYACETATE--Acetate PA	4	0.82	0.004 0.40	2.3	0.02 1.0	(1)3.0				
2039 ALLYL PHENYLACETATE	3	0.06 3.0	(1)8.0	14.	(1)40					
2040 ALLYL PROPIONATE	3	0.06 3.0	(1)16.	6.5	(1)10.					
2041 ALLYL SORBATE--2,4-Hexadienoate	3	0.86	(1)0.50	0.50 5.0	(1)1.0	(1)2.0				
2042 ALLYL SULFIDE--Thiallyl ether, Diallyl sulfide	10	0.04	0.06	0.07	0.05			*Condiments* 13.	*Meats* 3.7	
2043 ALLYL TIGLATE--Allyl *trans*-2-methyl-2-butenoate	3	0.28	0.50 0.50	0.50 3.0	0.50 3.0					
2044 ALLYL 10-UNDECENOATE--Allyl undecylenate	2	0.25 1.0	0.50 0.50	(1)0.50	(1)0.50					
2045 ALLYL *iso*-VALERATE	5	8.6	18.	22.	15. 48.	(1)1.0				
2046 *ALMONDS, BITTER, OIL (FFPA)--*Prunus amygdalus* Batsch var. *amara* (DC.) Focke	99	80.	66.	97.	96.	29.	330.	*Maraschino Cherries* 340.		
2047 ALOE, EXTRACT--*Aloe* spp.	4	5.0 2,000.	-	-	-			*Alcoholic Beverages* (1)130.		
2048 ALTHEA ROOT [*Althea officinalis* L.]--Marshmallow root	3	5.7 10.	-	-	-					
2049 *AMBERGRIS, TINCTURE	4	2.0	1.7	9.7	(1)0.10					
2050 *AMBRETTE, ABSOLUTE, OIL--*Hibiscus abelmoschus* L.	3	0.14	0.22	0.34	0.34					
2051 *AMBRETTE SEED, OIL--*Hibiscus abelmoschus* L.	3	0.30	0.30 0.50	0.80	0.80					
2052 *AMBRETTE, TINCTURE--*Hibiscus abelmoschus* L.	3	(1)5.0	1.0 5.0	0.04 10.	(1)10.			*Alcoholic Beverages* (1)10.		

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
2053 AMMONIUM SULFIDE	1	-	-	-	(1)5.0			*Condiments* (1)5.0		
2054 AMMONIUM *iso*-VALERATE	4	-	-	-	58			*Syrups* (1)0.20		
2055 *iso*-AMYL ACETATE * --common Amyl acetate, β-Methyl butyl acetate	72	28.	56.	190.	120.	100.	2,700.			
2056 AMYL ALCOHOL--Pentyl alcohol, 1-Pentanol	16	18.	15.	35.	24.	7.7 50.	150. 340.			
2057 *iso*-AMYL ALCOHOL--*iso*-Pentyl alcohol, 3 Methyl-1-butanol, *iso*-Butyl carbinol	21	17.	7.6	52.	24.	46.	(1)300.	*Alcoholic Beverages* (1)100.		
2058 *iso*-AMYL BENZOATE--*iso*-Pentyl benzoate	13	3.0	2.5	3.5	7.4	(1)1.6	(1)200			
2059 AMYL BUTYRATE--Pentyl butyrate	45	19.	32.	76.	43.	0.50 1.4	760.	*Syrups* (1)58.		
2060 *iso*-AMYL BUTYRATE--*iso*-Pentyl butyrate	37	13.	34.	79.	51.	60.	570.			
2061 α-AMYLCINNAMALDEHYDE--α-Pentylcinnamaldehyde, α-Amyl β-phenylacrolein, Buxine *	20	1.3	1.5	4.0	4.5	0.03 0.05	(1)15.			
2062 α-AMYLCINNAMALDEHYDE DIMETHYL ACETAL--1,1-Dimethoxy-2-amyl-3 phenyl 2 propene	5	0.80	1.5 2.0	(1)2.0	2.6					
2063 *iso* AMYL CINNAMATE--*iso*-Pentyl cinnamate	13	3.1	4.2	13.	13.					
2064 α-AMYLCINNAMYL ACETATE--α-Pentylcinnamyl acetate	4	0.92	3.5	3.5	3.0		(1)3.0			
2065 α-AMYLCINNAMYL ALCOHOL--α-Pentylcinnamyl alcohol	6	0.47	1.5	1.6	1.5		(1)2.0			
2066 α-AMYLCINNAMYL FORMATE--α-Pentylcinnamyl formate	3	0.17	0.93	1.5	1.5		(1)1.0			
2067 α-AMYLCINNAMYL *iso*-VALERATE--α-Pentylcinnamyl *iso*-valerate	4	0.36	1.2	1.3	1.7		(1)1.0			
2068 AMYL FORMATE--Pentyl formate	22	13.	11.	31.	8.0		170.			
2069 *iso*-AMYL FORMATE--*iso*-Pentyl formate	23	8.4	14.	22.	16.	2.0 28.	250.			
2070 *iso*-AMYL 2-FURANBUTYRATE--*iso*-Pentyl 2-furanbutyrate, α-*iso*-Amyl furfurylpropionate	5	0.03 5.0	(1)2.8	6.0	0.50 8.0	(1)5.0				
2071 *iso*-AMYL 2-FURANPROPIONATE--*iso*-Pentyl 2-furanpropionate, α-*iso*-Amyl furfurylacetate	4	0.02 0.33	0.33 0.65	1.6 3.6	1.6 3.6					
2072 AMYL 2-FUROATE--Pentyl 2-furoate	3	(1)5.0	-	1.5 6.0	(1)1.0			*Condiments* (1)10.		
2073 AMYL HEPTANOATE--Pentyl heptanoate	9	7.0	3.8	7.5	3.0	(1)3.5	(1)53.			
2074 AMYL HEXANOATE--Pentyl hexanoate	16	5.3	16.	22.	8.3	0.30 3.7	(1)110.			
2075 *iso*-AMYL HEXANOATE--*iso*-Pentyl hexanoate	19	7.8	14.	17.	15.	(1)3.7				
2076 2-AMYL-5 or 6-KETO-1,4-DIOXANE	1		(1)5.0	(1)5.0	(1)5.0			*Shortening* (1)5.0		
2077 *iso*-AMYL LAURATE--*iso*-Pentyl laurate, *iso*-Amyl dodecanoate	2	0.04 3.0	0.16 6.0	0.50 6.0	0.50 6.0					
2078 *iso*-AMYL NONANOATE--*iso*-Pentyl nonanoate	6	1.5	3.3	3.0	4.0					
2079 AMYL OCTANOATE--Pentyl octanoate	8	5.0	3.5	6.0	3.5	(1)2.1				
2080 *iso*-AMYL OCTANOATE--*iso*-Pentyl octanoate	10	6.6	5.1	7.4	3.5	(1)2.1				

* Throughout this report, the names "*iso*-amyl" and "amyl" are used in accord with the rules of chemical nomenclature. In commercial practice, however, "amyl" invariably means "*iso*-amyl" unless it is prefaced by the *n*- for normal.

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2081 *iso*-AMYL PHENYLACETATE--*iso*-Pentyl phenylacetate	22	5.0	16.	12.	14.	0.15 3.4		*Toppings* 0.25 0.80		
2082 *iso*-AMYL PROPIONATE--*iso*-Pentyl propionate	14	3.8	13.	38.	6.1	0.80 3.7	750.			
2083 *iso*-AMYL PYRUVATE--*iso*-Pentyl pyruvate	4	4.7	8.1	9.2	12.					
2084 *iso*-AMYL SALICYLATE--*iso*-Pentyl salicylate; *iso*-Amyl *o*-hydroxybenzoate, Orchidee	6	1.4	2.9	3.0	3.0					
2085 *iso*-AMYL *iso*-VALERATE--*iso*-Pentyl *iso*-valerate	35	8.5	14.	33.	41.	1.0 61.	390.	*Jellies* (1)10.		
2086 ANETHOLE--*p*-Propenylanisole; 1-Methoxy-4-propenylbenzene; Anise camphor	43	11.	26.	340.	150		1,500	*Alcoholic Beverages* 1,400.		
2087 *ANGELICA ROOT, EXTRACT--*Angelica archangelica* L.	12	49.	46.	44.	61.			*Syrups* 1.0 100.		
2088 *ANGELICA ROOT, OIL--*Angelica archangelica* L.	18	12.	0.99	0.86	1.0	0.03 5.0	(1)60.	*Alcoholic Beverages* 15.		
2089 *ANGELICA SEED, EXTRACT--*Angelica archangelica* L.	6	1,100.	-	19.	(1)50.			*Condiments* (1)10.	*Syrups* (1)100.	
2090 *ANGELICA SEED, OIL--*Angelica archangelica* L.	10	6.3	1.4	1.9	2.2	(1)5.0		*Alcoholic Beverages* 32.	?	
2091 *ANGELICA STEM, OIL--*Angelica archangelica* L.	2	0.50 1.5	0.50 10.	1.0 25.	1.0 24.	(1)0.50				
2092 *ANGOSTURA, EXTRACT--*Galipea officinalis* Hancock	8	18.	-	-	-			*Alcoholic Beverages* 1,700.		
2093 *ANISE--*Pimpinella anisum* L.	17	2.0 30.	1.0 4.0	3.0 4.0	490			*Condiments* 96. 5,000.	*Meats* 1,200.	
2094 *ANISE, OIL--*Pimpinella anisum* L.	83	7.5	67.	500.	120		3,200.	*Alcoholic Beverages* 45.	*Meats* 65.	
2095 *ANISE, STAR--*Illicium verum* Hook. f.	33	13.	18.	83.	140.			*Alcoholic Beverages* 40. 60.	*Meats* 500. 1,000.	
2096 ANISE, STAR, OIL--*Illicium verum* Hook. f.	46	12.	99.	190.	230.			*Alcoholic Beverages* 50.	*Meats* 20. 55.	*Syrups* (1)8.0
2097 ANISOLE--Methoxybenzene; Methylphenyl ether	6	9.0	16.	51.	34.					
2098 ANISYL ACETATE--*p*-Methoxybenzyl acetate	21	6.3	8.0	15.	12.	11.	(1)30.			
2099 ANISYL ALCOHOL--*p*-Methoxybenzyl alcohol; Anisic alcohol	15	7.4	8.0	11.	12.	(1)1.9				
2100 ANISYL BUTYRATE--*p*-Methoxybenzyl butyrate	4	3.1	5.7	10.	13.					
2101 ANISYL FORMATE--*p*-Methoxybenzyl formate	9	3.2	3.9	7.9	14.	(1)0.20				
2102 ANISYL PROPIONATE--*p*-Methoxybenzyl propionate	14	5.6	6.1	16.	20.	(1)0.25				
2103 ANNATTO, EXTRACT [*Bixa orellana* L.]--Annotta, extract, Annottu, extract	14	(1)25.	200.	-	2,000.			*Breakfast Cereals* (1)2,000.	*Margarine* 330.	
2104 ANNATTO, SEED [*Bixa orellana* L.]--Annotta, seed, Annotta, seed	3	-	-	-	100. 100.			-		
2105 *APRICOT KERNEL, OIL [*Prunus armeniaca* L.]--Persic, oil	9	130. 150.	3.4 400.	300. 360.	270.			*Soups* 1.0 500.		
2106 *ASAFETIDA, FLUID EXTRACT--*Ferula assa-foetida* L.	4	(1)4.0	(1)10.	(1)5.0	8.0 10.			*Condiments* (1)50.	*Meats* (1)10.	*Soups* (1)10.
2107 *ASAFETIDA, GUM--*Ferula assa-foetida* L.	5	(1)5.0	(1)10.	15. 25.	(1)15.			*Condiments* 5.0 160.		

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
2108 *ASAFETIDA, OIL--*Ferula assa-foetida* L.	3	-	-	1.0 15.	(1)1.0			*Condiments* (1)10.		
2109 ASCORBIC ACID--Vitamin C	34	130.	0.80 250.	4.0 6,000	1.0 500.			*Fruit Sauces* 280. 380.	*Meats* 0.49 470.	
2110 *ASH BARK, PRICKLY, EXTRACT--*Xanthoxylum americanum* L. or *Xanthoxylum clava-herculis* L.	4	59	-	(1)78.	(1)82.					
2111 *BALM [*Melissa officinalis* L.]--Balm, lemon	0	-	-	-	-					
2112 *BALM LEAVES, EXTRACT [*Melissa officinalis* L.]--Balm, lemon, extract; Melissa, extract	1	(1)2,000.	-	-	-					
2113 *BALM, OIL [*Melissa officinalis* L.]--Balm, lemon, oil; Melissa, oil	7	8.5	1.7 15.	20.	10. 60.					
2114 BALSAM FIR, OIL--*Abies balsamea* (L.) Mill.	7	4.5	0.50 1.5	5.2	5.2	0.50 1.0				
2115 BALSAM FIR, OLEORESIN--*Abies balsamea* (L.) Mill.	3	(1)0.20	(1)1.5	(1)5.0	(1)5.0					
2116 *BALSAM, PERU--*Myroxylon pereirae* Klotzsch	38	3.0	5.9	10.	32.	0.05 1.0	120.	*Syrups* 0.25 7.0		
2117 *BALSAM, PERU, OIL--*Myroxylon pereirae* Klotzsch	15	3.2	2.2	8.4	6.6					
2118 *BASIL--*Ocimum basilicum* L.	20	(1)2.5	(1)5.0	(1)5.0	680.			*Condiments* 500.	*Meats* 520.	
2119 *BASIL, OIL--*Ocimum basilicum* L.	21	2.0	2.7	6.2	4.2	(1)0.01		*Condiments* 15.	*Meats* 24.	
2120 *BASIL, OLEORESIN--*Ocimum basilicum* L.	3	-	-	-	(1)16.			*Condiments* 2.0 5.0		
2121 *BAY LEAVES, WEST INDIAN, EXTRACT--*Pimenta acris* Kostel.	8	0.67	(1)2.0	1.6 2.0	(1)2.0			*Meats* 54.	*Soups* (1)0.72	
2122 *BAY LEAVES, WEST INDIAN, OIL [*Pimenta acris* Kostel]--Myrcia, oil	16	1.5	2.3	4.4	4.6			*Condiments* 27.	*Meats* 15.	
2123 *BAY LEAVES, WEST INDIAN, OLEORESIN--*Pimenta acris* Kostel	3	-	-	-	-			*Meats* 25. 25.	*Soups* (1)72.	
2124 *BAY, SWEET--*Laurus nobilis* L.	18	0.36 2.5	(1)5.0	(1)5.0	5.0 400.			*Condiments* 130.	*Meats* 840.	
2125 *BAY, SWEET, OIL--*Laurus nobilis* L.	5	2.0	1.8	2.6	21.		(1)2.9	*Condiments* (1)30.		
2126 BEESWAX, WHITE [*Apis mellifera* L.]--Cire d'abeille absolute	5.	0.50 0.50	2.0	10.	10.			*Honey* (1)5.0		
2127 BENZALDEHYDE-Benzenecarbonal; Benzenemethylal; Benzoic aldehyde	78	36.	42.	120.	110.	160.	840.	*Alcoholic Beverages* 50. 60.		
2128 BENZALDEHYDE DIMETHYL ACETAL	5	26.	22.	56.	45.	(1)50.		*Alcoholic Beverages* (1)60.		
2129 BENZALDEHYDE GLYCERYL ACETAL--2-Phenyl-m-dioxan-5-ol	9	21.	24.	110.	73.	100.	(1)840.			
2130 BENZALDEHYDE PROPYLENE GLYCOL ACETAL--4-Methyl-2-phenyl-m-dioxolane	5	34.	27.	110.	96.	(1)50.				
2131 BENZOIC ACID--Benzenecarboxylic acid, Phenylformic acid, Dracylic acid	14	7.5	4.8	8.9	40.		20. 32.	*Icings* (1)250.		
2132 BENZOIN--2-Hydroxy-2-phenylacetophenone	3	4.5	0.54	2.0	1.4	(1)0.10				
2133 BENZOIN, RESIN [*Styrax benzoin* Dryander, *S. paralleloneurus* Perkins; *S. tonkinensis* (Pierre) Craib ex Hartwich, or other spp. of the Section *Anthostyrax* of the genus *Styrax*]--Gum Benjamin, Benzoe	16	15.	5.1	8.7	26.	(1)10.	(1)110.			

HELO

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2134 BENZOPHENONE--Diphenyl ketone, Benzoylbenzene	8	0.50	0.61	1.7	2.4					
2135 BENZYL ACETATE	38	7.8	14.	34.	22.	23.	760.			
2136 BENZYL ACETOACETATE--Benzyl acetyl acetate	5	2.7	6.0	13.	13.	0.50 10.	(1)50.			
2137 BENZYL ALCOHOL--Phenyl carbinol, Phenylmethanol, α-Hydroxytoluene	24	15.	160.	47.	220.	21. 45.	1,200.			
2138 BENZYL BENZOATE--Benzyl benzene carboxylate, Benzyl phenylformate	20	4.5	12.	39.	33.		280.			
2139 BENZYL BUTYL ETHER	2	0.50 2.0	(1)3.5	(1)8.0	2.0 8.0	(1)2.0				
2140 BENZYL BUTYRATE--Benzyl butanoate	24	4.5	6.9	7.7	9.9	(1)3.0	(1)310.			
2141 BENZYL iso-BUTYRATE--Benzyl 2-methyl propanoate	11	5.2	12.	12.	25.					
2142 BENZYL CINNAMATE--Benzyl β-Phenylacrylate; Cinnamein	20	1.4	2.5	6.7	6.6	3.0 5.0	5.3 120.			
2143 BENZYL 2,3 DIMETHYLCROTONATE--Benzyl methyl tiglate	4	0.75	2.8	1.8	1.5					
2144 BENZYL ETHYL ETHER	2	0.50 1.0	(1)2.5	(1)7.5	(1)7.5					
2145 BENZYL FORMATE	14	2.4	8.0	12.	8.6					
2146 3-BENZYL 4-HEPTANONE--Benzyl dipropyl ketone, Morellone	4	1.2	4.6	11	11.	(1)3.2				
2147 BENZYL MERCAPTAN--α-Toluenethiol, Benzylthiol	2	0.15 0.25	0.15 0.50	0.50 0.75	0.50 0.75					
2148 BENZYL METHOXYETHYL ACETAL--Acetaldehyde benzyl β-methoxyethyl acetal, 1-Benzyloxy-1-(β-methoxy)-ethoxy ethane	1	(1)0.50	(1)1.0	(1)1.0	(1)1.0					
2149 BENZYL PHENYLACETATE	9	1.3	2.6.	6.6	4.3					
2150 BENZYL PROPIONATE--Benzyl propanoate	20	4.1	5.8	19.	17.		Toppings (1)5.0			
2151 BENZYL SALICYLATE--Benzyl o-hydroxybenzoate	7	1.4	0.89	1.8	0.01 2.2	19. 150.	Icings (1)40.			
2152 BENZYL iso-VALERATE	16	2.2	3.4	16.	*9.4					
2153 *BERGAMOT, OIL [*Citrus aurantium* L. subsp. *bergamia* Wright et Arn.]--Bergamot orange, oil	29	8.9	7.9	27.	29.	(1)56.	(1)200.			
2154 BIRCH, SWEET, OIL [*Betula lenta* L.]--Birch, black, oil	97	48.	44.	310.	110.	5.3 90.	43.	Icings 1.0 130.		
2155 BLACKBERRY BARK, EXTRACT--*Rubus*, spp. of Section *Eubatus*	7	81	3.0 880	230.	3.0 660.	(1)0.07	4,300.	Syrups (1)5.0		
2156 *BOIS DE ROSE, OIL--*Aniba rosaeodora* Ducke	12	0.65	2.6	6.7	9.3			Alcoholic Beverages 150. 10,000		
2157 BORNEOL--Bornyl alcohol, 2-Hydroxycamphane, Borneocamphor, 2-Camphanol, dl-Camphanol	8	0.25 1.4	(1)1.4	3.7	5.1	(1)35.				
2158 iso-BORNEOL	8	6.2	23.	11.	8.3	(1)0.30	Syrups (1)0.30			
2159 BORNYL ACETATE	11	1.1	1.8	1.9	1.4	(1)0.80				
2160 iso-BORNYL ACETATE	8	9.6	12.	3.9	9.5	(1)70.	(1)0.30	Syrups (1)0.20		

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2161 BORNYL FORMATE	5	3.7	0.30 3.0	0.80 2.0	0.80 2.0			*Syrups* (1)0.04		
2162 *iso*-BORNYL FORMATE	3	0.06 1.0	0.03 1.0	0.74	(1)0.80					
2163 *iso*-BORNYL PROPIONATE	3	0.01 1.0	0.80 1.0	1.2	(1)1.8					
2164 BORNYL VALERATE	3	0.06 1.0	(1)0.30	0.90 2.0	(1)0.80					
2165 BORNYL *iso*-VALERATE Bornyval	3	0.06 1.0	0.40 1.0	0.90 2.0	0.90 2.0			*Syrups* (1)1.2		
2166 *iso*-BORNYL *iso*-VALERATE	3	0.60 1.0	0.30 1.0	0.90	0.80 2.0					
2167 BORONIA, ABSOLUTE--*Boronia megastigma* Nees	9	4.3	2.8	11.	10.					
2168 BROMINATED VEGETABLE OILS	62	170.	260.	-	35. 190.					
2169 BUCHU LEAVES, OIL--*Barosma betulina* Bartl. et Wendl., *B. crenulata* (L.) Hook., or *B. serratifolia* Willd.	12	1.9	6.8	8.5	5.2			*Alcoholic Beverages* (1)0.50	*Condiments* (1)7.0	
2170 2-BUTANONE--Methyl ethyl ketone; MEK	1	(1)70.	(1)270.	(1)100.	(1)100.					
2171 BUTTER ACIDS[b]	5	(1)2.0	(1)3.0	2,800.	8.3			Popcorn Oil (1)1,200.	Toppings (1)2.0	
2172 BUTTER ESTERS[c]	13	-	24.	78.	86.			*Shortening* 750. 12,000.		
2173 BUTTER STARTER DISTILLATE	10	-	20. 40.	420.	720.					
2174 BUTYL ACETATE	24	11.	16.	32.	32.	13.	220.			
2175 *iso* BUTYL ACETATE	28	11.	16.	36.	35.	170.	860.	*Icings* (1)5.5		
2176 BUTYL ACETOACETATE	3	4.2	7.3	26.	26.					
2177 *iso*-BUTYL ACETOACETATE	3	4.0	7.0	25.	25.			*Alcoholic Beverages* (1)1.0	*Cream* (1)4.0	
2178 BUTYL ALCOHOL--1-Butanol	9	12.	7.0	34.	32.					
2179 *iso*-BUTYL ALCOHOL--*iso*-Butanol	6	17.	7.0	30.	24.			*Icings* 3.0 100.		
2180 *iso*-BUTYL ANGELATE--*iso*-Butyl *cis*-2-methyl-2-butenoate	2	(1)1.5	(1)1.5	(1)5.0	-					
2181 BUTYL ANTHRANILATE	10	1.3	2.6	9.0	6.7		5.0			
2182 *iso*-BUTYL ANTHRANILATE	10	2.0	4.0	12.	12.		1,700.			
2183 BUTYLATED HYDROXYANISOLE--Mixture of 2-*tert*-Butyl-4-methoxyphenol and 3-*tert*-Butyl-4-methoxyphenol; BHA, Embanox	34	0.82	0.81	9.6	2.8	0.54	13.	*Potatoes* (1)5.0	*Shortening* 230.	
2184 BUTYLATED HYDROXYTOLUENE--2,6-di-*tert*-Butyl-*p*-cresol, BHT, 2,6-di-*tert*-Butyl-4-methylphenol; Ionol C.P., Impruvol, Vianol, Parabar	0	-	-	-	-	-				
2185 *iso*-BUTYL BENZOATE	5	2.0 9.0	7.9	12.	10. 23.					
2186 BUTYL BUTYRATE	21	8.6	22.	24.	22.	14.	150. 1,500.	*Alcoholic Beverages* (1)2.0		
2187 *iso*-BUTYL BUTYRATE	16	8.3	16.	25.	24.	14.	(1)2,000.			
2188 BUTYL *iso*-BUTYRATE	12	8.7	4.0 5.0	19.	39.		(1)2,000.			

[b] Assuming that these are a mixture consisting only of the saponified acids reported in the literature as derived from butter in the approximate proportions normally occurring.

[c] Assuming that these are the ethyl esters of butter acids (q.v.).

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2189 iso-BUTYL iso-BUTYRATE	10	7.5	7.4	16.	17.	3.3 10.		Alcoholic Beverages (1)2.0		
2190 BUTYL BUTYRYLLACTATE--Lactic acid, butyl ester, butyrate	15	13	9.0	44.	58					
2191 α-BUTYLCINNAMALDEHYDE	4	0.50 1.0	1.0 2.8	2.0 8.0	2.0 8.0			Alcoholic Beverages (1)2.0		
2192 BUTYL CINNAMATE	5	0.83	2.6	1.0 15.	1.0 15.			Alcoholic Beverages (1)2.0		
2193 iso-BUTYL CINNAMATE	7	1.3	3.4	5.4	5.4					
2194 BUTYL 2-DECENOATE--Butyl decylenate	2	(1)8.0	-	1.5 22.	(1)30.		(1)2,000.			
2195 BUTYL ETHYL MALONATE	2	(1)3.0	-	(1)0.13	-					
2196 BUTYL FORMATE	8	2.9	3.2	11.	9.1	(1)5.0				
2197 iso-BUTYL FORMATE--Tetryl formate	10	2.2	7.1	19.	8.2	(1)5.0				
2198 iso-BUTYL 2-FURANPROPIONATE--iso-Butyl furylpropionate	7	8.1	14.	17.	21.	4.0 30.	(1)12.	Icings (1)20.		
2199 BUTYL HEPTANOATE	2	0.50 1.0	2.0 10.	2.0 25.	2.0 25.					
2200 iso-BUTYL HEPTANOATE	2	0.50 1.5	2.4 10.	7.0 25.	7.0 25.					
2201 BUTYL HEXANOATE	6	1.7	3.9	7.6	10.					
2202 iso-BUTYL HEXANOATE	8	5.4	3.9	8.1	8.3		(1)2.0			
2203 BUTYL p-HYDROXYBENZOATE--Butyl parasept	2	(1)1,000.	-	-	(1)10.					
2204 2-BUTYL-5 or 6-KETO-1,4-DIOXANE	1	-	(1)5.0	(1)5.0	(1)5.0			Shortening (1)5.0		
2205 BUTYL LACTATE	3	0.66	2.8	6.5	7.7					
2206 BUTYL LAURATE--Butyl dodecanoate	4	0.40 3.0	(1)0.60	17.	1.0 40.					
2207 BUTYL LEVULINATE	3	0.20 1.0	2.1	4.6	4.6					
2208 α-iso-BUTYLPHENETHYL ALCOHOL--4-Methyl-1-phenyl-2-pentanol; Benzyl iso-butyl carbinol; Benzyl iso-amyl alcohol	3	1.0 10.	38.	54.	15. 50.			Alcoholic Beverages (1)50.		
2209 BUTYL PHENYLACETATE	7	0.50	2.1	4.5	4.6	(1)5.0		Maraschino Cherries (1)3.0		
2210 iso-BUTYL PHENYLACETATE	11	2.8	2.8	5.5	5.0	(1)5.0				
2211 BUTYL PROPIONATE	10	4.0	5.2	25.	27.					
2212 iso-BUTYL PROPIONATE	10	5.4	4.2	25.	35.					
2213 iso-BUTYL SALICYLATE	7	3.5	1.8	2.6	5.0			Alcoholic Beverages (1)5.0		
2214 BUTYL STEARATE--Butyl octadecanoate	5	(1)1.0	(1)2.0	190.	340.		(1)330.			
2215 BUTYL SULFIDE--Dibutyl sulfide	2	0.02 1.0	0.01 1.0	0.03 1.0	0.03 1.0					
2216 BUTYL 10-UNDECENOATE	5	0.90	2.0	6.6	7.8		0.40 60	Alcoholic Beverages (1)5.0	Icings (1)5.0	
2217 BUTYL VALERATE	5	3.0	2.6	8.0	6.8					
2218 BUTYL iso-VALERATE	7	4.6	12.	13.	15.	(1)50.				

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
2219 BUTYRALDEHYDE--Butanal; Butyl aldehyde, Butyric aldehyde	6	0.71	4.8	2.9	5.4			*Alcoholic Beverages* (1)0.50	*Icings* (1)0.25	
2220 *iso*-BUTYRALDEHYDE--2-Methyl propanal, *iso*-Butyl aldehyde; *iso*-Butyric aldehyde	4	0.30	0.25 0.50	0.67	0.50 1.0			*Alcoholic Beverages* (1)5.0		
2221 BUTYRIC ACID--Ethylacetic acid, Butanoic acid	54	5.5	6.5	82.	32.	0.19 45.	60. 270.	*Margarine* 18.		
2222 *iso*-BUTYRIC ACID--2-Methyl propanoic acid; *iso*-Propylformic acid	16	4.1	12.	41.	38.		(1)470.	*Margarine* (1)30.		
2223 (tri-)BUTYRIN--Glyceryl tributyrate; Butyrin	5	(1)0.10	(1)0.04	0.33 1,000.	290.	(1)0.36		*Margarine* 2.0 50.		
2224 CAFFEINE--1,3,7-Trimethylxanthine, 1,3,7-Trimethyl-2,6-dioxopurine, Coffeine, Theine, Guaranine, Methyltheobromine, No-Doz®	24	120.	-	-	-					
2225 CAJEPUT, OIL--*Melaleuca leucadendron* L.	5	0.50 2.0	(1)1.0	13.	11.			*Alcoholic Beverages* (1)30.	*Bitters* (1)3,000.	
2226 CALAMUS [*Acorus calamus* L.]--Sweet Flag	6	68.	(1)1.0	(1)10.	(1)15.			*Alcoholic Beverages* 5.0 15.		
2227 CALAMUS, OIL [*Acorus calamus* L.]--Sweet flag, oil	16	2.0	1.5	3.0	3.8	(1)0.02				
2228 CALCIUM ACETATE--Sorbo-Calcion, technical product known as Brown or Gray Acetate of Lime	2	(1)200.	-	-	(1)500.					
2229 CAMPHENE--2,2-Dimethyl-3-methylenenorbornane	4	40. 90.	(1)20.	(1)160.	27.					
2230 *d*-CAMPHOR	4	-	(1)0.10	1.1 25.	11.			*Condiments* (1)20.		
2231 CAMPHOR, JAPANESE, WHITE, OIL--*Cinnamomum camphora* (L.) Nees et Eberm.	5	5.4	-	-	1.6 48.			*Condiments* (1)15.		
2232 *CANANGA, OIL--*Cananga odorata* Hook. f. and Thoms.	4	7.0	(1)1.0	(1)2.0	(1)2.0					
2233 *CAPSICUM, EXTRACT--*Capsicum frutescens* L.; *C. annuum* L.	8	120.	(1)15.	(1)12.	12. 14.			*Condiments* 50. 100.	*Meats* (1)200.	
2234 *CAPSICUM, OLEORESIN--*Capsicum frutescens* L.; *C. annuum* L.	49	14.	-	11.	(1)14.		46.	*Condiments* 92.	*Meats* 50. 100.	
2235 CARAMEL COLOR	123	2,200.	590.	180.	220.			*Meats* 2,100.	*Syrups* 2,800.	
2236 *CARAWAY--*Carum carvi* L.	7	(1)63.	(1)63.	-	3,000. 10,000.			*Condiments* (1)96.		
2237 *CARAWAY, BLACK--*Nigella sativa* L.	0	-	-	-	-					
2238 *CARAWAY, OIL--*Carum carvi* L.	33	29.	49.	86.	150.		(1)0.80	*Alcoholic Beverages* 140.	*Condiments* 38.	*Meats* 34.
2239 CARBOXYMETHYLCELLULOSE	23	91.	670.	9.0	41.			*Condiments* (1)2,000.	*Icings & Toppings* 2,300.	*Meats* (1)100.
2240 *CARDAMOM--*Elletaria cardamomum* (L.) Maton	22	3.0	(1)2.0	(1)2.0	1,700.			*Condiments* (1)900.	*Meats* 570.	
2241 *CARDAMOM SEED, OIL--*Elletaria cardamomum* (L.) Maton	37	1.9	1.3	5.8	57.		(1)2.2	*Alcoholic Beverages* 10. 10.	*Pickles* 10. 16.	*Condiments* 8.0 *Meats* 36.
2242 CARMINE--*Coccus cacti* L.	5	-	-	10. 100.	7.0 300.			*Condiments* (1)200.	*Meats* (1)10.	
2243 *CAROB BEAN, EXTRACT [*Ceratonia siliqua* L.]--St. John's bread	33	66.	93.	180.	120.	600.		*Icings & Toppings* 500. 1,000.		
2244 *CARROT, OIL--*Daucus carota* L.	13	3.1	5.5	5.1	4.4	(1)0.02		*Condiments* (1)15.	*Soups* (1)1.0	

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2245 CARVACROL--2-*p*-Cymenol; 2-Methyl-5-*iso*-propylphenol; *iso*-Thymol, Cymophenol; 2-Hydroxy-*p*-cymene, *iso*-Propyl *o*-cresol	9	26.	34.	92.	120.			*Condiments* 37.		
2246 CARVACRYL ETHYL ETHER--2-Ethoxy-*p*-cymene; Ethyl carvacrol	3	2.0 13.	(1)10.	21.	3.0 39.					
2247 CARVEOL--*p*-Mentha-6,8-dien-2-ol	3	1.5 13.	(1)3.0	3.0 39.	3.0 5.0					
2248 4-CARVOMENTHENOL--1-*p*-Menthen-4-ol, 4-Terpinenol, 1-Methyl-4-*iso*-propyl-1-cyclohexene-4-ol; Origanol	3	1.0 21.	1.0 84.	7.0 63.	(1)7.0					
2249 CARVONE--6,8(9)-*p*-Menthadien-2-one; Carvol	20	850.	120.	180.	110.			*Alcoholic Beverages* 130.		
2250 CARVYL ACETATE	3	1.5 11.	3.0 44.	20.	20					
2251 CARVYL PROPIONATE	2	(1)1.0	(1)2.0	2.0 24.	2.0 24.					
2252 β-CARYOPHYLLENE	9	14.	(1)2.0	34.	27.		(1)200.	*Condiments* (1)50.		
2253 CASCARA, BITTERLESS, EXTRACT--*Rhamnus purshiana* DC.	4	(1)100.	(1)50.	-	(1)100.					
2254 *CASCARILLA BARK, EXTRACT--*Croton cascarilla* Benn. and *C. eluteria* Benn.	2	5.0 800.	-	-	-					
2255 *CASCARILLA BARK, OIL--*Croton cascarilla* Benn. and *C. eluteria* Benn.	9	2.3	3.0	8.7	13.			*Condiments* (1)50.		
2256 *CASSIA--*Cinnamomum cassia* Blume	33	9.2	5.1	130.	3,000.					
2257 *CASSIA BARK, EXTRACT--*Cinnamomum cassia* Blume	5	310.	(1)10.	(1)10.	(1)10.					
2258 *CASSIA BARK, OIL--*Cinnamomum cassia* Blume--	66	3.0	11.	150.	73.		1,900.	*Condiments* 140.	*Meats* 290.	
2259 *CASSIA BUDS [*Cinnamomum cassia* Blume] Cinnamon flowers; Cassia flowers	1	(1)1,000.	-	-	-					
2260 CASSIE, ABSOLUTE--*Acacia farnesiana* (L.) Willd.	7	0.96	1.2	4.1	4.1	(1)1.0				
2261 *CASTOREUM, EXTRACT--*Castor fiber* L. and *C. canadensis* Kuhl	19	5.0	5.6	12.	41.		(1)400.	*Condiments* (1)50.	*Toppings* (1)2.0	
2262 *CASTOREUM, LIQUID--*Castor fiber* L. and *C. canadensis* Kuhl	23	3.2	1.9	4.9	7.3	1.2 2.0	19. 60.	*Toppings* (1)2.0		
2263 CASTOR, OIL [*Ricinus communis* L.]--Ricinus, oil; Palma Christi, oil; Tangan-tangan, oil	11	1.5 140.	3.0 540.	3.0 410.	210.					
2264 CATECHU, EXTRACT [*Acacia catechu* Willd.]--Black cutch, extract; Cachou, extract; Pegu catechu, extract; Cashoo, extract	7	16.	21.	140.	140.					
2265 CATECHU, POWDER [*Acacia catechu* Willd.]--Gambir, gum; Pale catechu; Gambir catechu; Terra japonica	10	45.	27.	43.	37.		(1)15.			
2266 *CAYENNE--*Capsicum annuum* L. var. *longum* Sendt	22	(1)1.0	(1)2.0	(1)2.0	2.0 50.			*Condiments* 610.	*Meats* 910.	*Soups* (1)100.
2267 CEDAR LEAF, OIL[4] --*Thuja occidentalis* L.	11	0.01 0.50	0.01 1.0	12.	1.0 20.			*Alcoholic Beverages* (1)16.	*Meats* (1)15.	
2268 *CELERY SEED--*Apium graveolens* L.	29	0.37 1,000.	-	-	1,800.			*Condiments* 2,500.	*Meats* 1,400. *Soups* 37. 500.	*Pickles* 13. 590

[4] Provided it is used at levels such that no thujone is detectable in the finished food, using the standard AOAC method.

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2269 *CELERY SEED, EXTRACT--*Apium graveolens* L.	15	240.	(1)5.0	(1)10.	1,900.			*Condiments* (1)10.	*Meats* (1)100.	*Soups* 160. 500.
2270 *CELERY SEED, EXTRACT SOLID--*Apium graveolens* L.	5	(1)150.	-	(1)8.0	(1)12.			*Condiments* (1)7.0	*Syrups* (1)10.	
2271 *CELERY SEED, OIL--*Apium graveolens* L.	42	11.	3.0 13.	13.	12.		(1)28.	*Condiments* 40	*Meats* 40. *Pickles* 10. 35.	*Soups* (1)1.0
2272 *CHAMOMILE FLOWER, ENGLISH, OIL--*Anthemis nobilis* L.	4	4.1	0.10 0.50	1.3 5.0	0.10 5.0					
2273 *CHAMOMILE FLOWER, HUNGARIAN, OIL--*Matricaria chamomilla* L.	8	2.6	6.1	3.8	6.5		(1)0.80	*Alcoholic Beverages* (1)1.0		
2274 *CHAMOMILE FLOWER, ROMAN, EXTRACT--*Anthemis nobilis* L.	8	13.	9.3	6.7	16.					
2275 *CHAMOMILE FLOWER, ROMAN, OIL--*Anthemis nobilis* L.	10	2.3	3.3	4.3	4.3	(1)0.25		*Alcoholic Beverages* (1)20.		
2276 *CHERRY BARK, WILD, EXTRACT--*Prunus serotina* Ehrh.	35	120.	140.	200.	76.	(1)3.5		*Alcoholic Beverages* 300. 800.	*Syrups* (1)30.	
2277 CHERRY LAUREL, OIL (FFPA)--*Prunus laurocerasus* L.	5				(1)75.			*Maraschino Cherries* (1)77.	*Extracts* 50. 65.	
2278 CHERRY PITS, EXTRACT--*Prunus avium* L. (sweet cherry); *P. cerasus* L. (sour cherry)	6	80. 150.	50. 60.	-	-					
2279 *CHERVIL--*Anthriscus cerefolium* (L.) Hoffm.	4	(1)100.	(1)50.	-	(1)150.			*Condiments* (1)60.		
2280 *CHICORY, EXTRACT--*Cichorium intybus* L.	19	63.	58.	57.	100.					
2281 CINCHONA BARK, RED--*Cinchona succirubra* Pav. or its hybrids	9	1.5 3.7	(1)3.0	(1)3.0	27.			*Alcoholic Beverages* 20. 300.	*Bitters* (1)1,000.	
2282 CINCHONA BARK, RED, EXTRACT--*Cinchona succirubra* Pav. or its hybrids	3	(1)100.	(1)25.	-	(1)20.			*Condiments* (1)60.		
2283 *CINCHONA BARK, YELLOW--*Cinchona ledgeriana* Moens, *C. calisaya* Wedd., or hybrids of these with other spp. of *Cinchona*	4	-	-	-	-			*Alcoholic Beverages* (1)300.	*Bitters* (1)100.	
2284 CINCHONA BARK, YELLOW, EXTRACT--*Cinchona ledgeriana* Moens, *C. calisaya* Wedd., or hybrids of these with other spp. of *Cinchona*	4	(1)100.*	-	-	-					
2285 CINCHONA, EXTRACT [*Cinchona ledgeriana* Moens et Trimen; *C. succirubra* Pavon et Klotzsch or its hybrids; *C. calisaya* Wedd., or hybrids of these with other *Cinchona* spp.]--Quinine, extract	4	(1)10.	-	(1)1.0	-					
2286 CINNAMALDEHYDE--Cinnamic aldehyde, 3-Phenylpropenal; Phenylacrolein; Cinnamal	51	9.0	7.7	700.	180.		4,900.	*Condiments* (1)20.	*Meats* (1)60.	
2287 CINNAMALDEHYDE ETHYLENE GLYCOL ACETAL--Cinncloval	2	-	-	0.06 2.0	(1)2.0			*Condiments* (1)5.0		
2288 CINNAMIC ACID--β-Phenylacrylic acid, 3-Phenylpropenoic acid	10	31.	40.	30.	36.		(1)10.			
2289 *CINNAMON [*Cinnamomum zeylanicum* Nees, *C. loureiri* Blume, *C. cassia* Blume]--Ceylon cinnamon, Chinese cinnamon; Saigon cinnamon	43	5.6	53.	10. 4,000.	1,900.			*Apple Butter* 78. 450.	*Condiments* (1)110.	*Meats* 880.
2290 *CINNAMON BARK, EXTRACT--*Cinnamomum zeylanicum* Nees, *C. loureiri* Blume, *C. cassia* Blume	6	10. 13.	(1)8.5	-	(1)170.			*Condiments* 40. 200.	*Meats* (1)40.	

* Probably alcoholic beverages

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2291 *CINNAMON BARK, OIL--*Cinnamomum zeylanicum* Nees; *C. loureirii* Blume, *C. cassia* Blume	33	5.5	18.	80.	110.		620.	*Condiments* (1)25.	*Meats* (1)50.	
2292 *CINNAMON LEAF, OIL, [*Cinnamomum zeylanicum* Nees, *C. loureirii* Blume, *C. cassia* Blume]--Ceylon cinnamon leaf, oil; Chinese cinnamon leaf, oil; Saigon cinnamon leaf, oil	24	6.8	3.4	32.	54.	(1)0.20	160. 520.	*Condiments* 20 78.	*Pickles* 32. 48.	*Spiced Fruits* (1)3.0
2293 CINNAMYL ACETATE	32	2.7	6.5	16.	11.		(1)8.7	*Condiments* 2.0 2.0		
2294 CINNAMYL ALCOHOL--3-Phenyl-2-propen-1-ol; Cinnamic alcohol; Styryl carbinol; γ-Phenylallyl alcohol	19	8.8	8.7	17.	33.	(1)22.	720.	*Alcoholic Beverages* (1)5.0		
2295 CINNAMYL ANTHRANILATE	14	6.8	1.7	4.3	5.3	(1)28.	46. 730.			
2296 CINNAMYL BUTYRATE	11	1.6	8.5	7.6	11.	(1)1.2				
2297 CINNAMYL *iso*-BUTYRATE	16	1.5	5.0	7.7	8.5	0.02 1.2	(1)140.	*Toppings* (1)1.0		
2298 CINNAMYL CINNAMATE--Phenylallyl cinnamate; Styracin	4	0.81	1.5	10.	7.0					
2299 CINNAMYL FORMATE	10	1.3	9.1	6.9	8.0		(1)0.60			
2300 CINNAMYL PHENYLACETATE	5	2.7	0.25 2.0	7.3	7.3					
2301 CINNAMYL PROPIONATE--γ-Phenylallyl propionate, 3-Phenyl-2-propenyl propionate	22	1.0	4.3	7.5	8.8	2.4 4.0	20. 53.			
2302 CINNAMYL *iso*-VALERATE	21	2.2	2.6	4.1	3.6	11.	19. 30.			
2303 CITRAL--3,7-Dimethyl-2,6-octadienal; Geranial	78	9.2	23.	41.	43.		170			
2304 CITRAL DIETHYL ACETAL--3,7-Dimethyl-2,6-octadienal diethyl acetal	3	(1)0.03	-	(1)0.13	-			*Condiments* (1)110.		
2305 CITRAL DIMETHYL ACETAL--3,7-Dimethyl-2,6-octadienal dimethyl acetal	3	6.3	11.	60.	60.		(1)15.			
2306 CITRIC ACID--2-Hydroxy-1,2,3-propanetricarboxylic acid; β-Hydroxytricarballylic acid	89	2,500.	1,600.	4,300.	1,200.		3,600.			
2307 CITRONELLAL--3,7-Dimethyl-6-octenal; Rhodinal	16	4.0	1.3	4.5	4.7	(1)0.60	(1)0.30			
2308 *CITRONELLA, OIL--*Cymbopogon nardus* Rendle	6	17.	26.	25.	31.					
2309 *dl*-CITRONELLOL--3,7-Dimethyl-6-octen-1-ol (commercial Citronellol is largely *dl*-)	16	4.1	4.1	16.	18.	5.8	29. 52.			
2310 CITRONELLOXYACETALDEHYDE--6,10-Dimethyl-3-oxa-9-undecenal	3	0.005 1.0	1.4	4.1	4.3					
2311 CITRONELLYL ACETATE--3,7-Dimethyl-6-octen-1-yl acetate	13	3.4	4.2	7.5	9.7	0.71 3.7	6.9 600.			
2312 CITRONELLYL BUTYRATE--3,7-Dimethyl-6-octen-1-yl butyrate	13	3.8	11.	13.	11.	3.1 4.2	(1)2.3			
2313 CITRONELLYL *iso*-BUTYRATE--3,7-Dimethyl-6-octen-1-yl *iso*-butyrate	8	2.3	1.7	8.2	12.	(1)3.1				
2314 CITRONELLYL FORMATE--3,7-Dimethyl-6-octen-1-yl formate	7	14.	13.	19.	32.		63. 100.			
2315 CITRONELLYL PHENYLACETATE--3,7-Dimethyl-6-octen-1-yl phenylacetate	6	1.3	0.95	2.4	17.					

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2316 CITRONELLYL PROPIONATE--3,7-Dimethyl-6-octen-1-yl propionate	7	3.1	9.0	18.	19.		0.80 15.			
2317 CITRONELLYL VALERATE--3,7-Dimethyl-6-octen-1-yl valerate	4	1.0	2.5	3.0	7.7					
2318 *CITRUS PEELS, EXTRACT--*Citrus* spp.	10	190.	420.	480.	480.					
2319 *CIVET, ABSOLUTE--Civet cats: *Viverra civetta* Schreber and *V. zibetha* Schreber	10	1.0	3.0	3.7	2.8	(1)0.10	(1)2.2			
2320 *CLARY [*Salvia sclarea* L.]--Clary sage	2	-	-	-	-			*Alcoholic Beverages* (1)500.		
2321 *CLARY, OIL [*Salvia sclarea* L.]--Clary sage, oil	18	1.8	3.9	5.3	13.			*Alcoholic Beverages* (1)100.	*Condiments* (1)20.	
2322 *CLOVE BUD, EXTRACT--*Eugenia caryophyllata* Thunb. [*Eugenia aromatica* (L.) Baill.]	7	16.	19.	2.0 20.	48.			*Condiments* (1)150.	*Meats* 160. 250.	
2323 *CLOVE BUD, OIL--*Eugenia caryophyllata* Thunb. [*Eugenia aromatica* (L.) Baill.]	76	3.1	13.	320.	37.	0.33 5.0	1,800.	*Alcoholic Beverages* (1)300. *Meats* 75.	*Condiments* 55. *Spiced Fruits* 830.	*Jellies* (1)7.3
2324 *CLOVE BUD, OLEORESIN--*Eugenia caryophyllata* Thunb. [*Eugenia aromatica* (L.) Baill.]	5	-	- -	-	-			*Meats* 100.		
2325 *CLOVE LEAF, OIL--*Eugenia caryophyllata* Thunb. [*Eugenia aromatica* (L.) Baill.]	21	8.6	16.	22.	30.	(1)5.0		*Apple Butter* (1)2.0 *Pickles* 7.0 16.	*Condiments* 14. 40.	*Meats* 670.
2326 *CLOVER TOPS, RED, EXTRACT SOLID [*Trifolium pratense* L.]--Trifolium, extract solid	2	(1)2.0	(1)3.0	(1)20.	(1)9.0					
2327 *CLOVES--*Eugenia caryophyllata* Thunb. [*Eugenia aromatica* (L.) Baill.]	32	20. 1,000.	33.	-	1,200.			*Spiced Cherries* (1)500.	*Meats* 810.	
2328 *CLOVE STEM, OIL--*Eugenia caryophyllata* Thunb. [*Eugenia aromatica* (L.) Baill.]	11	5.9	4.0 7.0	91.	64.			*Condiments* 30. 70.		
2329 *COCA LEAF, EXTRACT (DECOCAINIZED)--*Erythroxylon coca* Lam.	7	200.	(1)540.	(1)400.	-					
2330 COCHINEAL--*Coccus cacti* L.	5	(1)100.	-	36.	(1)300.			*Condiments* (1)200.		
2331 *COGNAC, GREEN, OIL	44	5.2	8.2	12.	14.		56.	*Alcoholic Beverages* 390.	*Condiments* (1)1.0	
2332 *COGNAC, WHITE, OIL	19	5.6	14.	18.	24.	(1)0.10				
2333 *CORIANDER--*Coriandrum sativum* L.	20	7.4	(1)1.0	1.0 20.	880.			*Alcoholic Beverages* (1)1,000.	*Condiments* (1)54.	*Meats* 1,300.
2334 *CORIANDER, OIL--*Coriandrum sativum* L.	60	3.1	4.5	8.8	9.3		7.4	*Alcoholic Beverages* 10. 30.	*Condiments* 12.	*Meats* 47.
2335 *CORN SILK--*Zea mays* L.	4	16. 28.	5.5 10.	12. 18.	12. 21.					
2336 COSTUS ROOT, OIL [*Saussurea lappa* Clarke (*Aplotaxis lappa* Dec., *A. auriculata* DC., *Aucklandia costus* Falc.)]--Spiral flag, oil	7	0.08	0.90	1.9	1.2	(1)0.10				
2337 *p*-CRESOL--4-Cresol	3	0.67	0.01 1.0	0.01 2.0	0.01 2.0					
2338 CUBEBS--*Piper cubeba* L. f.	4	(1)800.	-	-	-				*Meats* 25.	
2339 CUBEBS, OIL--*Piper cubeba* L. f.	13	2.4	(1)0.25	1.8	4.6			*Condiments* 33.	30.	

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2340 *CUMIN--*Cuminum cyminum* L.	18	-	-	-	(1)2,500.			*Condiments* 300. 3,900.	*Meats* 1,000.	
2341 CUMINALDEHYDE--*p-iso*-Propyl benzaldehyde; Cuminal, Cuminic aldehyde; Cumaldehyde	16	3.1	3.2	4.0	4.0		0.40 0.50	*Condiments* (1)3.0		
2342 *CUMIN, BLACK--*Nigella sativa* L.	0	-	-	-	-					
2343 *CUMIN, OIL--*Cuminum cyminum* L.	20	0.48	0.66	7.3	10.		(1)0.20	*Condiments* 230.	*Meats* 40. 100.	*Pickles* (1)40.
2344 *CURACAO PEEL, EXTRACT--*Citrus aurantium* L.	3	1,700.	-	-	-					
2345 *CURACAO PEEL, OIL--*Citrus aurantium* L.	5	33.	0.80 20.	43.	4.0 100.					
2346 CURRANT BUDS, BLACK, ABSOLUTE [*Ribes nigrum* L.]--Cassis	4	4.7	(1)8.0	(1)20.	(1)20.	(1)5.0				
2347 CYCLOHEXANEACETIC ACID--Cyclonexylacetic acid	2	(1)1.0	(1)2.0	(1)2.0	(1)2.0					
2348 CYCLOHEXANEËTHYL ACETATE--Cyclohexylethyl acetate	1	(1)2.0	(1)3.0	(1)6.0	(1)26.					
2349 CYCLOHEXYL ACETATE	10	20.	115.	100.	110.					
2350 CYCLOHEXYL ANTHRANILATE	3	10.	3.7	3.7	2.0 10.	(1)1.0				
2351 CYCLOHEXYL BUTYRATE	14	3.9	5.7	9.2	23.	(1)0.54				
2352 CYCLOHEXYL CINNAMATE	4	2.0	1.0 5.0	4.0 10.	1.0 20.					
2353 CYCLOHEXYL FORMATE	4	11.	2.8	8.0	7.0 10.					
2354 CYCLOHEXYL PROPIONATE	3	2.4	2.7	2.0 3.0	(1)3.0	(1)5.0				
2355 CYCLOHEXYL *iso*-VALERATE	7	13.	7.0 25.	9.3	1.7 60.					
2356 *p*-CYMENE--Cymene; Cymol	9	3.3	5.3	10.	7.0		(1)250.	*Condiments* 10. 130.		
2357 *DANDELION, FLUID EXTRACT--*Taraxacum officinale* Weber, *T. erythrospermum* Andrz.	5	35.	6.0	2.0 8.0	53.					
2358 *DANDELION ROOT, EXTRACT SOLID--*Taraxacum officinale* Weber, *T. erythrospermum* Andrz.	5	10.	2.5 20.	8.0 40.	27.					
2359 DAVANA, OIL--*Artemisia pallens* Wall.	4	3.0	6.5	8.0	11.		(1)5.0			
2360 γ-DECALACTONE--4-Hydroxydecanoic acid, γ-lactone	10	2.0	4.5	5.7	7.1	0.08 8.0				
2361 δ-DECALACTONE--5-Hydroxydecanoic acid, δ-lactone	4	5.0 5.0	(1)10.	0.25 5.0	0.25 8.0			*Toppings* (1)5.0		
2362 DECANAL--Capraldehyde; Capric aldehyde; Caprinaldehyde; Aldehyde C-10	24	2.3	4.1	5.7	6.6	(1)3.0	0.60			
2363 DECANAL DIMETHYL ACETAL--1,1-Dimethoxydecane	3	1.0 2.0	(1)2.0	(1)8.0	(1)8.0	(1)3.0		*Alcoholic Beverages* (1)8.0		
2364 DECANOIC ACID--Capric acid; Decylic acid	14	9.9	3.1	4.5	7.8	0.10 3.0		*Shortening* (1)5.0		
2365 1-DECANOL--Decyl alcohol; Decylic alcohol; Nonylcarbinol; Alcohol C-10	16	2.1	4.6	5.2	5.2		(1)3.0			
2366 2-DECENAL--Decenaldehyde	5	3.4	6.0	9.0	9.0					
2367 DECYL ACETATE--Decanyl acetate; Acetate C-10	12	3.4	2.7	6.1	10.	(1)1.2	(1)12.			
2368 DECYL BUTYRATE	4	0.18	1.4	5.9	7.5					

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2369 DECYL PROPIONATE	4	0.81	1.4	5.9	7.5					
2370 DIACETYL--2,3-Butanedione; Biacetyl; Dimethyl ketone; Dimethylglyoxal; 2,3-Diketobutane	63	2.5	5.9	21.	44.	19.	35.	*Shortening* 11.		
2371 DIBENZYL ETHER	6	8.3	5.6	23.	25.		85. 160.			
2372 4,4-DIBUTYL-γ-BUTYROLACTONE--4,4-Dibutyl-4-hydroxybutyric acid, γ-lactone	2	-	2.8 3.5	4.4 15.	(1)15.					
2373 DIBUTYL SEBACATE--Butyl sebacate	2	1.0 5.0	2.0 5.0	(1)15.	(1)15.					
2374 DIETHYL MALATE--Ethyl malate	3	5.5	6.5	18.	44.	(1)1.5				
2375 DIETHYL MALONATE--Ethyl malonate, Malonic ester	15	5.6	17.	20.	19.	(1)20.				
2376 DIETHYL SEBACATE--Ethyl sebacate	22	4.1	9.1	21.	41.	3.2 19.	2.7 450.			
2377 DIETHYL SUCCINATE	11	7.3	11.	38.	45.					
2378 DIETHYL TARTRATE	1	(1)50.	(1)200.	(1)200.	(1)200.					
2379 DIHYDROCARVEOL--8-*p*-Menthen-2-ol; 6-Methyl-3-*iso*-propenylcyclohexanol	3	(1)84.	(1)300.	10. 250.	10. 250.			*Alcoholic Beverages* (1)500.		
2380 DIHYDROCARVYL ACETATE--8-*p*-Menthen-2-yl acetate, 6-Methyl-3-*iso*-propenylcyclohexyl acetate	4	2.0 5.0	(1)20.	22.	22.			*Condiments* (1)10.		
2381 DIHYDROCOUMARIN--Hydrocoumarin; 1,2-Benzodihydropyrone	26	7.8	21.	44.	28.	10.	78.			
2382 *DILL--*Anethum graveolens* L.	14	-	-	-	(1)4,800.			*Condiments* 1,400.	*Meats* 1,200.	*Pickles* 8,200.
2383 *DILL, OIL--*Anethum graveolens* L.	31	1.6	5.8	9.9	5.0	(1)20.	3.8 8.0	*Alcoholic Beverages* (1)5.0 *Pickles* 140.	*Condiments* 150.	*Meats* 51.
2384 DILL SEED, INDIAN--*Anethum sowa* Roxb. (*Peucedanum graveolens* Benth. et Hook.; *A. graveolens* L.)	9	-	-	-	(1)400.			*Condiments* (1)200.	*Meats* 3.3 100.	
2385 *m*-DIMETHOXYBENZENE--Resorcinol dimethyl ether; 1,3-Dimethoxybenzene; Dimethyl resorcinol	6	3.0	5.0	5.0	8.0					
2386 *p*-DIMETHOXYBENZENE--Hydroquinone dimethyl ether, Dimethyl hydroquinone	14	8.1	5.0	4.7	5.8			*Alcoholic Beverages* (1)1.0		
2387 2,4-DIMETHYLACETOPHENONE	6	0.78	0.77	3.9	2.7					
2388 α,α-DIMETHYLBENZYL *iso*-BUTYRATE--Phenyl dimethyl carbinyl *iso*-butyrate	3	(1)5.0	(1)40.	(1)30.	(1)20.					
2389 2,6-DIMETHYL-5-HEPTENAL--Melonal	11	2.8	1.7	8.4	19.	0.02 10.	(1)0.80			
2390 2,6-DIMETHYL OCTANAL--*iso*-Decylaldehyde	3	0.44	3.2	1.9	1.9					
2391 3,7-DIMETHYL-1-OCTANOL--Tetrahydrogeraniol	5	4.3	2.0 44.	15.	19.					
2392 α,α-DIMETHYLPHENETHYL ACETATE--Benzyl dimethyl carbinyl acetate, Benzylpropyl acetate	5	2.8	8.0	22.	19.		(1)2.9			
2393 α,α-DIMETHYLPHENETHYL ALCOHOL--Dimethyl benzyl carbinol, Benzylpropyl alcohol	8	3.3	3.2	4.0	4.9	(1)0.01	(1)100.	*Jellies* (1)3.2		
2394 α,α-DIMETHYLPHENETHYL BUTYRATE--Benzyl dimethyl carbinyl butyrate	1	(1)10.	(1)20.	(1)20.	-	(1)20.				

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category, Uses —		
2395 α,α-DIMETHYLPHENETHYL FORMATE--Benzyl dimethyl carbinyl formate	1	(1)2.0	(1)10.	(1)10.	-					
2396 DIMETHYL SUCCINATE	3	1.0 100.	(1)5.0	(1)15.	(1)15.		(1)5.0			
2397 1,3-DIPHENYL-2-PROPANONE--Dibenzyl ketone	4	1.7	4.5	9.5	13.					
2398 DISODIUM PHOSPHATE	18	350.	350.	-	(1)2,300.			*Meats* 50. 250.	*Toppings* 2,500.	
2399 DITTANY OF CRETE [*Origanum dictamnus* L.]--Cretan dittany; Spanish hops	4	(1)25.	-	-	(1)8.8					
2400 γ-DODECALACTONE--4-Hydroxydodecanoic acid, γ-lactone; γ-Octyl-γ-butyrolactone	7	3.3	4.3	13.	11.	0.15		*Jellies* (1)0.01		
2401 δ-DODECALACTONE--5-Hydroxydodecanoic acid, δ-lactone	2	-	-	(1)0.06	(1)0.06			*Toppings* (1)10.		
2402 2-DODECENAL	5	2.9	3.1	2.8	2.8					
2403 *DOGGRASS, EXTRACT [*Agropyron repens* (L.) Beauv.]--Agropyrum, Quack grass; Quick grass; Couch grass; Triticum, Graminis	2	(1)2.0	(1)4.0	(1)6.0	(1)6.0					
2404 DRAGON'S BLOOD, EXTRACT [*Dracomorops* spp. or other botanical sources]--Draco rubin, extract	2	(1)300.	-	-	-					
2405 *DULSE--*Rhodymenia palmata* (L.) Grev.	1	-	-	-	-					
2406 *ELDER FLOWERS--*Sambucus canadensis* L. or *S. nigra* L.	5	340.	(1)1.0	(1)1.0	(1)1.0			*Alcoholic Beverages* (1)25.		
2407 ELEMI, GUM--*Canarium commune* L. or *C. luzonicum* (Miq.) A. Gray	4	(1)0.13	(1)0.25	(1)0.25	(1)0.25					
2408 ELEMI, OIL--*Canarium commune* L. or *C. luzonicum* (Miq.) A. Gray	5	0.71	0.25 5.0	0.25 15.	7.5			*Soups* (1)10.		
2409 ERIGERON, OIL [*Erigeron canadensis* L.]--Fleabane, oil	5	0.13 4.8	0.25 3.5	0.25 30.	0.25 1.0			*Condiments* (1)2.0		
2410 ERYTHORBIC ACID--Araboascorbic acid; iso-Ascorbic acid	15	100.	(1)20.	-	(1)500.			*Meats* 380.		
2411 ESTRAGOLE--*p*-Allylanisole; Methyl chavicol; Chavicyl methyl ether, Esdragol; *p*-Methoxyallylbenzene	10	10.	11.	36.	41.		(1)150.	*Condiments* (1)2.0		
2412 *ESTRAGON, OIL [*Artemisia dracunculus* L.]--Tarragon, oil	18	0.79	0.50 0.70	0.85	17.			*Alcoholic Beverages* 1.0 3.0	*Condiments* 26.	*Meats* (1)40.
2413 *p*-ETHOXYBENZALDEHYDE	2	0.06 0.08	0.36 0.50	1.0 1.0	1.0 1.0					
2414 ETHYL ACETATE--Acetic ether; Vinegar naphtha	81	67.	99.	170.	170.	200.	1,400.	*Alcoholic Beverages* 50. 65.		
2415 ETHYL ACETOACETATE--Acetoacetic ester; Ethyl 3-oxobutanoate	21	17.	24.	110.	120.	93.	530.			
2416 ETHYL 2-ACETYL-3-PHENYLPROPIONATE--Ethyl benzyl acetoacetate, Ethyl α-acetylhydrocinnamate	2	0.10 5.0	(1)2.0	(1)7.0	-					
2417 ETHYL ACONITATE (Mixed esters)--Ethyl 1-propene 1,2,3-tricarboxylate	5	3.6	12.	55.	66.	(1)2.5				
2418 ETHYL ACRYLATE	5	0.13 0.26	0.06 1.0	1.1	1.1		0.10 0.10			

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
2419 ETHYL ALCOHOL--Ethanol	92	1,700.	2,000.	2,200.	1,800.	(1)230.	1,400.	*Maple Syrup* (1)610. *Alcoholic Beverages* 250. 450,000.	*Condiments* (1)1,000.	*Dairy Products* (1)30.
2420 ETHYL *p*-ANISATE	10	2.6	0.96	8.8	7.2					
2421 ETHYL ANTHRANILATE	21	5.9	7.6	19.	23.	14.	(1)79.	*Alcoholic Beverages* (1)0.50		
2422 ETHYL BENZOATE--Ethyl benzenecarboxylate	31	2.8	2.8	9.0	10.	(1)0.06	59.			
2423 ETHYL BENZOYLACETATE	1	(1)0.70	(1)5.0	(1)10.	(1)10.					
2424 α-ETHYL BENZYL BUTYRATE--α-Phenylpropyl butyrate	3	0.13 1.0	0.12 0.20	1.0	(1)0.14					
2425 2-ETHYL BUTYL ACETATE	2	(1)5.0	(1)2.0	0.03 7.0	-					
2426 2-ETHYLBUTYRALDEHYDE	2	(1)10.	(1)40.	0.12 25.	0.20 20.					
2427 ETHYL BUTYRATE	77	28.	44.	98.	93.	54.	1,400.			
2428 ETHYL *iso*-BUTYRATE	8	10.	25.	73.	(1)200.	(1)6.0		*Toppings* (1)1.5		
2429 2-ETHYLBUTYRIC ACID--α-Ethylbutyric acid	2	(1)5.0	(1)20.	20. 35.	(1)20.					
2430 ETHYL CINNAMATE--Ethyl 3-phenylpropenoate, Ethyl phenylacrylate	27	4.1	8.8	9.5	12.	2.4	11. 40.			
2431 ETHYL CYCLOHEXANEPROPIONATE--Ethyl cyclohexylpropionate	2	(1)9.0	-	0.03 30.	(1)24.			*Alcoholic Beverages* 3.0 10.		
2432 ETHYL DECANOATE	12	2.1	4.5	8.3	23.	5.3		*Ground Spices* 21.	*Whole Spices* 15. 45.	
2433 ETHYLENE OXIDE[1]--Epoxyethane	6	-	-	-	-					
2434 ETHYL FORMATE--Formic ether	49	9.4	21.	50.	98.	0.35 11.	430.	*Alcoholic Beverages* (1)10.		
2435 ETHYL 2-FURANPROPIONATE--Ethyl furylpropionate, Ethyl furfurylhydracrylate	8	1.6	1.6	5.6	7.5					
2436 4-ETHYLGUAIACOL--4-Ethyl-2-methoxyphenol	2	(1)0.05	(1)1.1	-	-	(1)0.23		*Alcoholic Beverages* 8.5 20.		
2437 ETHYL HEPTANOATE	39	6.8	7.5	17.	24.	0.06 350.	340.			
2438 2-ETHYL-2-HEPTENAL--2-Ethyl-3-butylacrolein	2	(1)0.40	-	0.03 2.0	-					
2439 ETHYL HEXANOATE--Caproic ether absolute	26	7.0	18.	12.	12.	(1)10.	32.	*Jellies* (1)1.3		
2440 ETHYL LACTATE--Ethyl α-hydroxypropionate	25	5.4	17.	28.	71.	8.3	580. 3,100.	*Alcoholic Beverages* (1)1,000.	*Syrups* (1)35.	
2441 ETHYL LAURATE--Ethyl dodecanoate	15	1.7	3.7	17.	17.	4.4	(1)39.	*Alcoholic Beverages* (1)3.0		
2442 ETHYL LEVULINATE	6	5.8	11.	12.	12.					
2443 ETHYL 2-METHYLBUTYRATE	1	(1)0.50	(1)3.0	(1)5.0	-					
2444 ETHYL METHYL PHENYLGLYCIDATE--Ethyl 3-methyl-3-phenylglycidate, Strawberry aldehyde, Aldehyde C-16 Pure (so-called)	58	5.6	6.7	21.	18.	13.	470.	*Alcoholic Beverages* (1)30.		
2445 ETHYL MYRISTATE--Ethyl tetradecanoate	7	6.7	8.0	10.	14.					

1 As a fumigant for whole and ground spices, provided that residues of ethylene oxide do not exceed 50 p.p.m.

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2446 ETHYL NITRITE--Spirit of nitrous ether; Sweet spirit of nitre	11	3.0	4.5	0.10 8.0	(1)0.10		(1)3.9	*Icings* (1)13	*Syrups* (1)52.	
2447 ETHYL NONANOATE	28	3.9	4.0	14.	15.	(1)15.	(1)580.	*Alcoholic Beverages* 20.	*Icings* (1)39.	
2448 ETHYL 2-NONYNOATE--Ethyl octyne carbonate	5	0.56	0.55	0.52	1.2					
2449 ETHYL OCTANOATE	16	4.1	2.4	9.0	11.	0.10 2.7	4.0 60.			
2450 ETHYL OLEATE	2	(1)0.10	(1)0.10	0.10 40.	0.10 55.	(1)0.10				
2451 ETHYL PALMITATE--Ethyl hexadecanoate	1	-	(1)20.	(1)40.	(1)20.					
2452 ETHYL PHENYLACETATE--α-Toluic acid, ethyl ester	14	2.4	5.2	8.1	6.0			*Syrups* (1)24.		
2453 ETHYL 4-PHENYLBUTYRATE	2	0.06 1.0	-	(1)0.06	-					
2454 ETHYL 3-PHENYLGLYCIDATE	17	4.6	12.	18.	20.	10. 70.				
2455 ETHYL 3-PHENYLPROPIONATE--Ethyl hydrocinnamate	5	1.8	1.0	2.5	0.50 3.0					
2456 ETHYL PROPIONATE	33	7.7	29.	78.	110.	10. 15.	1,100.			
2457 ETHYL PYRUVATE	4	(1)50.	20. 150.	35.	40.					
2458 ETHYL SALICYLATE--Salicylic ether; Sal ethyl	15	2.8	11.	10.	16.	(1)0.04	(1)16.			
2459 ETHYL SORBATE--Ethyl 2,4-hexadienoate	5	5.5	14.	15.	18.					
2460 ETHYL TIGLATE--Ethyl *trans*-2-methyl-2-butenoate	8	5.3	6.0	20.	6.5					
2461 ETHYL 10-UNDECENOATE	5	1.7	8.7	10.	11.			*Alcoholic Beverages* (1)5.0		
2462 ETHYL VALERATE	24	4.2	4.4	15.	8.3	5.5	200.			
2463 ETHYL *iso*-VALERATE--Ethyl β-methylbutyrate	33	4.9	7.5	29.	27.	5.0	80. 430.	*Condiments* (1)1.0		
2464 ETHYL VANILLIN--3-Ethoxy-4-hydroxybenzaldehyde; Ethovan; Vanillal	110	20.	47.	65.	63.	74.	110.	*Alcoholic Beverages* (1)100. *Chocolate* 250.	*Imitation Vanilla Extract* 28,000.	*Icings & Toppings* 140 200.
2465 EUCALYPTOL--1,8-Epoxy-*p*-menthane, Cineole; Cajeputol	9	(1)0.13	(1)0.50	15.	0.50 4.0		190.			
2466 EUCALYPTUS, OIL--*Eucalyptus globulus* Labille	14	1.7	0.50 50.	130.	76.			*Alcoholic Beverages* (1)1.0		
2467 EUGENOL--4-Allyl-2-methoxyphenol; 4-Allylguaiacol; 4-Hydroxy-3-methoxy-1-allylbenzene	36	1.4	3.1	32.	33.	(1)0.60	500.	*Condiments* 9.6 100.	*Meats* 40. 2,000.	
2468 *iso*-EUGENOL--2-Methoxy-4-propenylphenol; 4-Propenylguaiacol	15	3.7	3.8	5.0	11.		0.30 1,000.	*Condiments* (1)1.0		
2469 EUGENYL ACETATE--Acetyl eugenol	12	0.43	3.3	20.	10.		25. 100.	*Condiments* 2.0 2.0		
2470 *iso*-EUGENYL ACETATE--2-Methoxy-4-propenylphenyl acetate, Acetyl *iso*-eugenol	6	0.44	2.1	17.	17.		(1)100.			
2471 EUGENYL BENZOATE--4-Allyl-2-methoxyphenyl benzoate, Benzoyl eugenol	2	0.03 0.13	0.25 2.0	0.25 10.	0.13 10.					
2472 *iso*-EUGENYL ETHYL ETHER--2-Ethoxy-5-propenylanisole; Ethyl *iso*-eugenol	2	(1)7.8	(1)0.50	(1)17.	1.0 3.5					
2473 EUGENYL FORMATE--4-Allyl-2-methoxyphenyl formate	1	-	-	-	-			*Condiments* (1)0.20		

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2474 *iso*-EUGENYL FORMATE--2-Methoxy-4-propenylphenyl formate	1	-	-	-	-			*Condiments* (1)0.20		
2475 EUGENYL METHYL ETHER--4-Allylveratrole; Methyl eugenol, 1,2-Dimethoxy-4-allylbenzene	8	10.	4.8	11.	13.			*Jellies* (1)52.		
2476 *iso*-EUGENYL METHYL ETHER--4-Propenylveratrole, Methyl *iso*-eugenol	10	4.0	7.7	13.	18.	(1)0.10	(1)110.			
2477 *iso* EUGENYL PHENYLACETATE--2-Methoxy-4-propenylphenyl phenylacetate	2	(1)0.05	(1)0.20	(1)3.0	2.0 3.0					
2478 FARNESOL--3,7,11-Trimethyl-2,6-10-dodecatrien-1-ol	7	0.76	0.40	1.4	1.7	(1)0.10				
2479 *d*-FENCHONE--*d*-1,3,3-Trimethyl-2-norbornanone	4	0.13 0.80	(1)0.25	0.25 30.	(1)0.25			*Alcoholic Beverages* (1)5.0		
2480 FENCHYL ALCOHOL--1,3,3-Trimethyl 2-norbornanol, 2-Fenchanol; Fenchol	3	1.8	(1)0.25	4.7	(1)0.25					
2481 *FENNEL, COMMON--*Foeniculum vulgare* Mill.	9	(1)800.	-	-	300. 6,500.			*Condiments* (1)50.	*Meats* 2,400.	
2482 *FENNEL, SWEET [*Foeniculum vulgare* Mill. var. *dulce* (DC.) Alef.]--Finochin; Florence fennel	4	(1)11.	(1)44.	(1)33.	(1)33.			*Meats* 40. 300.		
2483 *FENNEL, SWEET, OIL--*Foeniculum vulgare* Mill. var. *dulce* (DC.) Alef.	31	3.9	0.38	22.	19.	0.10 10.		*Alcoholic Beverages* 10. 20.	*Condiments* (1)2.0	*Meats* 40. 100.
2484 *FENUGREEK--*Trigonella foenum graecum* L.	13	(1)470.	15. 560.	600.	570.			*Condiments* 420. 800.	*Meats* (1)250.	*Syrups* (1)450.
2485 *FENUGREEK, EXTRACT--*Trigonella foenum-graecum* L.	51	50.	85.	280.	99.	30.	(1)7.6	*Alcoholic Beverages* (1)20. *Pickles* (1)90.	*Condiments* (1)150. *Syrups* 170.	*Icings* 37. *Meats* 40. 60.
2486 *FENUGREEK, OLEORESIN--*Trigonella foenum-graecum* L.	18	290.	72.	90.	72.	(1)500.		*Syrups* 300.		
2487 FORMIC ACID	2	(1)1.0	(1)5.0	5.0 18.	5.0 6.1					
2488 FUMARIC ACID--*trans*-Butenedioic acid; *trans*-1,2-Ethylenedicarboxylic acid; Allomaleic acid; Boletic acid	3	(1)50.	-	-	(1)1,300.	(1)3,600.				
2489 FURFURAL--2-Furaldehyde; Pyromucic aldehyde	16	4.0	13.	12.	17.	(1)0.80	45.	*Alcoholic Beverages* (1)10.	*Syrups* (1)30.	
2490 FURFURYL ACETATE	4	11.	17.	37.	1.0 40.		(1)500.			
2491 FURFURYL ALCOHOL--2-Furancarbinol; α-Furylcarbinol; 2-Furylcarbinol; 2-Hydroxymethylfuran; Furfuralcohol	5	19.	88.	59.	110.			*Alcoholic Beverages* (1)10.		
2492 2-FURFURYLIDENE BUTYRALDEHYDE--2-Ethyl-3-furylacrolein	2	(1)0.50	0.50 3.0	2.0 6.0	2.0 6.0					
2493 FURFURYL MERCAPTAN--2-Furanmethanethiol	18	0.52	0.78	2.0	2.1	(1)0.10		*Icings* (1)0.50		
2494 FURYL ACROLEIN--2-Furanacrolein	6	(1)0.17	6.1	36.	21.	0.10 0.56				
2495 4-(2-FURYL)-3-BUTEN-2-ONE- Furfurylidene acetone, Furfuralacetone	4	4.2	4.8	33.	46.	(1)1.6				
2496 (2-FURYL)-2-PROPANONE--Furyl acetone	3	-	(1)5.0	3.8 20.	2.0 20.					
2497 FUSEL, OIL, REFINED [a] --Amyl alcohol, commercial	20	21.	4.1	30.	34.	(1)4.0	(1)270.	*Alcoholic Beverages* (1)2.5		
2498 *GALANGAL ROOT--*Alpinia officinarum* Hance; *A. galanga* Willd.	4	750.	-	-	-					

[a] Assuming that refined fusel oil is mixed amyl alcohols, predominantly 3-Methyl-1-butanol

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
2499 *GALANGAL ROOT, EXTRACT- *Alpinia officinarum* Hance; *A. galanga* Willd.	5	0.10 350.	(1)0.30	(1)0.20	(1)0.10			*Alcoholic Beverages* (1)350.	*Bitters* (1)80.	
2500 *GALANGAL ROOT, OIL--*Alpinia officinarum* Hance; *A. galanga* Willd.	6	0.27	0.40	1.5	2.3			*Alcoholic Beverages* (1)1.0	*Condiments* (1)2.0	
2501 GALBANUM, OIL--*Ferula galbaniflua* Boiss. et Buhse and other *Ferula* spp.	5	0.16	0.84	1.7	1.8					
2502 GALBANUM, RESIN--*Ferula galbaniflua* Boiss. et Buhse and other *Ferula* spp.	5	0.04 0.25	0.05 0.50	1.7	2.4			*Condiments* (1)50.		
2503 *GARLIC, OIL--*Allium sativum* L.	20	0.01 0.30	(1)0.04	2.9	6.0		(1)12.	*Condiments* 16.		
2504 GENET, ABSOLUTE--*Spartium junceum* L.	5	0.83	0.50 1.0	1.7	1.0 2.0		(1)12.			
2505 GENET, EXTRACT [*Spartium junceum* L.]--Broom, extract	4	1.4	-	-	-					
2506 GENTIAN ROOT, EXTRACT--*Gentiana lutea* L.	22	26.	47.	120.	160.			*Alcoholic Beverages* 13.		
2507 GERANIOL--*trans*-3,7-Dimethyl-2,6-octadien-1-ol	36	2.1	3.3	10.	11.	2.0 2.0	0.80 2.9	*Toppings* (1)1.0		
2508 *GERANIUM, ROSE, OIL--*Pelargonium graveolens* L'Her.	36	1.6	2.8	6.9	8.1	1.1 2.0	(1)210.	*Jellies* (1)5.2		
2509 GERANYL ACETATE	37	1.6	6.5	15.	17.	6.8 7.5	0.30 1.2	*Syrups* (1)1.0		
2510 GERANYL ACETOACETATE	2	0.50 0.50	1.0 1.0	1.0 3.0	1.0 10.					
2511 GERANYL BENZOATE	3	0.10 0.13	0.16 0.25	(1)0.50	(1)0.50					
2512 GERANYL BUTYRATE	17	1.6	2.8	10.	10.	5.3	0.30 1.5			
2513 GERANYL *iso*-BUTYRATE	8	1.0	0.80	5.0	4.9	(1)0.60	(1)15.			
2514 GERANYL FORMATE	17	1.9	1.6	7.5	4.1	3.4	(1)0.80			
2515 GERANYL HEXANOATE	8	1.3	0.90	3.2	(1)2.0					
2516 GERANYL PHENYLACETATE	10	1.1	3.1	6.7	4.7		(1)11.			
2517 GERANYL PROPIONATE	12	1.5	1.3	3.7	4.9	(1)3.0	30. 70.			
2518 GERANYL *iso*-VALERATE	9	4.2	11.	10.	6.8					
2519 *GHATTI, GUM [*Anogeissus latifolia* Wall.]--Indian gum	4	(1)2,100.	(1)800.	-	-					
[illegible]	40	44.	220.	-	2,500.			*Meats* 1,500.		
[illegible]	32	65.	43.	83.	100.			*Condiments* (1)15.	*Meats* 56.	
[illegible]	12	17.	20.	14.	47.			*Condiments* 13.	*Meats* 12.	
[illegible]		79.	36. 65.	27.	52.			*Condiments* 10. 1,000.	*Meats* 30 250.	
[illegible]		(1)100.	-	-	(1)1,500.					
[illegible]		570.	500.	980.	1,300.	(1)360.	17. 6,000.	*Extracts* (1)100,000.	*Meats* (1)40.	*Toppings* (1)25,000
[illegible]		(1)15.	-	(1)8.0	30. 2,000.			*Shortening* (1)600.		
[illegible] FORMATE 4-Allyl-2-methoxyphenyl formate		-	2,000.	(1)100.	1,100.		(1)1,600.			

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2528 GLYCYRRHIZIN, AMMONIATED--*Glycyrrhiza glabra* L. and other spp. of *Glycyrrhiza*	15	51.	-	5.0 62.	(1)5.0					
2529 *GRAINS OF PARADISE--*Aframomum melegueta* (Rosc.) K. Schum	6	43.	(1)120.	(1)120.	-					
2530 *GRAPEFRUIT, OIL--*Citrus paradisi* Macf.	53	160.	180.	630.	370.	(1)250.	1,500.	*Toppings* (1)400.		
2531 GUAIAC GUM, EXTRACT--*Guaiacum officinale* L.; *G. sanctum* L.	2	(1)600.	-	-	-					
2532 GUAIACOL--o-Methoxyphenol; o-Hydroxyanisole; Methylcatechol	11	0.95	0.52	0.96	0.75					
2533 GUAIAC WOOD, EXTRACT--*Guaiacum officinale* L.; *G. sanctum* L.; *Bulnesia sarmienti* Lorentz	6	760.	(1)4.0	(1)8.0	(1)70.					
2534 GUAIAC WOOD, OIL--*Guaiacum officinale* L.; *G. sanctum* L.; *Bulnesia sarmienti* Lorentz	25	1.1	4.1	9.2	8.1	4.2	(1)60			
2535 GUAIACYL PHENYLACETATE	4	0.38	1.0	2.2	3.2			*Toppings* (1)1.0		
2536 GUARANA, GUM--*Paullinia cupana* HBK.	7	12.	-	(1)10.	-					
2537 GUAR, GUM [*Cyamopsis tetragonolobus* (L.) Taub.]--Guar flour, Gum cyamopsis, Jaguar, Regonol, Burtonite V-7-E	8	47. 100.	(1)5,000.	(1)10.	(1)2,900.			*Meats* (1)2,500.		
2538 HAW BARK, BLACK, EXTRACT--*Viburnum prunifolium* L.	5	5.0 6.0	(1)2.5	(1)6.0	(1)6.0					
3034 HEMLOCK, OIL (See SPRUCE, OIL)-- *Tsuga canadensis* (L.) Carr., *T. heterophylla* (Raf.) Sarg.; *Picea mariana* (Mill.); *P. glauca* (Moench) Voss.										
2539 γ-HEPTALACTONE--4-Hydroxyheptanoic acid, γ-lactone	4	18.	40.	28.	26.					
2540 HEPTANAL--Enanthaldehyde, Heptaldehyde; Heptyl aldehyde, Enanthal, Aldehyde C-7	10	4.9	1.2	2.0	2.6			*Alcoholic Beverages* (1)4.0		
2541 HEPTANAL DIMETHYL ACETAL	3	0.10 0.13	(1)0.25	(1)0.25	(1)0.25			*Condiments* (1)1.0		
2542 HEPTANAL GLYCERYL ACETAL (Mixed 1,2 and 1,3 acetals)	2	(1)5.0	(1)10.	(1)10.	(1)10.			*Condiments* (1)100.		
2543 2,3-HEPTANEDIONE--Acetyl valeryl; Acetyl pentanoyl	14	0.96	3.1	8.2	7.9		(1)1.7			
2544 2-HEPTANONE--Methyl amyl ketone; Ketone C-7	11	2.7	6.0	6.4	13.			*Condiments* 10. 25.		
2545 3-HEPTANONE--Ethyl butyl ketone	3	0.13 2.0	0.25 170.	67.	0.25 130.					
2546 4-HEPTANONE--Dipropyl ketone; Butyrone	8	7.8	11.	19.	27.	0.60 8.0				
2547 HEPTYL ACETATE	6	4.1	3.3	4.9	4.8					
2548 HEPTYL ALCOHOL--1-Heptanol, Enanthyl alcohol; *pri*-Heptyl alcohol, Hydroxyheptane, Enanthic alcohol; Alcohol C-7	3	0.90	1.0 5.0	3.0	3.0					
2549 HEPTYL BUTYRATE	6	0.66	0.74	2.7	2.4					
2550 HEPTYL *iso*-BUTYRATE	5	1.2	0.82	2.6	3.0					
2551 HEPTYL CINNAMATE	4	3.3	1.0 2.0	1.0 6.0	(1)1.0		(1)270.			
2552 HEPTYL FORMATE	5	1.7	0.87	3.6	3.3					

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Usep—		
2553 HEPTYL OCTANOATE	1	(1)1.0	-	-	-					
2554 1-HEXADECANOL--Cetyl alcohol, Alcohol C-16	1	-	(1)2.0	(1)2.0	-					
2555 ω-6-HEXADECENLACTONE--16-Hydroxy-6 hexadecenoic acid,ω-lactone, 6-Hexadecenolide, Ambretolide	6	0.32	0.18	0.16	0.19	(1)0.007	(1)0.70			
2556 γ-HEXALACTONE--4-Hydroxyhexanoic acid, γ-lactone, Ethyl butyrolactone, Tonkalide	3	7.0	0.07 84.	21.	21.					
2557 HEXANAL--Hexaldehyde, Hexoic aldehyde, Caproic aldehyde; Caproaldehyde	10	1.3	2.8	3.6	4.2	2.0 2.5	(1)3.0			
2558 2,3-HEXANEDIONE--Acetyl butyryl	8	6.6	4.8	7.3	6.6					
2559 HEXANOIC ACID--Caproic acid, Hexoic acid	30	1.8	4.3	28.	22.		(1)1.5	Condiments (1)150.		
2560 2-HEXENAL	8	3.1	0.70	15.	16.					
2561 cis-3-HEXENAL	1	(1)0.20	(1)5.0	(1)5.0	-					
2562 2-HEXEN-1-OL	7	1.0	0.63	3.8	4.1					
2563 3-HEXEN-1-OL--Leaf alcohol, Blatteralkohol	6	1.0	3.7	5.0	5.0					
2564 2-HEXEN-1-YL ACETATE	4	0.28	0.40	1.7	1.7					
2565 HEXYL ACETATE	10	4.6	4.6	26.	26.		(1)3.0			
2566 2-HEXYL-4-ACETOXYTETRAHYDROFURAN	1	(1)1.0	(1)3.0	(1)3.0	(1)3.0					
2567 HEXYL ALCOHOL--1-Hexanol	10	6.6	26.	21.	18.	0.22 0.28				
2568 HEXYL BUTYRATE	6	2.6	2.1	7.8	8.6					
2569 α-HEXYLCINNAMALDEHYDE	5	0.80	2.6	6.5	2.4	(1)0.05				
2570 HEXYL FORMATE	5	12.	45	39.	52.			Condiments (1)0.20		
2571 HEXYL 2-FUROATE	2	-	-	(1)0.31	-					
2572 HEXYL HEXANOATE	3	2.5 3.0	(1)2.5	3.6 10.	(1)10.					
2573 2-HEXYLIDENE CYCLOPENTANONE	1	(1)1.0	(1)5.0	(1)10	-					
2574 2-HEXYL-5 or 6-KETO-1,4-DIOXANE--(1 or 2-Hexyl-2-hydroxyethoxy)acetic acid, δ-lactone	1	-	(1)5.0	(1)5.0	(1)5.0			Margarine (1)5.0		
2575 HEXYL OCTANOATE	2	(1)1.0	-	-	-	(1)0.70				
2576 HEXYL PROPIONATE	5	5.7	23.	21.	22.			Alcoholic Beverages (1)70.	Condiments 65.	
2577 *HICKORY BARK, EXTRACT--*Carya* spp.	5	21. 40.	0.01 25.	(1)48.	(1)48.					
2578 *HOPS, EXTRACT--*Humulus lupulus* L.	5	100.	-	-	-					
2579 *HOPS, EXTRACT SOLID--*Humulus lupulus* L.	4	8.0	20. 75.	0.70 50.	0.80 40.			Condiments 20		
2580 *HOPS, OIL--*Humulus lupulus* L.	13	1.7	1.7	2.5	2.9		(1)2.2	35.		
2581 *HOREHOUND (HOARHOUND), EXTRACT--*Marrubium vulgare* L.	10	8.7	(1)2.0	680.	(1)2.0					
2582 *HORSEMINT LEAVES, EXTRACT--*Monarda* spp.	2	(1)600.	-	-	-					

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2583 HYDROXYCITRONELLAL--3,7-Dimethyl-7-hydroxy-octanal; Laurine[R]	17	3.5	13.	9.4	10	(1)0.30	16.			
2584 HYDROXYCITRONELLAL DIETHYL ACETAL	4	2.7	0.50 1.0	7.3	2.2					
2585 HYDROXYCITRONELLAL DIMETHYL ACETAL	4	10.	(1)0.50	24.	0.50 20.					
2586 HYDROXYCITRONELLOL--3,7-Dimethyl-1,7-octanediol	9	2.0	1.6	3.6	3.5	(1)0.30	(1)0.30			
2587 5-HYDROXY-4-OCTANONE--Butyroin	6	0.50 5.0	1.0 20.	10.	7.8					
2588 4-(p-HYDROXYPHENYL)-2-BUTANONE--p-Hydroxy-benzyl acetone	9	16.	34.	44.	54.	5.0 50.	40. 320.			
2589 *HYSSOP--*Hyssopus officinalis* L.	2	-	-	-	-			*Bitters* (1)600.		
2590 *HYSSOP, EXTRACT--*Hyssopus officinalis* L.	2	(1)13.	(1)13.	-	-			*Alcoholic Beverages* (1)50.		
2591 *HYSSOP, OIL--*Hyssopus officinalis* L.	6	4.7	(1)0.25	14.	0.25 33.			*Alcoholic Beverages* 5.0 50.		
2592 *IMMORTELLE, EXTRACT--*Helichrysum angustifolium* DC.	7	5.2	16.	11.	15.	(1)0.01	(1)0.50			
2593 INDOLE--2,3-Benzopyrrole	18	0.26	0.28	0.50	0.58	0.02 0.40				
2594 α-IONONE--4-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-3-buten-2-one; α-Irisone[R]	33	2.5	3.6	12.	6.7	3.6	39.	*Icings* (1)50.		
2595 β-IONONE--4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-one, β-Irisone[R]	35	1.6	3.4	7.6	5.2	5.8	89.	*Maraschino Cherries* (1)10.		
2596 *IRISH MOSS, EXTRACT [*Chondrus crispus* (L.) Stackh. or *Gigartina mamillosa* (Gooden. et Woodw.) J. Ag.]--Carrageen, extract; Chondrus, extract	24	300.	390.	3.0 500.	1,300.	1,700. 20,000.		*Jellies* (1)200.	*Syrups* 1,300.	
2597 α-IRONE--4-(2,5,6,6-Tetramethyl-2-cyclohexen-1-yl)-3-buten-2-one, 6-Methylionone	13	1.2	2.3	4.1	5.4		(1)1.4			
2598 *JASMINE, ABSOLUTE--*Jasminum grandiflorum* L.	15	0.41	1.3	0.80	2.9	0.10 0.50	(1)30.			
2599 *JASMINE, CONCRETE--*Jasminum grandiflorum* L.	5	0.70	1.0 1.5	1.0 3.4	1.0 15.	(1)1.0				
2600 *JASMINE, OIL--*Jasminum grandiflorum* L.	13	0.63	1.6	3.0	9.3	0.50 1.0	(1)1.4	*Jellies* (1)0.25		
2601 *JASMINE, SPIRITUS--*Jasminum grandiflorum* L.	3	(1)1.0	(1)0.75	(1)3.0	(1)4.0	(1)1.0		*Maraschino Cherries* (1)10.		
2602 *JUNIPER BERRIES--*Juniperus communis* L.	3	-	-	-	-			*Condiments* (1)60.	*Alcoholic Beverages* 60. 2,000.	
2603 *JUNIPER, EXTRACT--*Juniperus communis* L.	4	53.	(1)5.0	(1)5.0	(1)5.0					
2604 *JUNIPER, OIL--*Juniperus communis* L.	25	32.	1.9	4.3	11.	(1)0.01	(1)0.10	*Alcoholic Beverages* 95.	*Meats* (1)20.	
2605 *KARAYA, GUM [*Sterculia urens* Roxb.]--Sterculia, Indian tragacanth, Kadaya; Kullo, Katilo, Mucara; Kuteemi	20	13.	1,300.	1.0 44.	36.			*Emulsions* 20. 18,000.	*Meats* (1)40.	*Toppings* (1)3,500.
2606 *KELP--Atlantic: *Laminaria digitata*, *L. saccharina*; Pacific: *Macrocystis pyrifera* (L.) C. Agardh	1	-	-	-	-					
2607 *KOLA NUT, EXTRACT--*Cola acuminata* Schott et Endl.	34	120.	220.	160.	150.					
2608 LABDANUM, ABSOLUTE--*Cistus* spp.	8	2.8	9.8	5.6	23.	(1)0.06	1.0 19.			
2609 LABDANUM, OIL [*Cistus* spp.]--Ambreine, oil	5	0.41	0.78	2.0	0.75					
2610 LABDANUM, OLEORESIN--*Cistus* spp.	6	2.7	(1)2.0	5.5	(1)4.0					

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2611 LACTIC ACID	25	34.	66.	130.	89.	14. 25.	(1)610.	*Pickles & Olives* 1,200. 24,000.	*Toppings* (1)300.	
2612 *LAUREL BERRIES--*Laurus nobilis* L.	2	(1)450.	-	-	-			*Spiced Vegetables* (1)5.0		
2613 *LAUREL LEAVES, EXTRACT--*Laurus nobilis* L.	1	-	-	-	-					
2614 LAURIC ACID--Dodecanoic acid; Laurosteanic acid; Dodecoic acid	1	(1)15.	(1)16.	(1)2.4	(1)39.	(1)25.				
2615 LAURIC ALDEHYDE--Dodecanal; Lauraldehyde; Aldehyde C-12 Lauric	21	0.93	1.5	2.4	2.8	(1)0.10	0.20 110.			
2616 LAURYL ACETATE--Dodecyl acetate; Dodecanyl acetate; Acetate C-12	9	2.3	1.7	4.6	5.6					
2617 LAURYL ALCOHOL--Dodecyl alcohol; 1-Dodecanol; Alcohol C-12	11	2.0	1.0	2.8	1.7		16. 27.	*Syrups* (1)7.0		
2618 *LAVANDIN, OIL--Hybrids between *Lavandula officinalis* Chaix and *L. latifolia* Vill.	5	5.5	12.	18.	18.		(1)0.30			
2619 *LAVENDER--*Lavandula officinalis* Chaix	1	(1)0.08	-	-	-					
2620 *LAVENDER, ABSOLUTE--*Lavandula officinalis* Chaix	2	0.20 7.5	(1)0.40	2.0 14.	2.0 6.3					
2621 *LAVENDER, CONCRETE--*Lavandula officinalis* Chaix	2	0.01 0.20	(1)0.08	0.03 0.25	(1)0.25					
2622 *LAVENDER, OIL--*Lavandula officinalis* Chaix	8	2.9	7.8	5.5	8.3		(1)220.			
2623 *LEMON, EXTRACT--*Citrus limon* (L.) Burm. f.	12	1,000.	540. 4,000.	400. 12,000.	8,900.			*Icings* (1)10,000.		
2624 *LEMON-GRASS, OIL--*Cymbopogon citratus* DC. and *C. flexuosus* Stapf	9	4.4	9.2	38.	38.	(1)290.	(1)220.			
2625 *LEMON, OIL--*Citrus limon* (L.) Burm. f.	120	230.	380.	1,100.	580.	340.	1,900.	*Breakfast Cereals* (1)140. *Meats* 25. 40.	*Condiments* 10. 80. *Syrups* (1)65.	*Icings* 65. 600.
2626 *LEMON, OIL, TERPENELESS [*Citrus limon* (L.) Burm. f.]--Cedro, oil	64	13.	25.	68.	50.	80.	110. 670.	*Toppings* (1)1,000.		
2627 LEVULINIC ACID--3-Acetylpropionic acid; 4-Oxovaleric acid	3	14.	14.	53.	53.	(1)4.0				
2628 *LICORICE, EXTRACT [*Glycyrrhiza glabra* L. and other spp. of *Glycyrrhiza*]--Glycyrrhiza, extract	28	33.	39.	130.	84.	(1)4.0	(1)29,000.	*Syrups* (1)50.		
2629 *LICORICE, EXTRACT POWDER--*Glycyrrhiza glabra* L.	7	110.	(1)200.	6,500.	(1)200.		22,000. 22,000.			
2630 *LICORICE ROOT [*Glycyrrhiza glabra* L.]--Glycyrrhiza	13	130.	-	(1)460.	(1)75.		(1)3,200.			
2631 *LIME, OIL--*Citrus aurantifolia* (Christman) Swingle	97	130.	160.	680.	370.	200.	3,100.	*Condiments* (1)20.		
2632 *LIME, OIL, TERPENELESS--*Citrus aurantifolia* (Christman) Swingle	42	15.	17.	37.	22.	26.	(1)0.10	*Syrups* (1)8.0		
2633 d-LIMONENE--d p-Mentha-1,8-diene; Cinene; Dipentene; Cajeputene; Kautschin	14	31.	68.	49.	120.	48. 400.	2,300.			
2634 LINALOE WOOD, OIL--*Bursera delpechiana* Poiss. and other *Bursera* spp.	10	4.3	3.8	16.	15.			*Alcoholic Beverages* (1)1.0		
2635 LINALOÖL--3,7-Dimethyl-1,6-octadien-3-ol; Linalol; Licareol	35	2.0	3.6	8.4	9.6	2.3	0.80 90.	*Meats* (1)40.		

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2636 LINALYL ACETATE--Bergamol	29	1.9	3.8	11.	8.9	3.8	13.			
2637 LINALYL ANTHRANILATE	8	1.8	0.72	4.7	0.20 8.0					
2638 LINALYL BENZOATE	5	0.31	0.42	1.2	1.6	(1)0.28				
2639 LINALYL BUTYRATE	13	1.2	4.3	2.2	13.	(1)0.09				
2640 LINALYL *iso*-BUTYRATE	12	1.7	2.8	4.9	13.					
2641 LINALYL CINNAMATE	6	0.57	0.59	2.0	2.1					
2642 LINALYL FORMATE	11	1.3	12.	3.8	13.		(1)1.2			
2643 LINALYL HEXANOATE	3	3.2	6.0	11.	(1)15.					
2644 LINALYL OCTANOATE	3	1.3	0.50 3.0	0.50	0.60 15.					
2645 LINALYL PROPIONATE	11	4.9	3.6	5.3	12.	(1)4.4				
2646 LINALYL *iso*-VALERATE	13	0.96	0.91	5.7	5.6	(1)0.10				
2647 *LINDEN FLOWERS--*Tilia glabra* Vent.	5	12. 2,000.	-	-	-					
2648 *LOCUST, GUM--*Ceratonia siliqua* L.	21	2,900.	1,200.	-	(1)150.			*Condiments* (1)980.	*Toppings* 1,500. 6,000.	
2649 LOVAGE [*Levisticum officinale* Koch]--Smallage; Smellage	11	4.0 30.	18.	28.	25.			*Syrups* (1)0.08		
2650 LOVAGE, EXTRACT [*Levisticum officinale* Koch]--Smellage, extract; Smallage, extract	20	8.8	18.	26.	24.			*Condiments* (1)40.	*Icings* (1)0.07	*Syrups* 66.
2651 LOVAGE, OIL [*Levisticum officinale* Koch]--Smellage, oil, Smallage, oil	26	1.3	0.60	0.83	2.4			*Condiments* 3.7	*Icings* (1)10.	*Syrups* 6.8
2652 *MACE--*Myristica fragrans* Houtt.	28	350.	(1)40.	-	1,300.			*Condiments* 43.	*Meats* 500 2,000.	
2653 *MACE, OIL--*Myristica fragrans* Houtt.	45	6.0	4.5	23.	68.		(1)37.	*Condiments* 12.	*Meats* 33.	
2654 *MACE, OLEORESIN--*Myristica fragrans* Houtt.	7	-	-	-	360.			*Condiments* (1)800.	*Meats* 100. 600.	*Pickles* (1)35.
2655 *l*-MALIC ACID--*l*-Hydroxysuccinic acid; Apple acid	10	380.	390.	420.	0.60 1.5					
2656 MALTOL--3-Hydroxy-2-methyl-4*H*-pyran-4-one; 3-Hydroxy-2-methyl-γ-pyrone; Larixinic acid; Corps Pipline; Palatone	23	4.1	8.7	31.	30.	7.5	90.	*Jellies* (1)15.		
2657 *MANDARIN, OIL--*Citrus reticulata* Blanco	44	62.	160.	350.	190.	(1)30.	83.			
2658 *MARIGOLD, POT [*Calendula officinalis* L.]--Calendula	1	(1)11.	(1)14.	(1)33.	(1)33.					
2659 *MARJORAM, OLEORESIN--*Majorana hortensis* Moench (*Origanum majorana* L.)	7	-	-	-	-			*Condiments* (1)75.	*Meats* 37.	
2660 *MARJORAM, POT--*Origanum vulgare* L.	0	-	-	-	-					
2661 *MARJORAM SEED--*Majorana hortensis* Moench (*Origanum majorana* L.)	7	(1)750.	-	-	-			*Condiments* 70. 700.	*Meats* 200. 3,500.	
2662 *MARJORAM, SWEET--*Majorana hortensis* Moench (*Origanum majorana* L.)	23	(1)1.9	-	-	2,000.			*Condiments* (1)200.	*Meats* 510.	*Soups* (1)150.
2663 *MARJORAM, SWEET, OIL--*Majorana hortensis* Moench (*Origanum majorana* L.)	23	4.2	1.0	4.0	15.			*Condiments* 8.0		

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2664 *p*-MENTHA-1,8 DIEN-7-OL--Perillyl alcohol, 1-Hydroxymethyl-4-*iso*-propenyl-1-cyclohexene	3	0.50 1.0	0.50 1.0	20.	10. 50.					
2665 MENTHOL--5-Methyl-2-*iso*-propylcyclohexanol; Hexahydrothymol, 3-*p*-Menthanol, 5-Methyl-2-*iso*-propyl hexahydrophenol; *l*-Menthol, Peppermint camphor	31	35.	68.	400.	130.		1,100.			
2666 (*d*)-*neo*-MENTHOL--2-*iso*-Butyl-5-methyl cyclohexanol	4	10.	31.	50.	48.					
2667 MENTHONE--*p*-Menthan-3-one	10	7.7	33.	71.	52.		(1)8.7			
2668 MENTHYL ACETATE--*p*-Menth-3-yl acetate	9	5.5	4.0	26.	24.		(1)5.2			
2669 MENTHYL *iso*-VALERATE--*p*-Menth-3-yl *iso*-valerate, Validol	8	5.0	2.9	7.4	18.					
2670 *p*-METHOXYBENZALDEHYDE--*p*-Anisaldehyde; Aubepine liquid	32	6.3	5.6	14.	16.	0.50 30.	18. 76.			
2671 2-METHOXY-4 METHYLPHENOL--4-Methylguaiacol; 2-Methoxy-*p*-cresol, 4-Hydroxy-3 methyl-1-methylbenzene; Creosol	6	10. 21.	0.05	0.77	1.0			*Alcoholic Beverages* (1)0.02		
2672 4-(*p*-METHOXYPHENYL)-2-BUTANONE--Anisyl acetone	4	12.	10. 12.	28.	26.	(1)25.				
2673 1-(*p*-METHOXYPHENYL)-1-PENTEN-3-ONE--α-Methyl anisylidene acetone; Ethone	8	2.3	2.3	28.	12.					
2674 1-(*p*-METHOXYPHENYL)-2-PROPANONE--Anisyl methyl ketone; Anisic ketone, *p*-Methoxyphenyl acetone	2	0.60 2.8	1.2 2.8	4.4 6.0	4.4 6.0					
2675 2-METHOXY-4-VINYLPHENOL--*p*-Vinylguaiacol; 4-Hydroxy-3-methoxystyrene	2	0.25 3.0	0.25 11.	1.0 8.0	1.0 8.0					
2676 METHYL ACETATE	4	28.	29.	11.	14.	(1)0.10		*Alcoholic Beverages* (1)0.20		
2677 4'-METHYL ACETOPHENONE--Methyl *p*-tolyl ketone; *p*-Methyl acetophenone, *p*-Acetyl toluene, 1-Methyl-4-acetyl benzene	18	1.1	1.6	5.2	4.9		(1)870.	*Maraschino Cherries* (1)8.0	*Condiments* (1)5.8	
2678 2-METHYLALLYL BUTYRATE--2-Methyl-2-propen-1-yl butyrate	2	(1)0.20	-	-	(1)0.20					
2679 METHYL ANISATE	7	2.7	3.0	8.0	6.2					
2680 *o*-METHYLANISOLE--*o*-Cresyl methyl ether; Methyl *o*-tolyl ether, 2-Methoxytoluene	3	1.7	(1)1.0	2.3 4.0	(1)4.0					
2681 *p*-METHYLANISOLE--*p*-Cresyl methyl ether, *p*-Methoxytoluene, 4-Methoxytoluene	19	2.7	2.7	4.8	7.6	0.50 4.0		*Condiments* (1)2.0	*Syrups* (1)8.0	
2682 METHYL ANTHRANILATE--*o*-Aminomethyl benzoate; Methyl 2-aminobenzoate	67	16.	21.	56.	20.	23.	2,200.	*Alcoholic Beverages* (1)0.20		
2683 METHYL BENZOATE--Oil of Niobe	15	2.2	4.5	8.4	9.9		(1)61.			
2684 α-METHYLBENZYL ACETATE--Styralyl acetate, Methyl phenylcarbinyl acetate, Styrolene acetate, Gardenol	11	3.9	5.4	12.	17.		(1)0.80	*Toppings* (1)30.		
2685 α-METHYLBENZYL ALCOHOL--Styralyl alcohol, 1-Phenylethanol, α-Phenylethyl alcohol; Methyl phenylcarbinol	6	4.6	3.8	6.8	9.0	(1)4.0	(1)0.30			
2686 α-METHYLBENZYL BUTYRATE--Styralyl butyrate; Methyl phenylcarbinyl butyrate	2	4.0 5.0	4.0 10.	10. 20.	10. 20.					

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2687 α-METHYLBENZYL *iso*-BUTYRATE--Styralyl *iso*-butyrate; Methyl phenylcarbinyl *iso*-butyrate	1	(1)2.0	(1)10.	(1)10.	(1)10.					
2688 α-METHYLBENZYL FORMATE--Styralyl formate, Methyl phenylcarbinyl formate	2	2.0 5.0	3.0 5.0	10. 20.	10. 20.					
2689 α-METHYLBENZYL PROPIONATE--Styralyl propionate; Methyl phenylcarbinyl propionate	2	4.0 5.0	4.0 5.0	10. 15.	10. 15.					
2690 METHYL *p-tert*-BUTYLPHENYLACETATE	3	(1)0.50	0.35 1.0	2.0	2.0					
2691 2-METHYLBUTYRALDEHYDE--2-Methylbutanal; Methyl ethyl acetaldehyde	3	1.5 2.0	2.0 8.0	6.6	5.7					
2692 3-METHYLBUTYRALDEHYDE--*iso*-Valeraldehyde, *iso*-Pentaldehyde; 3-Methylbutanal, *iso*-Valeric aldehyde, *iso*-Amyl aldehyde; *iso*-Valeral	10	0.63	1.4	2.8	3.1	(1)3.0				
2693 METHYL BUTYRATE	4	17.	31.	86.	48. 200.					
2694 METHYL *iso*-BUTYRATE	3	22.	38.	48. 200.	48. 200.					
2695 2-METHYLBUTYRIC ACID	1	(1)0.50	(1)3.0	(1)5.0	-					
2696 METHYL CELLULOSE--Cellulose methyl ether	5	90.	0.50 1,700.	0.50 30.	(1)0.65			*Toppings* (1)3,000.		
2697 α-METHYLCINNAMALDEHYDE	4	0.50 11.	1.0 15.	26.	27.		(1)430			
2698 METHYL CINNAMATE	23	1.9	3.8	8.7	13.	1.7 14.	2.7 40	*Condiments* (1)0.40		
2699 6-METHYLCOUMARIN	10	5.2	4.8	21.	24.	39.	0.80 15.			
2700 METHYLCYCLOPENTENOLONE--3-Methylcyclopentane-1,2-dione; Cyclotene; Kentonarome	22	11.	5.6	18.	13.	(1)14.	8.0 15.	*Syrups* 10. 30.		
2701 4-(3,4-METHYLENEDIOXYPHENYL)-2-BUTANONE--Piperonyl acetone	7	8.2	45.	40.	40.					
2702 5-METHYLFURFURAL	2	(1)0.13	(1)0.13	0.03 0.13	(1)0.03					
2703 METHYL 2-FUROATE--Methyl pyromucate	4	0.61	0.06 1.3	0.66	1.0 1.3			*Condiments* (1)0.02		
2704 2-METHYL-3-FURYLACROLEIN--α-Methyl furylacrolein	5	0.60	0.65	0.68	0.92					
2705 METHYL HEPTANOATE	5	0.80	0.83	0.33	0.50 0.60					
2706 2-METHYLHEPTANOIC ACID--2-Methyloenanthic acid; Methylamylacetic acid	1	(1)1.0	(1)10.	(1)10.	(1)10.					
2707 6-METHYL-5-HEPTEN-2-ONE	12	1.1	1.1	1.1	1.3	(1)1.3				
2708 METHYL HEXANOATE	4	4.1	8.5	5.3	(1)20.					
2709 METHYL 2-HEXENOATE	2	0.03 0.12	-	(1)0.03		-				
2710 METHYL *p*-HYDROXYBENZOATE--Methylparaben; Methyl parasept, Nipagin, Tegosept M	2	(1)5.0	(1)5.0	(1)5.0	5.0 8.0					
2711 METHYL-α-IONONE--5-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-4-penten-3-one, Raldeine®, α-Cetone	14	1.7	2.4	6.6	6.5		(1)0.60	*Jellies* (1)0.21		
2712 METHYL β-IONONE--5-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-4-penten-3-one, Raldeine®, β-Cetone	11	2.0	2.2	7.5	5.9					
2713 METHYL-δ-IONONE--5-(2,6,6-Trimethyl-3-cyclohexen-1-yl)-4-penten-3-one	9	0.61	0.89	3.2	2.8					

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2714 α-iso-METHYLIONONE--4-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-3-methyl-3-buten-2-one; Methyl-γ-ionone (so-called)	14	0.97	0.98	4.9	4.3	(1)0.05	(1)0.80			
2715 METHYL LAURATE--Methyl dodecanoate	3	0.50 5.0	0.50 5.0	0.02 0.50	(1)1.0					
2716 METHYL MERCAPTAN--Methanethiol	3	0.56	0.13 1.0	0.13 1.0	0.15 1.0					
2717 METHYL o-METHOXYBENZOATE--o-Methoxy methyl benzoate	1	(1)12.	(1)9.0	(1)30.	(1)40.					
2718 METHYL N-METHYLANTHRANILATE--Dimethyl anthranilate; 2-Methylamino methylbenzoate	22	5.1	5.0	18.	17.		*Jellies* (1)4.0			
2719 METHYL 2-METHYLBUTYRATE	1	(1)5.0	(1)10.	(1)10.	(1)10.					
2720 METHYL 2-METHYLTHIOPROPIONATE--Methyl β-methyl mercaptopropionate, Methyl β-methiopropionate	10	0.35	0.37	0.74	1.0			*Syrups* (1)0.05		
2721 METHYL 4 METHYLVALERATE--Methyl 4-methylpentanoate; Methyl *iso*-caproate, Methyl *iso*-butylacetate	1	(1)11.	(1)11.	(1)33.	(1)33.					
2722 METHYL MYRISTATE--Methyl tetradecanoate	4	0.25 0.50	0.25 0.50	2.4	0.30 2.0	(1)0.24				
2723 METHYL β-NAPHTHYL KETONE--2'-Acetonaphthone; Orange crystals; Cetone D	12	0.50	0.75	5.3	2.5	2.2 3.0	480. 700.			
2724 METHYL NONANOATE	6	3.9	3.6	6.2	7.1					
2725 METHYL 2-NONENOATE--Neofolione	6	3.2	12	9.9	13.					
2726 METHYL 2-NONYNOATE--Methyl octyne carbonate	9	0.69	0.28	0.61	2.2	0.02 0.12		*Condiments* (1)10		
2727 2-METHYLOCTANAL--Methyl hexyl acetaldehyde	2	(1)1.0	(1)1.0	(1)2.0	(1)2.0					
2728 METHYL OCTANOATE	4	0.02 1.0	1.0 10.	13.	1.0 40.					
2729 METHYL 2-OCTYNOATE--Methyl heptine carbonate; Folione®	24	0.15	0.30	1.4	1.4	1.7 1.7	13. 20.	*Jellies* (1)0.23		
2730 4-METHYL-2,3-PENTANEDIONE--Acetyl *iso*-butyryl	13	7.6	5.6	6.2	8.3	1.2 18.				
2731 4-METHYL-2-PENTANONE--Methyl *iso*-butyl ketone	1	(1)6.3	(1)6.3	(1)6.3	(1)6.3					
2732 β-METHYLPHENETHYL ALCOHOL--Hydratropyl alcohol; 2-Phenyl-1-propanol	4	1.1	0.42	1.2	0.92					
2733 METHYL PHENYLACETATE--Methyl α-toluate	21	3.9	2.5	13.	12.	(1)0.10	(1)11.	*Syrups* (1)37.		
2734 3-METHYL-4-PHENYL-3-BUTENE-2-ONE--Benzylidene acetone methyl	5	0.59	2.0	2.8	2.0					
2735 2-METHYL-4-PHENYL-2-BUTYL ACETATE--Dimethyl phenethyl carbinyl acetate	4	1.8	(1)0.50	0.50 10.	0.50 10.					
2736 2-METHYL-4 PHENYL 2 BUTYL *iso*-BUTYRATE--Dimethyl phenethyl carbinyl *iso*-butyrate	3	0.50 11.	1.0 41.	11.	2.0 30.					
2737 2-METHYL-4-PHENYLBUTYRALDEHYDE	1	(1)0.02	(1)0.50	(1)0.50	(1)0.50					
2738 3-METHYL-2 PHENYLBUTYRALDEHYDE--α *iso*-Propyl phenylacetaldehyde	2	(1)0.10	(1)0.50	0.32 0.50						
2739 METHYL 4 PHENYLBUTYRATE	4	0.56	0.52	1.6	1.4					
2740 4 METHYL-1-PHENYL-2 PENTANONE- Benzyl *iso*-butyl ketone	2	(1)1.0	(1)5.0	0.06 5.0	(1)5.0					

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2741 METHYL 3-PHENYLPROPIONATE--Methyl hydrocinnamate	4	0.46	0.56	1.7	0.70 4.0					
2742 METHYL PROPIONATE	4	20.	29.	96.	130.					
2743 2-METHYL-3-(*p*-*iso*-PROPYLPHENYL) PROPIONALDEHYDE--*α*-Methyl-*p*-*iso*-propyl hydrocinnamaldehyde, Cyclamen aldehyde, Cyclamal, *α*-Methyl-*p*-*iso*-propyl phenylpropyl aldehyde, Violet Alpine, oil	10	0.30	0.45	0.99	1.2					
2744 4-METHYLQUINOLINE--Lepidine, *p*-Methylquinoline	4	0.22	1.4	1.8	1.8					
2745 METHYL SALICYLATE	76	59.	27.	840.	54.		8,400.	*Syrups* (1)200.		
2746 METHYL SULFIDE--Dimethyl sulfide	6	1.1	0.30	1.4	1.6	(1)0.13		*Syrups* (1)0.50		
2747 2-METHYLTHIOPROPIONALDEHYDE--Methional, *β*-Methiopropionaldehyde, Methylmercapto propionaldehyde	8	0.35	0.01 1.0	0.01 1.0	0.66			*Condiments* 0.62	*Meats* (1)1.9	
2748 2-METHYL-3-TOLYL PROPIONALDEHYDE (Mixed *o*-, *m*-, *p*-)	1	(1)0.05	(1)1.0	(1)1.0	(1)1.0					
2749 2-METHYLUNDECANAL--Methyl nonyl acetaldehyde, 2-Methylhendecanal, Aldehyde C-12 MNA	17	0.31	0.11	0.94	1.3	0.50 2.5	(1)0.20	*Jellies* (1)0.33		
2750 METHYL 9-UNDECENOATE	4	3.7	6.7	22.	22.					
2751 METHYL 2-UNDECYNOATE--Methyl decyne carbonate	3	0.10 5.0	(1)20.	(1)15.	(1)15.					
2752 METHYL VALERATE	4	9.1	25.	28.	39.					
2753 METHYL *iso*-VALERATE	6	9.3	26.	26.	30.		(1)35.			
2754 2-METHYLVALERIC ACID--2-Methylpentanoic acid	1	-	-	(1)0.80	-					
2755 MIMOSA, ABSOLUTE--*Acacia decurrens* Willd. var. *dealbata*	8	0.79	21.	1.7	17.					
2756 MONOSODIUM GLUTAMATE	29	-	-	(1)1.3	61.			*Condiments* 1,900. *Soups* 4,300.	*Meats* 2,900.	*Pickles* (1)130.
2757 MOUNTAIN MAPLE, EXTRACT SOLID--*Acer spicatum* Lam.	4	(1)100.	(1)8.0	2.0 60.	44.					
2758 MUSK AMBRETTE--2,6-Dinitro-3-methoxy-1-methyl-4-*tert*-butylbenzene	13	0.43	0.26	4.8	0.41	(1)0.01	(1)9.0			
2759 *MUSK TONQUIN--*Moschus moschiferus* L.	9	0.67	0.62	2.0	2.7			*Syrups* (1)3.0		
2760 *MUSTARD, BROWN [*Brassica juncea* (L.) Coss. (brown); *B. nigra* (L.) Koch (blk.)]--Mustard, black	6	-	-	-	-			*Condiments* 5,200.	*Meats* 2,300.	
2761 *MUSTARD, YELLOW [*Brassica hirta* Moench (*B. alba* (L.) Boiss.)]--Mustard, white	23	(1)350.	-	-	(1)20			*Condiments* 8,200.	*Meats* 1,400.	*Pickles* 2,500. 38,000.
2762 MYRCENE--7-Methyl-3-methylene-1,6-octadiene	5	4.4	6.4	0.50 13.	4.9					
2763 MYRISTALDEHYDE--Tetradecanal, Tetradecyl aldehyde, Aldehyde C-14 (Myristic)	5	2.7	0.06 8.0	1.9	0.08 24.	(1)0.15				
2764 MYRISTIC ACID--Tetradecanoic acid	6	5.3	2.6 10.	4.1	5.3	(1)0.10				
2765 MYRRH, GUM *Commiphora molmol* Engler, *C. abyssinica* (Berg) Engler, and other *Commiphora* spp.	5	84.	(1)0.13	0.13 10.	(1)0.15		(1)1.2	*Soups* (1)10.		
2766 MYRRH, OIL--*Commiphora molmol* Engler, *C. abyssinica* (Berg) Engler, and other *Commiphora* spp.	4	3.3	8.3	13.	13.					
2767 *β*-NAPHTHYL ANTHRANILATE--2-Naphthyl anthranilate	9	2.3	1.1	16.	19.					

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2768 β-NAPHTHYL ETHYL ETHER--2-Ethoxynaphthalene, Ethyl 2-naphthyl ether, Nerolin, Neroline	14	0.65	0.74	2.8	3.6	(1)0.12		*Alcoholic Beverages* (1)0.20		
2769 *KARINGEN, EXTRACT--*Citrus paradisi* Macf.	9	71.	5.7	-	-					
2770 NEROL--*cis*-3,7-Dimethyl 2,6-octadien-1-ol; Nemsol	10	1.4	3.9	16.	19.	1.0 1.3	(1)0.80			
2771 *NEROLI BIGARDE, OIL [*Citrus aurantium* L.]--Orange flowers, bitter, oil	36	2.0	3.2	8.9	16.		(1)10.			
2772 NEROLIDOL--3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol; Peruviol	5	0.91	0.92	3.5	2.0 8.0					
2773 NERYL ACETATE	11	1.3	1.6	5.1	15.					
2774 NERYL BUTYRATE	6	6.2	22.	16.	25					
2775 NERYL *iso*-BUTYRATE	6	1.2	1.8	3.3	5.4					
2776 NERYL FORMATE	6	5.3	23.	17.	22.					
2777 NERYL PROPIONATE	6	6.3	23.	21.	21.					
2778 NERYL *iso*-VALERATE	5	0.97	1.6	4.0	5.7					
2779 *NITROUS OXIDE	1	-	-	-	-					
2780 2,6-NONADIEN-1-OL	2	(1)0.01	(1)0.05	0.05 0.50	(1)0.01			*Alcoholic Beverages* (1)0.01		
2781 γ-NONALACTONE--4-Hydroxynonanoic acid, γ-lactone; γ-Amyl butyrolactone; Aldehyde C-18 (so-called); Prunolide; Coconut Aldehyde	38	11.	14.	33.	55.	28	(1)15.	*Icings* 25.		
2782 NONANAL--Pelargonic aldehyde; Pelargonaldehyde; Aldehyde C-9	17	1.3	1.3	4.1	2.3	(1)6.0	0.20 38.			
2783 1,3-NONANEDIOL ACETATE (Mixed esters)--Octyl crotonyl acetate; Hexylene glycol diacetate; Jasmonyl; Diasmol	2	0.30 1.0	0.50 1.0	1.5 6.0	1.5 4.8					
2784 NONANOIC ACID--Pelargonic acid; Nonoic acid; Nonylic acid	5	1.8	7.8	6.6	13.			*Shortening* (1)10.		
2785 2-NONANONE--Methyl heptyl ketone	5	0.55	0.10 1.0	0.40 4.0	0.40 4.0					
2786 3-NONANON-1-YL ACETATE--Methylol methyl hexyl ketone acetate; Ketone alcohol ester; Compound 1051	2	(1)0.30	(1)0.30	0.80 5.0	(1)1.0			*Condiments* (1)10.		
2787 NONANOYL 4-HYDROXY-3-METHOXYBENZYLAMIDE--Pelargonyl vanillylamide; N-(4-Hydroxy-3-methoxybenzyl) nonanamide; N-Nonanoyl vanillylamide	1	-	-	(1)10.	(1)10.			*Condiments* (1)10.		
2788 NONYL ACETATE--Acetate C-9	6	0.81	0.81	1.9	3.1					
2789 NONYL ALCOHOL--1-Nonanol; Alcohol C-9; Nonalol	9	0.70	0.61	2.0	1.9		(1)18.			
2790 NONYL OCTANOATE	2	(1)2.0	-	-	(1)0.06					
2791 NONYL *iso*-VALERATE	3	0.50 1.0	0.50	1.0 2.0	1.4					
2792 *NUTMEG--*Myristica fragrans* Houtt.	44	700.	550.	-	2,000			*Condiments* (1)100.	*Meats* 670.	*Pickles* (1)100.
2793 *NUTMEG, OIL--*Myristica fragrans* Houtt.	75	14.	13.	19.	75.		1.2 640.	*Condiments* 21. *Syrups* (1)16.	*Icings* 2.0 30.	*Meats* 150.

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								Alcoholic Beverages		
2794 OAK CHIPS, EXTRACT--*Quercus alba* L.	5	2.5 21.	2.5 84.	2.5 63.	(1)63.			1,000. 1,000.		
2795 OAK MOSS, ABSOLUTE--*Evernia prunastri* (L.) Ach., *E. furfuracea* (L.) Mann, and other lichens	8	1.8	0.41	0.81	2.5	(1)0.15		*Condiments* (1)40.	*Soups* (1)0.50	
2796 γ-OCTALACTONE--4-Hydroxyoctanoic acid, γ-lactone	9	4.8	16.	16.	17.	(1)15.		*Syrups* (1)57.		
2797 OCTANAL--Caprylaldehyde, Caprylic aldehyde; Octylaldehyde; Aldehyde C-8	18	1.4	1.6	3.4	4.4	3.0 6.1	(1)0.10			
2798 OCTANAL DIMETHYL ACETAL--1,1-Dimethoxy-octane; C-8 Dimethylacetal	4	0.74	0.78	2.8	2.8			*Alcoholic Beverages* (1)3.0		
2799 OCTANOIC ACID--Caprylic acid; Octoic acid, C-8	11	2.9	2.0	13.	18.			*Condiments* (1)12.		
2800 1-OCTANOL--Octyl alcohol; Heptyl carbinol, *pri*-Octyl alcohol; Capryl alcohol, Caprylic alcohol, Alcohol C-8	12	2.9	0.91	2.8	3.0	(1)1.5	16. 57.			
2801 2-OCTANOL--secondary Octyl alcohol; secondary Capryl alcohol; Methyl hexyl carbinol	1	-	(1)0.60	(1)3.0	(1)4.0	(1)2.0				
2802 2-OCTANONE--Methyl hexyl ketone	7	0.10 1.0	0.20 1.0	0.40 4.0	0.40 4.0					
2803 3-OCTANONE--Ethyl amyl ketone, EAK	8	3.3	10.	11.	11.					
2804 3-OCTANON-1-OL--Methylol methyl amyl ketone, Ketone alcohol, Compound 1010	2	(1)0.20	(1)0.30	(1)0.80	0.60 0.80			*Condiments* (1)1.0		
2805 1-OCTEN-3-OL--Amyl vinyl carbinol	1	(1)0.20	(1)1.0	(1)2.0	(1)6.0			*Condiments* (1)6.0	*Soups* (1)6.0	
2806 OCTYL ACETATE--2-Ethyl hexyl acetate, Acetate C-8	8	1.6	0.87	4.7	6.0					
2807 OCTYL BUTYRATE	6	0.59	1.3	2.9	2.9					
2808 OCTYL *iso*-BUTYRATE	6	2.0	2.4	3.5	3.5		(1)0.50			
2809 OCTYL FORMATE	4	0.01 1.0	(1)1.0	5.0	7.0					
2810 OCTYL HEPTANOATE	2	0.13 1.0	0.13 1.0	0.13 2.0	0.20 2.0					
2811 OCTYL OCTANOATE	2	0.50 1.0	0.50 1.0	0.50 2.0	0.50 2.0					
2812 OCTYL PHENYLACETATE	4	1.3	(1)1.0	0.20 4.0	(1)4.0					
2813 OCTYL PROPIONATE	5	0.84	0.57	3.6	2.0 4.0					
2814 OCTYL *iso*-VALERATE	4	0.90	0.80 1.0	1.0 4.0	1.0 4.0					
2815 OLEIC ACID--9-Octadecenoic acid, Oleinic acid	10	0.25 0.40	30.	3.5	25.			*Condiments* (1)0.02		
2816 OLIBANUM, OIL [*Boswellia carteri* Birdw. and other *Boswellia* spp.]--Frankincense	4	0.60	1.2	3.3	3.7					
2817 *ONION, OIL--*Allium cepa* L.	20	(1)0.50	(1)0.50	(1)0.50	1.9			*Condiments* 2.2	*Meats* 10.	*Pickles* (1)16.
2818 *ORANGE BLOSSOMS, ABSOLUTE--*Citrus aurantium* L.	3	1.7	7.3	5.7	1.0 15.		(1)10.			
2819 *ORANGE FLOWERS--*Citrus aurantium* L.	2	100. 2,000.	-	-	-					
2820 *ORANGE LEAF, ABSOLUTE--*Citrus aurantium* L.	1	(1)0.02	(1)0.10	(1)0.25	(1)0.25					
2821 *ORANGE, OIL, DISTILLED--*Citrus sinensis* (L.) Osbeck	19	130.	140.	690.	440.	45. 500.	(1)930.			

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
2822 *ORANGE, OIL, TERPENELESS--*Citrus sinensis* (L.) Osbeck	49	10.	17.	38.	25.	230.	13. 160.	*Condiments* (1)25.		
2823 *ORANGE PEEL, BITTER, OIL--*Citrus aurantium* L.	29	67.	71.	150.	110.	(1)300.	(1)500.	*Alcoholic Beverages* (1)4.0		
2824 *ORANGE PEEL, SWEET, EXTRACT--*Citrus sinensis* (L.) Osbeck	10	99.	170.	320. 330.	320. 330.					
2825 *ORANGE PEEL, SWEET, OIL--*Citrus sinensis* (L.) Osbeck	104	210.	330.	1,000.	430.	1,300.	4,200.	*Alcoholic Beverages* (1)5.0 *Condiments* (1)32.	*Breakfast Cereals* (1)49. *Icings* 190.	*Meats* (1)10. *Syrups* (1)0.34
2826 *ORANGE PEEL, SWEET, OIL, TERPENELESS--*Citrus sinensis* (L.) Osbeck	6	43.	83.	190.	240.	(1)600.				
2827 *OREGANO [Mexican: *Lippia* spp., usually *L. graveolens* HBK European: *Origanum* spp. Elsewhere: Other genera, including *Coleus*, *Lantana*, and *Hyptis*]--Oregano, Mexican oregano, Mexican sage, Origan	22	(1)320.	-	-	(1)400.			*Condiments* 2,800.	*Meats* 540.	
2828 *ORIGANUM, OIL (EXTRACTIVE) [*Thymus capitatus* Hoffm. et Link (syn. *Coridothymus capitatus* Reichb.)]--Spanish origanum	12	(1)0.50	(1)0.50	(1)0.50	0.66 33.			*Condiments* 30.	*Meats* 37.	
2829 ORRIS, CONCRETE, LIQUID, OIL[h]--*Iris florentina* L.	41	1.7	0.52	1.1	1.3	(1)0.56	8.8	*Icings* (1)4.0		
2830 ORRIS ROOT, EXTRACT[h] [*Iris florentina* L.]--White flag, extract	22	9.2	29.	56.	31.	(1)2.0	10. 120.			
2831 *PALMAROSA, OIL--*Cymbopogon martini* (Roxb.) Stapf	7	4.2	1.7	12.	13.					
2832 PALMITIC ACID--Hexadecanoic acid, Hexadecylic acid, Cetylic acid	1	-	-	-	-			*Condiments* (1)1.0		
2833 *PAPRIKA--*Capsicum annuum* L.	31	-	-	-	1,900.			*Condiments* 970.	*Meats* 7,400.	*Soups* 1,000. 7,500.
2834 *PAPRIKA, OLEORESIN--*Capsicum annuum* L.	17	1.0 25.	(1)1.0	0.56	(1)1.2			*Condiments* 100	*Meats* 96.	
2835 *PARSLEY--*Petroselinum crispum* (Miller) Nyman (*P. sativum* Hoffm.)	18	(1)3,000.	-	-	(1)850.			*Condiments* 2,700.	*Meats* 1,000.	*Soups* 200 500.
2836 *PARSLEY, OIL--*Petroselinum crispum* (Miller) Nyman (*P. sativum* Hoffm.)	17	1.4	0.20 0.25	4.1	8.5			*Condiments* 4.9		
2837 *PARSLEY, OLEORESIN--*Petroselinum crispum* (Miller) Nyman (*P. sativum* Hoffm.)	2	-	-	-	-			*Condiments* 5.0 30.		
2838 PATCHOULY, OIL--*Pogostemon cablin* Benth. and *P. heyneanus* Benth.	12	0.88	1.1	6.3	10.		43. 220.			
2839 PENNYROYAL, OIL [*Hedeoma pulegioides* (L.) Pers. (American), *Mentha pulegium* L. var. *eriantha* (European and North African)]--Hedeoma, oil	4	1.5 5.0	3.7	14.	20. 24.					
2840 ω-PENTADECALACTONE--15-Hydroxypentadecanoic acid ω-lactone, Cyclopentadecanolide, 14-Hydroxytetradecanoic acid, Thibetolide, Angelica lactone, Exaltolide, Pentadecanolide	8	0.27	0.68	1.4	1.5	(1)0.10		*Alcoholic Beverages* (1)0.50		
2841 2,3-PENTANEDIONE--Acetyl propionyl	16	0.60	3.3	5.9	9.6	0.28		*Toppings* (1)0.30		
2842 2-PENTANONE--Methyl propyl ketone, Ethyl acetone	3	13.	34.	32.	32.					
2843 4-PENTENOIC ACID--Allyl acetic acid	1	(1)1.0	(1)2.0	(1)5.0	(1)5.0			*Margarine* (1)2.0		

[h] The expert panel can find no evidence for oral sensitivity to orris root, extract, and orris concrete.

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2844 *PEPPER, BLACK--*Piper nigrum* L.	33	(1)30.	-	-	1,200.			*Condiments* 690. *Meats* 1,700.	*Pickles* 7.2 230.	*Soups* 27 100.
2845 *PEPPER, BLACK, OIL--*Piper nigrum* L.	15	2.7	0.10 20.	5.3	8.5			*Condiments* 17.	*Meats* (1)140.	
2846 *PEPPER, BLACK, OLEORESIN--*Piper nigrum* L.	21	15.	1.0 20.	1.0 15.	1,600.			*Condiments* 370.	*Meats* 230.	
2847 *PEPPERMINT LEAVES--*Mentha piperita* L.	5	-	-	-	-					
2848 *PEPPERMINT, OIL--*Mentha piperita* L.	81	99.	110.	1,200.	300.	75. 200.	8,300.	*Alcoholic Beverages* 240. *Meats* (1)8.0	*Icings* 5.0 54.	*Toppings* 650.
2849 *PEPPER, RED--*Capsicum frutescens* L. (*C. annuum* L.)	28	15. 240.	-	-	270.			*Condiments* 630.	*Meats* 310.	*Pickles* 11. 59.
2850 *PEPPER, WHITE--*Piper nigrum* L.	22	5.9 140.	-	-	(1)450.			*Condiments* 2,700.	*Meats* 600.	*Soups* (1)500.
2851 *PEPPER, WHITE, OIL--*Piper nigrum* L.	3	-	-	-	(1)0.60					
2852 *PEPPER, WHITE, OLEORESIN--*Piper nigrum* L.	6	-	-	-	-			*Meats* (1)50.		
2853 *PETITGRAIN, LEMON, OIL--*Citrus limon* (L.) Burm. f.	3	8.6	9.3	35.	35.					
2854 *PETITGRAIN, MANDARIN, OIL--*Citrus reticulata* Blanco var. *mandarin*	14	4.3	4.1	4.5	11.	(1)0.43				
2855 *PETITGRAIN, OIL--*Citrus aurantium* L.	27	1.5	1.4	5.3	17.	(1)0.20	4.1	*Condiments* (1)15.		
2856 α-PHELLANDRENE--*p*-Mentha-1,5-diene, 2-Methyl-5-*iso*-propyl-1,3-cyclohexadiene	11	10.	28.	130.	41.					
2857 PHENETHYL ACETATE--2-Phenylethyl acetate, Benzyl carbinyl acetate	17	1.4	2.2	4.2	5.6					
2858 PHENETHYL ALCOHOL--β-Phenylethyl alcohol; 2-Phenylethyl alcohol, Benzyl carbinol	30	1.5	8.3	12.	16.	(1)0.15	21. 80.			
2859 PHENETHYL ANTHRANILATE--2-Phenylethyl anthranilate	9	1.4	1.9	6.2	5.8					
2860 PHENETHYL BENZOATE--2-Phenylethyl benzoate	5	1.0	1.0	2.0	(1)4.0		(1)3.8			
2861 PHENETHYL BUTYRATE--2-Phenylethyl butyrate	10	3.2	8.9	13.	13.					
2862 PHENETHYL *iso*-BUTYRATE--2-Phenylethyl *iso*-butyrate	12	3.4	4.0	13.	11.					
2863 PHENETHYL CINNAMATE--2-Phenylethyl cinnamate	7	1.7	0.80	3.2	3.1	(1)0.10				
2864 PHENETHYL FORMATE--2-Phenylethyl formate	8	1.3	11.	13.	15.					
2865 PHENETHYL 2-FUROATE--2-Phenylethyl 2-furoate	2	(1)0.03	-	(1)0.03	(1)0.03					
2866 PHENETHYL PHENYLACETATE--2-Phenylethyl phenylacetate	13	2.3	4.2	4.8	5.3			*Maraschino Cherries* (1)10.		
2867 PHENETHYL PROPIONATE--2-Phenylethyl propionate	10	3.6	11.	12.	16.					
2868 PHENETHYL SALICYLATE--2-Phenylethyl salicylate	5	0.75	0.67	1.5	2.0 2.0					
2869 PHENETHYL SENECIOATE--Phenethyl 3,3-dimethylacrylate, 2-Phenylethyl senecioate; Phenethyl 3-methylcrotonate	1	-	(1)5.0	(1)5.0	-			*Alcoholic Beverages* (1)5.0		

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2870 PHENETHYL TIGLATE--2-Phenylethyl tiglate	5	0.80 0.90	4.3	10.	10.				
2871 PHENETHYL *iso*-VALERATE--2-Phenylethyl *iso*-valerate, Phenethyl 3-methylbutyrate	13	1.3	2.5	5.9	6.1		0.80 45.		
2872 PHENOXYACETIC ACID--Phenoxyethanoic acid; O-Phenylglycolic acid, Phenylum	4	0.37	1.0	2.2	2.2				
2873 2-PHENOXYETHYL *iso*-BUTYRATE	2	0.90 5.0	5.0 30.	15. 30.	15. 30.				
2874 PHENYLACETALDEHYDE--α-Toluic aldehyde; α-Tolualdehyde; Hyacinthin	8	0.68	0.75	1.6	2.0		1.7 87.		
2875 PHENYLACETALDEHYDE 2,3 BUTYLENE GLYCOL ACETAL	2	-	-	(1)4.0	-				
2876 PHENYLACETALDEHYDE DIMETHYL ACETAL--Viridine	6	0.40	0.78	1.4	8.8		(1)1.0		
2877 PHENYLACETALDEHYDE GLYCERYL ACETAL	2	(1)5.0	(1)20.	0.06 20.	-				
2878 PHENYLACETIC ACID--α-Toluic acid	25	1.8	5.3	5.9	12.	(1)27.	5.4 11.	*Alcoholic Beverages* (1)0.10	*Syrups* (1)0.10
2879 4-PHENYL-2-BUTANOL--Phenylethyl methyl carbinol	3	0.12 0.90	0.60 6.0	1.5 15.	1.5 15.				
2880 4-PHENYL-3-BUTEN-2-OL--Methyl styryl carbinol	2	(1)2.0	(1)20.	0.03 20.	(1)20.				
2881 4-PHENYL-3-BUTEN-2-ONE--Benzilidene acetone; Methyl styryl ketone; Benzylacetone	12	0.82	0.84	3.7	4.5	(1)2.1		*Shortening* (1)0.20	
2882 4-PHENYL-2-BUTYL ACETATE--Phenylethyl methyl carbinyl acetate	3	0.10 3.0	(1)3.0	(1)3.0	0.50 3.0				
2883 1-PHENYL-3-METHYL-3-PENTANOL--Phenylethyl methyl ethyl carbinol	1	(1)0.16	-	(1)0.16	-	(1)0.60			
2884 1-PHENYL-1-PROPANOL--Phenyl ethyl carbinol	1	(1)0.50	(1)0.50	(1)1.5	(1)1.5				
2885 3-PHENYL-1-PROPANOL--Hydrocinnamyl alcohol; Benzylethyl alcohol; Phenylpropyl alcohol	8	0.73	1.4	2.8	3.3		(1)4.3	*Alcoholic Beverages* (1)5.0	
2886 2-PHENYLPROPIONALDEHYDE--Hydratropaldehyde; 2-Phenylpropanal; α-Methyl phenylacetaldehyde; α-Methyl tolualdehyde	5	0.61	0.30	0.85	0.85				
2887 3-PHENYLPROPIONALDEHYDE--Hydrocinnamaldehyde; Phenylpropyl aldehyde; Benzylacetaldehyde	10	1.0	1.7	5.0	5.5	(1)4.3			
2888 2-PHENYLPROPIONALDEHYDE DIMETHYL ACETAL--Hydratropaldehyde dimethyl acetal	11	0.26	0.51	1.5	3.1		(1)5.0	*Condiments* (1)5.0	
2889 3-PHENYLPROPIONIC ACID--Hydrocinnamic acid, Benzylacetic acid	4	0.02 1.0	0.48 1.0	0.80 4.0	17.	(1)1.2		*Dairy Products* (1)2.0	*Toppings* (1)1.0
2890 3-PHENYLPROPYL ACETATE--Hydrocinnamyl acetate	14	3.2	4.8	4.6	6.3		(1)10.	*Condiments* (1)0.10	
2891 2-PHENYLPROPYL BUTYRATE--β-Methylphenethyl butyrate, Hydratropyl butyrate, α-Phenylpropyl alcohol, butyric ester	1	(1)1.0	(1)1.0	(1)2.0	(1)2.0				
2892 2-PHENYLPROPYL *iso*-BUTYRATE--Hydratropyl *iso*-butyrate, α-Phenylpropyl alcohol, *iso*-butyric ester	1	(1)5.0	(1)20.	(1)20.	-				
2893 3-PHENYLPROPYL *iso*-BUTYRATE--Hydrocinnamyl *iso*-butyrate	7	1.3	3.0	5.0	5.8				
2894 3-PHENYLPROPYL CINNAMATE--Hydrocinnamyl cinnamate	7	3.4	4.1	4.3	5.3				

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2895 3-PHENYLPROPYL FORMATE--Hydrocinnamyl formate	3	1.3	0.90 1.5	3.0 5.0	2.7					
2896 3-PHENYLPROPYL HEXANOATE--Hydrocinnamyl hexanoate	4	0.67	1.3	3.3	3.7					
2897 3-PHENYLPROPYL PROPIONATE--Hydrocinnamyl propionate	4	0.49	0.52	2.0	2.4		0.80 50.			
2898 2-(3-PHENYLPROPYL)-TETRAHYDROFURAN--2-Hydrocinnamyl tetrahydrofuran	2	(1)0.50	(1)2.0	0.03 2.0	-	(1)2.0	(1)2.3			
2899 3-PHENYLPROPYL *iso*-VALERATE--Hydrocinnamyl *iso*-valerate	4	0.90	0.90	1.8	1.7					
2900 PHOSPHORIC ACID	43	510.	660.	(1)5,000.	(1)1,500.					
2901 *PIMENTA LEAF, OIL--*Pimenta officinalis* Lindl.	25	2.8	1.3	35.	32.	(1)0.06	(1)80.	*Condiments* 80.	*Meats* 160.	
2902 α-PINENE--2-Pinene, 2,6,6-Trimethylbicyclo-(3.1.1)-2-heptene	10	16. 54.	(1)64.	(1)48.	160.			*Condiments* 2.6 150.		
2903 β-PINENE--2(10) Pinene, Nopinene	3	0.05 16.	(1)64.	48. 600.	48. 600.					
2904 PINE NEEDLE, DWARF, OIL [*Pinus mugo* Turra var. *pumilio* (Haenke) Zenari]--Pinus pumilio, oil, Pine, mountain, oil	5	0.39	0.63	1.9	1.9					
2905 PINE NEEDLE, OIL [*Abies sibirica* Ledeb.; *A. alba* Mill.; *A. sachalinensis* Masters; *A. mayriana* Miyabe and Kudo]--Siberian fir, oil	5	1.5	0.62	5.2	2.7					
2906 PINE, SCOTCH, OIL--*Pinus sylvestris* L.	3	(1)6.0	-	(1)3.0	(1)2.0					
2907 PINE TAR, OIL [*Pinus palustris* Mill. and other spp. of *Pinus*]--Tar, oil	3	-	(1)2.0	(1)10.	-					
2908 PIPERIDINE--Hexahydropyridine	2	(1)3.0	-	(1)5.0	0.05 5.0			*Condiments* (1)0.05	*Meats* (1)0.05	*Soups* (1)0.05
2909 PIPERINE--Piperoylpiperidine	1	(1)0.01	-	-	-					
2910 *d*-PIPERITONE--*p*-Menth-1-en-3-one; 1-Methyl-4-*iso*-propyl-1-cyclohexen-3-one	5	1.0 11.	18.	18.	18.					
2911 PIPERONAL--Heliotropine, Piperonyl aldehyde, Dioxymethylene protocatechuic aldehyde; 3,4-Methylenedioxybenzaldehyde	48	6.0	7.0	7.4	18.	5.8	36.			
2912 PIPERONYL ACETATE--Heliotropyl acetate	2	27. 50.	80. 110.	70. 80.	55. 80.					
2913 PIPERONYL *iso*-BUTYRATE	3	0.05 1.0	(1)0.05	0.05 3.5	0.10 3.5					
2914 *PIPSISSEWA LEAVES, EXTRACT--*Chimaphila umbellata* Nutt.	6	41.	-	(1)75.	-					
2915 POLYSORBATE 20--Polyoxyethylene (20) sorbitan monolaurate, Tween® 20	6	180.	(1)500.	200. 1,000.	200. 1,000.			*Condiments* (1)380.		
2916 POLYSORBATE 60--Polyoxyethylene (20) sorbitan monostearate, Tween® 60	11	110.	150.	280.	1,600.	(1)100.	(1)28.	*Soups* (1)4,000.	*Toppings* 5,000. 17,000.	
2917 POLYSORBATE 80--Polyoxyethylene (20) sorbitan monooleate, Tween® 80	35	170.	200.	300.	320.			*Soups* (1)200.	*Pickles* 100. 120.	*Toppings* (1)8,000.
2918 *POMEGRANATE BARK, EXTRACT--*Punica granatum* L.	0	-	-	-	-					
2919 *POPPY SEED--*Papaver somniferum* L.	10	-	-	-	8,600.					

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2920 POTASSIUM ACETATE	1	(1)1.5	-	-	-					
2921 POTASSIUM SORBATE	12	110.	(1)580.	-	100. 1,000.			*Cottage Cheese* (1)500.	*Meats* (1)20.	
2922 PROPENYLGUAETHOL--6-Ethoxy-*m*-anol, 2-Propenyl-6-ethoxyphenol, 1-Ethoxy-2-hydroxy-4-propenylbenzene, Hydroxymethyl anethole, Vanitrope®	17	5.9	6.3	20.	20.	(1)2.5				
2923 PROPIONALDEHYDE--Propanal, Methylacetaldehyde, Propyl aldehyde	5	3.9	12.	11.	13.					
2924 PROPIONIC ACID--Propanoic acid, Methylacetic acid, Ethylformic acid	6	1.2 5.8	6.0	14.	20.			*Cheese* (1)600.		
2925 PROPYL ACETATE	12	4.0	16.	12.	14.					
2926 *iso*-PROPYL ACETATE	5	16.	17.	58.	75.					
2927 *p-iso*-PROPYLACETOPHENONE	2	(1)0.08	(1)0.10	(1)0.50	(1)1.0			*Pickles* (1)5.0		
2928 PROPYL ALCOHOL--1-Propanol, Propylic alcohol; Optal	2	0.50 5.0	(1)0.50	(1)0.50	(1)0.65					
2929 *iso*-PROPYL ALCOHOL--2-Propanol; *iso*-Propanol; Petrohol; Dimethylcarbinol	10	(1)25.	-	10. 75.	(1)75.					
2930 *p*-PROPYL ANISOLE--Dihydroanethole, Propyl methoxybenzene	4	4.3	9.9	64.	67.					
2931 PROPYL BENZOATE	1	(1)11.	(1)44.	(1)33.	(1)33					
2932 *iso*-PROPYL BENZOATE	1	(1)0.50	(1)1.0	(1)1.0	(1)1.0					
2933 *p-iso*-PROPYLBENZYL ALCOHOL--*p*-Cymen-7-ol; Cuminic alcohol, Cumin alcohol, Cuminyl alcohol, Cuminol	5	11.	0.47	33.	35.					
2934 PROPYL BUTYRATE	9	6.8	4.6	24.	16.					
2935 *iso*-PROPYL BUTYRATE	5	9.7	21.	39.	39.					
2936 PROPYL *iso*-BUTYRATE	8	6.8	4.8	24.	20.					
2937 *iso*-PROPYL *iso*-BUTYRATE	3	12. 25.	18. 25.	58. 100.	60. 100.					
2938 PROPYL CINNAMATE	7	2.6	2.9	4.9	4.3	(1)0.07				
2939 *iso*-PROPYL CINNAMATE	5	0.52	0.75	1.3	2.3					
2940 PROPYLENE GLYCOL--1,2-Propanediol, Methyl glycol, 1,2-Dihydroxypropane	89	690.	810.	1,300.	1,300.	250. 290.	1,800.	*Maraschino Cherries* (1)300. *Pickles* (1)20. *Syrups* (1)7,000. *Condiments* 1,000. 50,000.	*Icings* (1)0.08 *Soups* (1)20. *Icings* (1)150.	*Meats* 10. 40. *Toppings* 3,500.
2941 PROPYLENE GLYCOL ALGINATE	6	(1)200.	800. 2,100.	-	-					
2942 PROPYLENE GLYCOL STEARATE--Propylene glycol octadecanoate	2	-	-	17. 5,000.	43. 20,000.			*Toppings* (1)100,000		
2943 PROPYL FORMATE	5	20.	57.	65.	85.					
2944 *iso*-PROPYL FORMATE	2	18. 25.	18. 25.	55. 100.	60. 100					
2945 PROPYL 2-FURANACRYLATE--Propyl 3-(2-furyl) acrylate	2	(1)3.0	-	(1)0.03	-					

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2946 PROPYL 2-FUROATE	2	-	-	(1)0.03	(1)0.03			*Condiments* (1)0.20		
2947 PROPYL GALLATE--Tenox P G[a]	9	0.08	0.05	0.16	0.97	(1)0.03				
2948 PROPYL HEPTANOATE	4	3.8	5.1	5.9	18.			*Alcoholic Beverages* (1)3.0		
2949 PROPYL HEXANOATE	5	2.2	3.0	8.0	-					
2950 *iso*-PROPYL HEXANOATE	2	(1)0.50	5.5 10.	20 40.	20. 40.					
2951 PROPYL *p*-HYDROXYBENZOATE--Propylparaben; Propylparasept; Nipasol	2	0.20 32.	(1)130.	(1)96.	(1)96.					
2952 3-PROPYLIDENEPHTHALIDE	1	-	(1)5.0	(1)5.0	(1)5.0					
2953 α-PROPYLPHENETHYL ALCOHOL--1-Phenyl-2-pentanol; Benzylpropyl carbinol; Benzylbutyl alcohol	1	(1)1.0	(1)5.0	(1)5.0	-	(1)5.0				
2954 *p*-*iso*-PROPYLPHENYLACETALDEHYDE--*p*-Cymen-7-carboxaldehyde	1	(1)0.10	(1)0.50	(1)0.50	-					
2955 PROPYL PHENYLACETATE	4	0.30 1.0	0.30 1.5	2.7	1.0 5.0					
2956 *iso*-PROPYL PHENYLACETATE	3	0.20 0.50	1.8	0.50 8.0	3.0 8.0					
2957 3-(*p*-*iso*-PROPYL)PHENYL PROPIONALDEHYDE--*p*-*iso*-Propyl hydrocinnamaldehyde; Cuminyl acetaldehyde	3	(1)0.80	(1)0.80	(1)1.3	(1)1.0		(1)5.0			
2958 PROPYL PROPIONATE	9	6.0	12.	25.	25.					
2959 *iso*-PROPYL PROPIONATE	3	9.7	5.0 50	40. 50.	30. 50.					
2960 PROPYL *iso*-VALERATE	7	5.0	16.	17.	20.					
2961 *iso*-PROPYL *iso*-VALERATE	5	3.4	3.4	11.	11.					
2962 *iso*-PULEGOL--*p*-Menth-8-en-3-ol	4	7.4	29.	23.	23.					
2963 PULEGONE--*p*-Menth-4(8)-en-3-one; Δ-4(8)-*p*-Menthen-3-one; 1-Methyl-4-*iso*-propylidene-3-cyclohexanone	3	5.0 8.0	5.0 32.	17.	24. 25.					
2964 *iso*-PULEGONE--*p*-Menth-8-en-3-one; Δ-8(9)-*p*-Menthen-3-one; 1-Methyl-4-*iso*-propenyl-3-cyclohexanone	3	4.0	12.	16.	16.					
2965 *iso*-PULEGYL ACETATE	4	5.8	22	19.	19.					
2966 PYRIDINE	3	(1)1.0	0.02 0.12	(1)0.40	(1)0.40					
2967 PYROLIGNEOUS ACID[a]	16	10	15.	51.	33.	(1)30.		*Meats* 30. 300.		
2968 PYROLIGNEOUS ACID, EXTRACT[a]	4	-	-	-	50 200.			*Alcoholic Beverages* (1)20.	*Meats* 100. 300.	
2969 PYRUVALDEHYDE--Pyruvic aldehyde; Acetylformaldehyde; 2-Ketopropionaldehyde; 2-Oxopropanal	2	(1)1.0	(1)1.0	0.03 5.0	0.03 5.0					
2970 PYRUVIC ACID--Pyroracemic acid; Acetylformic acid; 2-Ketopropionic acid; 2-Oxopropanoic acid; α-Ketopropionic acid	5	(1)0.25	0.25 20.	27.	30.		(1)110.			
2971 QUASSIA, EXTRACT [*Picrasma excelsa* (Sw.) Planch., *Quassia amara* L.]--Bitter wood, extract; Bitter ash, extract	12	3.4	-	-	(1)50			*Alcoholic Beverages* 3.4		

[a] Prior Sanction - *Federal Register*, November 30, 1957, Page 9594, Section 3.201

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	—Other Category Uses—		
2972 QUEBRACHO BARK, EXTRACT--*Aspidosperma quebracho-blanco* Schlecht., or *Schinopsis lorentzii* (Gurgeb.) Engl.	4	11.	23.	27.	28.					
2973 QUILLAIA [*Quillaja saponaria* Molina]--Soap bark, Saponin, China bark, extract	22	95.	(1)0.12	(1)18.	-			*Syrups* (1)6.8		
2974 *QUINCE SEED, EXTRACT--*Cydonia oblonga* Mill. (*C. vulgaris* Pers.)	3	0.01 40.	0.06 20.	-	(1)1.0					
2975 QUININE BISULFATE	2	95. 100.	-	-	-					
2976 QUININE HYDROCHLORIDE	19	110.	-	-	-					
2977 QUININE SULFATE	6	100.	-	-	-					
2978 *iso*-QUINOLINE	3	(1)0.25	(1)0.25	(1)1.0	0.004 1.0					
2979 RHATANY, EXTRACT [*Krameria triandra* Ruiz et Pavon (Peruvian), *K. argentea* Martius (Brazilian)]--Krameria, extract	7	11.	31.	40.	8.0 63.			*Alcoholic Beverages* (1)10.		
2980 RHODINOL--3,7-Dimethyl-7-octen-1 ol; (Commercial Rhodinol is largely *l*-Citronellol)	28	2.0	2.1	7.6	8.1	(1)2.9	(1)31.	*Jellies* (1)0.92		
2981 RHODINYL ACETATE	10	2.8	1.4	9.4	18.					
2982 RHODINYL BUTYRATE	7	0.94	1.1	3.0	9.7		(1)1.1			
2983 RHODINYL *iso*-BUTYRATE	9	1.1	1.8	3.3	4.5	(1)0.01				
2984 RHODINYL FORMATE	9	1.3	1.8	4.3	4.9	(1)0.08				
2985 RHODINYL PHENYLACETATE	7	1.2	1.2	3.8	4.4					
2986 RHODINYL PROPIONATE	7	1.8	2.4	4.9	5.8					
2987 RHODINYL *iso*-VALERATE	5	2.0	2.3	7.2	7.2					
2988 *ROSE, ABSOLUTE--*Rosa alba* L.; *R. centifolia* L. and varieties of these spp.	11	0.63	1.2	2.0	1.6					
2989 *ROSE, BULGARIAN, TRUE OTTO, OIL [*Rosa damascena* Mill.]--Attar of roses	24	0.51	0.68	2.6	1.2	0.01 0.50	15.	*Jellies* (1)0.05		
2990 *ROSE HIPS, EXTRACT [*Rosa canina* L., *R. gallica* L., *R. candita* Scop.; *R. rugosa* Thunb.; and other *Rosa* spp.]--Hipberries, extract	1	-	-	-	-					
2991 *ROSEMARY--*Rosmarinus officinalis* L.	15	(1)700.	-	-	-			*Condiments* 680.	*Meats* 380.	
2992 *ROSEMARY, OIL [*Rosmarinus officinalis* L.]--Garden rosemary, oil	17	3.6	0.50 4.0	7.5	6.3			*Condiments* 2.9	*Meats* (1)40.	
2993 *ROSE WATER, STRONGER--*Rosa centifolia* L.	2	(1)100.	-	-	-					
2994 *RUE--*Ruta graveolens* L.	2	-	-	-	(1)6.0					
2995 *RUE, OIL--*Ruta graveolens* L.	13	1.2	1.3	4.1	3.3			*Condiments* (1)1.0		
2996 RUM ETHER[1]--Ethyl oxyhydrate	53	67.	110.	320.	230.	(1)1.7	380.	*Alcoholic Beverages* 80. 1,600.		
2997 SACCHARINE, SODIUM SALT--1,2-Benzisothiazolin-3-one, 1,1-dioxide, sodium salt, Kristallose, Crystallose, Saccharin soluble	8	72.	(1)150.	2,100. 2,600	(1)12.					

[1] Rum ether shall consist of at least 99 per cent water, ethyl alcohol, ethyl acetate, methanol, ethyl formate, acetone, acetaldehyde, and formaldehyde. It shall all distill at a temperature not exceeding 100°C at atmospheric pressure, and shall leave no residue on evaporation. The methanol and formaldehyde contents, combined, shall not exceed 5 per cent.

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
2998 *SAFFRON--*Crocus sativus* L.	11	(1)1.3	-	-	(1)10.			*Alcoholic Beverages* (1)200.	*Meats* 260.	
2999 *SAFFRON, EXTRACT [*Crocus sativus* L.]--Crocus, extract	5	1.3 7.5	1.3 9.0	6.3	1.9 14.			*Condiments* (1)50		
3000 *SAGE--*Salvia officinalis* L.	25	(1)300.	-	-	170.			*Meats* 1,500.		
3001 *SAGE, OIL--*Salvia officinalis* L.	22	3.7	16.	11.	14.		(1)30.	*Condiments* 14.	*Meats* 110.	*Pickles* (1)2.4
3002 *SAGE, OLEORESIN--*Salvia officinalis* L.	7	-	-	-	-			*Condiments* (1)100.	*Meats* 100.	
3003 *SAGE, SPANISH, OIL --*Salvia lavandulaefolia* Vahl.	8	2.0 11.	2.0 44.	20.	20.			*Condiments* (1)50.	*Meats* 40. 40.	
3004 SALICYLALDEHYDE--o-Hydroxybenzaldehyde	10	0.55	1.1	1.8	6.3		11. 18.	*Alcoholic Beverages* (1)5.0	*Condiments* (1)2.0	
3005 SANDALWOOD, YELLOW, OIL [*Santalum album* L.]--Sandalwood, East Indian, oil; Saunders, white, oil; Atheol	8	2.4	7.5	7.7	6.6		(1)47.			
3006 SANTALOL (α-and β-)--Atgeol	2	0.06 2.0	0.35 2.0	1.0 10.	1.0 8.0		(1)0.20			
3007 SANTALYL ACETATE	4	0.53	0.78	2.0	2.0		(1)2.3			
3008 SANTALYL PHENYLACETATE	3	1.0	0.95	2.0	2.0					
3009 SARSAPARILLA, EXTRACT [b]--*Smilax* spp.	9	190.	130.	(1)1,000.	(1)2,000.					
3010 SASSAFRAS BARK, EXTRACT (Safrol-free)--*Sassafras albidum* (Nutt.) Nees	5	290.	(1)10.	(1)100.	(1)50.					
3011 SASSAFRAS LEAVES (Safrol-free)--*Sassafras albidum* (Nutt.) Nees	2	-	-	-	-			*Soups* (1)30,000.		
3012 *SAVORY, SUMMER--*Satureja hortensis* L.	8	-	-	-	800. 850.			*Condiments* (1)200.	*Meats* 1,100.	
3013 *SAVORY, SUMMER, OIL--*Satureja hortensis* L.	2	-	-	(1)4.0	(1)4.0			*Condiments* 10. 50.		
3014 *SAVORY, SUMMER, OLEORESIN--*Satureja hortensis* L.	2	-	-	(1)4.0	(1)4.0			*Condiments* 35. 50.		
3015 *SAVORY, WINTER--*Satureja montana* L.	0	-	-	-	-					
3016 *SAVORY, WINTER, OIL--*Satureja montana* L.	1	-	-	(1)4.0	(1)4.0			*Condiments* (1)50.		
3017 *SAVORY, WINTER, OLEORESIN--*Satureja montana* L.	1	-	-	(1)4.0	(1)4.0			*Condiments* (1)50.		
3018 *SCHINUS MOLLE, OIL [*Schinus molle* L.]--Pepper tree, oil	4	-	-	(1)10.	(1)10.			*Condiments* (1)3.0		
3019 SKATOLE--3-Methylindole; β-Methylindole	8	0.75	1.0	0.78	0.80	(1)0.01	(1)0.10			
3020 *SLOE BERRIES [*Prunus spinosa* L.]--Blackthorn berries	2	-	-	-	-					
3021 *SLOE BERRIES, EXTRACT [*Prunus spinosa* L.]--Blackthorn berries, extract	7	110.	50. 100.	(1)40.	(1)45.			*Alcoholic Beverages* 43,000.		
3022 *SLOE BERRIES, EXTRACT SOLID [*Prunus spinosa* L.]--Blackthorn berries, extract solid	0	-	-	-	-					
3023 SNAKEROOT, CANADIAN, OIL [*Asarum canadense* L.]--Wild ginger, Canadian, oil	9	1.9	1.0 5.0	8.3	8.3			*Condiments* 1.4 4.0		
3024 SODIUM ACETATE	4	(1)1.5	(1)15.	(1)200.	(1)15.			*Breakfast Cereals* (1)60.		

[b] Judged solely on the basis of common use.

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
3025 SODIUM BENZOATE	89	350.	39.	350.	300.		(1)12.			
3026 SODIUM CITRATE--(Trisodium citrate), Citratin, Citrosodine	47	490.	(1)15.	(1)40.	220.			*Meats* 40. 600.	*Toppings* 50. 3,900.	
3027 SODIUM HEXAMETAPHOSPHATE--Sodium metaphosphates, Calgon, Giltex, Quadrafos, Micromet, Hagan phosphate	6	-	-	-	-	500. 7,000.		*Breakfast Cereals* (1)3,000.		
3028 SORBITAN MONOSTEARATE--Span® 60	6	140.	(1)5.0	9.0 7,500.	1,400.			*Cheese* (1)8.0	*Condiments* (1)8.0	
3029 d-SORBITOL--d-Glucitol; Sorbit, Sorbol, Sorbo; Nevitin, Karion; Sionon; Dinkarmon	24	1,300.	70,000.	21,000.	50,000.	(1)8,000.		*Icings* (1)500.	*Toppings* (1)280,000.	
3030 *SPEARMINT--*Mentha spicata* L.	5	(1)500.	-	-	-			*Condiments* (1)1,000.	*Meats* (1)500.	
3031 *SPEARMINT, EXTRACT--*Mentha spicata* L.	4	2,100.	(1)100.	(1)0.20	-					
3032 *SPEARMINT, OIL--*Mentha spicata* L.	47	100.	81.	830.	270.	(1)75.	6,200.	*Alcoholic Beverages* (1)100.	*Jellies* 72. 1,900.	
3033 *SPIKE LAVENDER, OIL--*Lavandula latifolia* Vill. (*L. spica* DC.)	5	10. 11.	10. 44.	18.	33. 50.					
3034 SPRUCE, OIL [*Tsuga canadensis* (L.) Carr.; *T. heterophylla* (Raf.) Sarg.; *Picea mariana* (Mill.), *P. glauca* (Moench) Voss]--Hemlock, oil	15	6.2	15.	11.	2.0 4.0	(1)1.0	(1)44.			
3035 STEARIC ACID--Octadecanoic acid	4	2.0 10.	-	(1)4,000.	(1)3.5					
3036 STORAX [*Liquidambar orientalis* Mill.; *L. styraciflua* L.]--Styrax, gum	8	2.0	2.0	13.	23.		(1)300.	*Toppings* (1)15.		
3037 STYRAX, EXTRACT--*Liquidambar orientalis* Mill.; *L. styraciflua* L.	5	0.84	0.25 0.60	3.5	4.0 6.0	(1)0.04				
3038 SUCROSE OCTAACETATE	2	0.35 20.	-	-	-					
3039 SULFUR DIOXIDE--Sulfurous anhydride; Sulfurous oxide	10	180.	(1)2.5	-	-			*Condiments* (1)400.	*Dehydrated Potatoes* (1)60.	*Soups* (1)20.
3040 TAGETES, OIL [*Tagetes erecta* L.; *T. patula* L.; or *T. glandulifera* Schrank]--Marigold, oil	8	4.1	7.4	9.0	13.	(1)7.0		*Condiments* (1)20.		
3041 *TANGERINE, OIL--*Citrus reticulata* Blanco	43	90.	160.	160.	250.	(1)20.	810.			
3042 TANNIC ACID [Nut galls of *Quercus infectoria* Oliv. and related spp. of *Quercus*]--Gallotannic acid, Tannin	5	1.1 45.	(1)160.	0.20 100.	40.			*Alcoholic Beverages* 6.0 1,000.		
3043 *TARRAGON--*Artemisia dracunculus* L.	16	-	-	-	(1)20.			*Condiments* 23.	*Meats* 260.	
3044 TARTARIC ACID (*d-, l-, dl-, meso-*)--Racemic acid	45	960.	570.	5,400.	1,300.	(1)60.	(1)3,700.	*Condiments* (1)10,000.		
3045 α-TERPINEOL--*p*-Menth-1-en-8-ol	24	5.4	16.	14.	19.	12. 16.	40.	*Condiments* (1)38.		
3046 TERPINOLENE--*p*-Menth-1,4(8)-diene; 1,4(8)-Terpadiene	2	(1)16.	(1)64.	0.12 48.	(1)49.					
3047 TERPINYL ACETATE--*p*-Menth-1-en-8-yl acetate	19	3.5	3.2	9.9	15.		14. 260.	*Condiments* (1)15.	*Meats* 1.7 40.	
3048 TERPINYL ANTHRANILATE--*p*-Menth-1-en-8-yl anthranilate	3	1.1	1.5 2.6	6.3	6.0 6.0					
3049 TERPINYL BUTYRATE--*p*-Menth-1-en-8-yl butyrate	4	6.4	9.2	11.	9.5		(1)210.			
3050 TERPINYL *iso*-BUTYRATE--*p*-Menth-1-en-8-yl *iso*-butyrate	2	0.90 2.4	(1)5.0	4.0 15.	5.0 15.					

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
3051 TERPINYL CINNAMATE--*p*-Menth-1-en-8-yl cinnamate	1	(1)0.50	(1)2.6	(1)6.0	(1)6.0					
3052 TERPINYL FORMATE--*p*-Menth-1-en-8-yl formate	3	0.50 3.0	2.6 5.0	6.0 10.	6.0 10.			*Alcoholic Beverages* (1)1.0		
3053 TERPINYL PROPIONATE--*p*-Menth-1-en-8-yl propionate	3	1.5	2.6 3.0	6.0 10.	6.0 10.					
3054 TERPINYL *iso*-VALERATE--*p*-Menth-1-en-8-yl *iso*-valerate	2	0.50 5.0	2.6 5.0	6.0 10.	6.0 10.					
3055 TETRAHYDROFURFURYL ACETATE	3	1.3 2.0	(1)8.0	1.0 20.	1.0 20.					
3056 TETRAHYDROFURFURYL ALCOHOL (Tetrahydro-2-furanmethanol, Tetrahydro-2-furylmethanol, Tetrahydro-2-furancarbinol, THFA	2	0.03 14.	(1)0.03	0.03 18.	(1)0.04					
3057 TETRAHYDROFURFURYL BUTYRATE	1	(1)0.90	(1)6.0	(1)15.	(1)15.					
3058 TETRAHYDROFURFURYL PROPIONATE	3	1.3 2.0	(1)8.0	1.0 20.	1.0 20.					
3059 TETRAHYDRO-*pseudo*-IONONE--6,10-Dimethyl-9-undecen-2-one	4	0.50 0.50	0.60 2.4	14.	14.					
3060 TETRAHYDROLINALOÖL--3,7-Dimethyloctan-3-ol	5	1.3	2.7	5.6	5.6					
3061 TETRAMETHYL ETHYLCYCLOHEXENONE (Mixture of isomers)	1	(1)5.0	(1)30.	(1)30.	(1)30.					
3062 2-THIENYL MERCAPTAN--2-Thienylthiol	1	-	-	(1)0.10	(1)0.10					
3063 *THYME--*Thymus vulgaris* L.	24	(1)13.	-	(1)5.0	550.			*Meats* 360.	*Soups* 500. 1,000.	
3064 *THYME, OIL--*Thymus vulgaris* L.	14	1.0 5.0	(1)20.	1.0 15.	1.5 5.3		(1)100.	*Condiments* 18.	*Meats* 33.	*Soups* (1)0.13
3065 *THYME, WHITE, OIL--*Thymus vulgaris* L.	9	0.01 1.0	(1)0.01	27.	5.4			*Alcoholic Beverages* (1)5.0	*Condiments* 4.0 8.0	*Meats* 15.
3066 THYMOL--3-*p*-Cymenol, 5-Methyl-2-*iso*-propylphenol; Thyme camphor	10	2.5 11.	(1)44.	9.4	5.0 6.5		(1)100.			
3067 TOLUALDEHYDE GLYCERYL ACETAL (Mixed *o*-, *m*-, *p*-)	3	0.08 6.0	6.0 8.0	12. 15.	12. 15.					
3068 TOLUALDEHYDES (Mixed *o*-, *m*-, *p*-)	27	11.	16.	25.	28.	8.3	430.	*Maraschino Cherries* (1)100.		
3069 TOLU, BALSAM, EXTRACT--*Myroxylon balsamum* L. Harms (*M. toluiferum* HBK.)	8	32.	150.	57.	71.		2.0 38.			
3070 TOLU, BALSAM, GUM--*Myroxylon balsamum* L. Harms (*M. toluiferum* HBK.)	7	2.6	13.	5.2	8.0			*Syrups* (1)3.0		
3071 *p*-TOLYLACETALDEHYDE--*p*-Methylphenylacetaldehyde	2	-	(1)2.0	0.03 2.0	(1)2.0					
3072 *o*-TOLYL ACETATE--*o*-Cresyl acetate, Acetyl *o*-cresol, *o*-Cresylic acetate	5	2.8	2.6	11.	9.0 10.	(1)1.0	0.30 220.			
3073 *p*-TOLYL ACETATE--*p*-Cresyl acetate; Acetyl *p*-cresol, *p*-Cresylic acetate	6	0.50 1.0	1.3	4.3	4.4		0.30 220.	*Condiments* (1)10.		
3074 4-(*p*-TOLYL)-2-BUTANONE--*p*-Methylbenzyl acetone	2	(1)1.0	(1)1.5	(1)6.0	(1)6.0					
3075 *p*-TOLYL *iso*-BUTYRATE--*p*-Cresyl *iso*-butyrate	2	0.10 4.0	(1)0.05	0.12 6.0	0.12 7.0					
3076 *p*-TOLYL LAURATE--*p*-Tolyl dodecanoate, *p*-Cresyl dodecanoate, *p*-Cresyl laurate	1	(1)1.0	(1)1.0	(1)2.0	(1)2.0					
3077 *p*-TOLYL PHENYLACETATE--*p*-Cresyl phenylacetate	9	1.6	0.87	4.8	5.4					

FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
3078 2-(p-TOLYL)-PROPIONALDEHYDE--*p*-Methylhydratropaldehyde	2	(1)0.13	(1)0.13	(1)0.13	(1)0.20			*Alcoholic Beverages* (1)0.005		
3079 *TRAGACANTH, GUM--*Astragalus gummifer* Lab. or other Asiatic spp. of *Astragalus*	70	42.	65.	67.	140.	(1)2,000.	(1)170.	*Condiments* 470.	*Meats* 50. 60.	
3080 TRIBUTYL ACETYLCITRATE--Citroflex A-4	1	(1)0.40	-	-	-					
3081 TRICALCIUM PHOSPHATE	25	1,000.	46.	50. 60.	80.	700.		*Condiments* (1)540.	*Meats* 360.	
3082 2-TRIDECENAL	2	0.10 0.30	1.6 6.0	4.0 6.0	4.0 6.0		(1)0.10			
3083 TRIETHYL CITRATE--Ethyl citrate	13	13.	47.	180.	230.	(1)10.				
3084 *TUBEROSE, OIL--*Polianthes tuberosa* L.	5	0.26	0.45	1.5	1.7					
3085 *TURMERIC--*Curcuma longa* L.	22	-	-	-	-	(1)0.05		*Condiments* 760. *Soups* 30. 50.	*Meats* 200.	*Pickles* 690.
3086 *TURMERIC, EXTRACT--*Curcuma longa* L.	13	(1)0.78	-	-	-			*Condiments* 59. *Soups* 30. 40.	*Meats* 43. *Pickles* (1)40.	
3087 *TURMERIC, OLEORESIN--*Curcuma longa* L.	13	-	-	-	-			*Condiments* 640.	*Meats* 20. 100.	*Pickles* 200.
3088 TURPENTINE, GUM--*Pinus palustris* Mill. and other *Pinus* spp.	5	-	-	-	(1)15.					
3089 TURPENTINE, STEAM DISTILLED--*Pinus palustris* Mill. and other *Pinus* spp.	6	-	-	11.	10. 20.		(1)7.1			
3090 2,3-UNDECADIONE--Acetyl nonyryl; Acetyl pelargonyl	1	(1)1.5	(1)3.0	(1)3.0	(1)3.0					
3091 γ-UNDECALACTONE--4-Hydroxyundecanoic acid, γ-lactone, γ-Undecyl lactone, γ-Heptyl butyrolactone; Aldehyde C-14 pure (so-called); Peach aldehyde	46	4.4	3.0	11.	7.1	7.5	90.			
3092 UNDECANAL--Undecylic aldehyde; Aldehyde C-11 Undecylic; Hendecanal	6	0.95	3.1	2.0	2.4		(1)56.			
3093 2-UNDECANONE--Methyl nonyl ketone	13	2.8	0.54	2.6	3.1	(1)5.0				
3094 9-UNDECENAL--Undecylenic aldehyde; Hendecen-9-al; Aldehyde C-11 Undecylenic	9	4.8	4.2	4.5	4.6					
3095 10-UNDECENAL	2	0.05 1.0	(1)0.20	(1)0.20	-					
3096 10-UNDECEN-1-yl ACETATE--10-Hendecenyl acetate; Undecenyl acetate; Undecylenic acetate; Acetate C-11	3	3.7	15.	12.	12.					
3097 UNDECYL ALCOHOL--1-Undecanol; Alcohol C-11 Undecylic	4	2.9	15.	12.	12.					
3098 VALERALDEHYDE--Pentanal; Valeric aldehyde; Valeral; Amyl aldehyde	5	1.3	5.0	4.2	5.4					
3099 VALERIAN ROOT, EXTRACT--*Valeriana officinalis* L.	22	25.	35.	65.	69.			*Condiments* (1)24.		
3100 VALERIAN ROOT, OIL--*Valeriana officinalis* L.	18	0.52	0.36	2.6	3.1	0.02 1.5				
3101 VALERIC ACID--Pentanoic acid, Propylacetic acid	16	1.2	1.8	2.5	8.0					

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FEMA No. and Substance	No. of Reports	Beverages	Ice Cream, Ices, Etc.	Candy	Baked Goods	Gelatins and Puddings	Chewing Gum	— Other Category Uses —		
3102 *iso*-VALERIC ACID--β-Methylbutyric acid, active Valeric acid, Valerianic acid, *iso*-Propylacetic acid; Delphinic acid, *iso*-Butyl formic acid	19	1.2	14.	12.	5.5			*Cheese* (1)2.4		
3103 γ-VALEROLACTONE--4-Hydroxypentanoic acid, γ-lactone; 3-Methylbutyrolactone; 3-Valerolactone	1	(1)4.0	(1)20.	(1)50.	(1)50.					
3104 *VANILLA--*Vanilla planifolia* Andrews or *V. tahitensis* J. W. Moore	40	420.	600.	490.	530.	630.		*Icings* 200. 630.	*Syrups* (1)10.	*Toppings* (1)300.
3105 *VANILLA, EXTRACT--*Vanilla planifolia* Andrews or *V. tahitensis* J. W. Moore	68	200.	3,000.	4,000.	1,900.			*Icings* 2,000. 4,800.	*Syrups* 8.5 54.	*Toppings* 2,700.
3106 *VANILLA, OLEORESIN--*Vanilla planifolia* Andrews or *V. tahitensis* J. W. Moore	26	190.	290.	210.	300.	(1)230.		*Condiments* (1)200.		
3107 VANILLIN--Methylprotocatechuic aldehyde, Vanillic aldehyde, 4-Hydroxy-3-methoxybenzaldehyde	127	63.	95.	200.	220.	120.	270.	*Chocolate* 970. *Toppings* 150.	*Margarine* (1)0.20	*Syrups* 330. 20,000.
3108 VANILLIN ACETATE--Acetyl vanillin	3	11.	11.	28.	28.					
3109 VERATRALDEHYDE--Vanillin methyl ether, Veratric aldehyde; 3,4-Dimethoxybenzenecarbonal; Dimethyl ether protocatechualdehyde	12	9.0	9.2	32	30.	(1)15.				
3110 *VIOLET LEAVES, ABSOLUTE--*Viola odorata* L.	5	2.3	8.4	7.6	2.0 24.					
3111 WALNUT HULL, EXTRACT--*Juglans nigra* L. or *J. regia* L.	3	43. 90.	100. 170.	(1)130.	100. 130.					
3112 WINTERGREEN, EXTRACT [*Gaultheria procumbens* L.]--Checkerberry, extract	4	10. 10.	-	900. 5,000.	-					
3113 WINTERGREEN, OIL [*Gaultheria procumbens* L.]--Checkerberry, oil	22	56.	44.	260.	1,500.		3,900.			
3114 WORMWOOD[1] [*Artemisia absinthium* L.]--Absinthium	4	360.	-	-	-			*Alcoholic Beverages* (1)5.0		
3115 WORMWOOD, EXTRACT[1] [*Artemisia absinthium* L.]--Absinthium, extract	8	15. 43.	(1)170.	(1)130.	-			*Alcoholic Beverages* 10. 40.		
3116 WORMWOOD, OIL[1] [*Artemisia absinthium* L.]--Absinthium, oil	11	14.	0.50 32.	9.0	(1)2.0			*Alcoholic Beverages* 11.		
3117 YARROW, HERB [*Achillea millefolium* L.]--Milfoil	6	29.	-	-	-			*Alcoholic Beverages* 5.0 40.		
3118 YERBA SANTA, FLUID EXTRACT--*Eriodictyon californicum* (Hook. and Arn.) Torr.	2	(1)25.	(1)200.	(1)400.	(1)400.					
3119 *YLANG YLANG, OIL--*Canangia odorata* Hook. f. and Thomson	16	0.95	1.4	2.9	2.9		18. 25.	*Icings* (1)0.75		
3120 YUCCA, JOSHUA TREE--*Yucca brevifolia* Engelm.	9	120.	(1)20.	-	-					
3121 YUCCA, MOHAVE, EXTRACT [*Yucca schidigera* Roezl ex Ortgies (*Y. mohavensis* Sarg.)]--Cactus root, extract, Plantarome	10	150.	-	-	-					
3122 *ZEDOARY--*Curcuma zedoaria* (Berg.) Rosc.	4	7.5 2,000.	-	-	-					
3123 *ZEDOARY BARK, EXTRACT--*Curcuma zedoaria* (Berg.) Rosc.	0	-	-	-	-					
3124 ZINGERONE--4-(4-Hydroxy-3-methoxyphenyl)-2-butanone; Zingiberone	6	6.9	7.8	11.	11.		(1)15.			

[1] Provided it is used at levels such that no thujone is detectable in the finished food, using the standard AOAC method.

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THE DIKETONE PRODUCED WHEN BUTTER CULTURES ARE STEAM DISTILLED WITH FERRIC CHLORIDE ADDED*

B. W. HAMMER

Iowa State College, Ames, Iowa

The relationship of diacetyl to the desirable flavor of butter has been established by the work of various investigators. When a satisfactory butter culture is steam distilled and the distillate treated with suitable reagents, a brick red precipitate is formed that is commonly considered to be nickel dimethylglyoximate, a derivative of diacetyl. Much more precipitate is formed if ferric chloride is added to the butter culture before distillation, and this is interpreted as indicating the formation of relatively large amounts of diacetyl through an oxidation of acetylmethylcarbinol present in the culture.

Since certain homologues of diacetyl have been assumed to be valuable for the purpose of giving oleomargarine a desirable flavor and aroma (3) and also because these homologues are similar to diacetyl from various standpoints, it seemed advisable to determine whether or not the diketone obtained on distilling a butter culture with ferric chloride is diacetyl. The general procedure followed was to prepare the usual nickel salts from the distillates of butter cultures and then determine the nickel contents for comparison with the theoretical nickel content of nickel dimethylglyoximate.

METHODS

A relatively large amount of each butter culture was steam distilled, after adding ferric chloride, and the distillate treated with hydroxylamine hydrochloride, sodium acetate, and nickel chloride solutions; 200 ml. of 40 per cent ferric chloride solution (40 gm. made up to 100 ml. with distilled water) were used per liter of butter culture, and the other reagents were employed in amounts sufficient to insure complete precipitation of the diketone present. Each precipitate was washed repeatedly with distilled water by decantation and then collected, with suction, on a crucible having a fused-in sintered filter disc; the precipitate was also thoroughly washed on the crucible. The salt was removed from the crucible and dried at slightly more than 100° C. During the decantation there was some loss of the precipitate, so the salt was determined quantitatively on a 200-gm. portion of each butter culture, using the procedure followed by Michaelian

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and Hammer (2) for determining both the diketone present originally and that resulting from an oxidation with ferric chloride.

The nickel content of each salt was determined by decomposing, with sulfuric and nitric acids, a portion that had been thoroughly dried and precipitating the nickel as dimethylglyoximate, which was collected and weighed.

RESULTS OBTAINED

The results obtained are given in Table 1. The percentages of nickel in the various salts prepared from butter cultures ranged from 20.14 to 20.30, all of the values being close to the theoretical for nickel dimethylglyoximate[1] and distinctly higher than the theoretical values for the higher homologues. An average nickel content of 20.30 per cent was obtained on the nickel salt made from a commercial preparation of diacetyl. When citric acid was added to the milk used in preparing the butter culture, the salt obtained had the same nickel content as the salts obtained when no citric acid was added to the milk. The amounts of nickel salt obtained quantitatively per 200 gm. of the various cultures were normal for butter cultures of a satisfactory quality (1).

TABLE 1

Nickel content of salts obtained when butter cultures were steam distilled with ferric chloride and the distillates treated with hydroxylamine hydrochloride, sodium acetate and nickel chloride

Butter cultures developed at 21° C.

Butter culture			Gm. of purified Ni salt obtained	% Ni in Ni salt	Mg. Ni salt obtained quantitatively from 200 gm. culture
No.	General quality	Ml. distilled			
15	excellent	3,000	0.5558	20.24, 20.20 } 20.22	45.8
146	fair	2,750	0.3524	20.29, 20.31 } 20.30	33.2
15	good	8,500	1.1838	20.25, 20.20 } 20.225	39.6
232	good	10,000	1.7534	20.17, 20.11 } 20.14	43.5
15*	**	10,000	3.2555	20.22, 20.25 } 20.235	84.1

* 0.15% citric acid crystals added to milk from which butter culture was made.

** Flavor and aroma were typical of a butter culture made from milk to which citric acid had been added.

[1] The theoretical Ni content of nickel dimethylglyoximate = 20.32 per cent; of nickel ethylmethylglyoximate = 18.52 per cent; of nickel diethylglyoximate = 17.02 per cent.

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From the data presented it appears that the diketone steam distilled from butter cultures after the addition of ferric chloride is diacetyl rather than one of the homologues and that if homologues are present they are limited to relatively non-significant amounts.

REFERENCES

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(3) U. S. Patent 1,945,347.

(0759)

FACTORS AFFECTING SURVIVAL OF *SALMONELLA* IN CHEDDAR AND COLBY CHEESE

R. E. HARGROVE, F. E. MCDONOUGH, AND W. A. MATTINGLY

Dairy Products Laboratory
Eastern Utilization Research and Development Division[1]
Washington, D. C. 20250

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ABSTRACT

Factors affecting the growth and survival of salmonellae in the experimental manufacture of 7 lots of Colby and 65 lots of Cheddar cheese were studied. Milk was artificially contaminated just prior to making of cheese. Manufacturing variables tested included effects of milk pasteurization, size of starter inoculum, titratable acidity and cheese pH, type of lactic culture, chemical additives, salt and moisture content, supplemental cheese microorganisms, and curing temperatures. The rate and amount of acid produced during making, the pH of the cheese, and the type and size of starter inoculum had significant effect in suppressing the growth and survival of salmonellae. Salt, moisture, chemical additives, and pasteurization of the milk, had little or no effect on the salmonellae. Addition of large numbers of *Propionibacterium* and *Leuconostoc* seemed to favor survival of salmonellae. Lactobacilli and enterococci tested had no effect.

Salmonellosis is a continuing health problem and its apparent increase can be attributed to an increasing awareness and more extensive surveillance. Salmonellae contamination in manufactured dairy products has become of increasing concern since 1966 when 11 serotypes were isolated from nonfat dry milk (NDM) from nine states *(8)*. Consequently, the Food and Drug Administration has indicated that it plans to screen all dairy products. In anticipation, these laboratories began a survey of a variety of dairy products to determine which manufacturing variables contribute to growth and dissemination of salmonellae and how procedures could be modified to insure safety of the product. Reports for Cottage cheese *(5)* and NDM *(6)* have been published.

A review of cheese-borne infections up to 1947 is given by Fabian *(1)*. More recently Price et al. *(7)* reported an outbreak from infected Cup cheese. In all instances for which background information is available, the cheeses were made from raw milk or from milk which was recontaminated after pasteurization. Pasteurization of cheese milk is not required providing the cheese is aged for at least 60 days prior to selling. Tucker et al. *(9)* reported survival of *Salmonella typhimurium* in Colby cheese for 302 days at 43 to 48 F. Goepfert et al. *(2)* reported survival of the same species in stirred curd Cheddar cheese for at least 12 weeks at 7.5 to 13 C.

Information is generally lacking concerning the effects of the many variables on salmonellae survival during manufacture and curing of Cheddar and Colby cheese. This paper reports the results of a study to determine such effects.

MATERIALS AND METHODS

Cultures

Four test species of *Salmonella* were selected on the basis of their heat resistance and frequency of occurrence in dairy products. They were *Salmonella senftenberg* 775W, *Salmonella typhimurium* TM1, *Salmonella new brunswick* 1608, and *Salmonella newport*. In most trials, 18-hr cultures of each, grown in trypticase soy broth, were added to the cheese milk 1 hr prior to manufacture. The inoculum ranged from 300 to 5000 per ml of milk.

Commercial mixed-strain lactic cultures were used in most instances as the starter. Additional cultures, used singly or as supplementals, included one or more species of *Leuconostoc*, *Streptococcus*, *Lactobacillus*, *Micrococcus*, *Pseudomonas*, and *Propionibacterium*. All lactic cultures were subcultured in sterile 10% NDM prior to use. The *Propionibacterium* was grown in lactose broth and the *Micrococcus* and *Pseudomonas* in nutrient broth.

Salmonellae determinations

Analyses for salmonellae were made on the following: *(a)* cheese milk at time of addition of salmonellae and lactic starter, *(b)* milk at setting (addition of coagulating enzyme), *(c)* curd and whey at cutting, *(d)* whey at cooking, *(e)* whey at draining, *(f)* curd at draining, *(g)* curd at salting, *(h)* curd at hooping (packing curd in molds), and *(i)* the 21-hr cheese. The cheeses were subsequently tested in two weeks and at monthly intervals thereafter.

Two procedures were employed to detect and enumerate salmonellae. When counts exceeded 10/g, direct plating on a selective, modified, lysine iron agar (Difco) was used. The antibiotic novobiocin[2] (5-10 μg/ml) was added aseptically to the cooled lysine agar just prior to plating to suppress the growth of many gram-positive microorganisms and select strains of *Proteus* and *Escherichia coli*. A very thin underlayment and overlayer were used in conjunction with conventional pour techniques. Plates were incubated at 37 C for 24 to 48 hr. All salmonellae used in this study grew readily in the agar, producing hydrogen sulfide and developing into large black lens-shaped colonies. Samples were prepared for plating by blending 11 g of cheese with 99 ml w/v of a sterile 0.2% sodium citrate solution. When counts were lower than 10/g, quantities of the blended samples were preenriched in a selective lysine-iron broth

[1]Agricultural Research Service, U. S. Department of Agriculture.

[2]Novobiocin, sodium (Albamycin UpJohn Co.)

(3) and identified by standard cultural procedure.

Make procedure

Seven lots of Colby cheese and 65 lots of Cheddar cheese were made in small steam-jacketed vats, each having a capacity of 50 lb of milk. Manufacturing variables studied included the effects of: milk pasteurization, size of starter inoculum, titratable acidity (TA) and cheese pH, type of lactic culture, chemical additives, salt and moisture content, supplemental cheese microorganisms, and curing temperatures. The manufacturing methods were essentially those prescribed by Lochry et al. (4) for Cheddar cheese and Wilster (10) for Colby cheese. Variations in the "make" procedure were necessary to compensate for some of the test variables.

Fresh whole milk, standardized to 3.3% fat, was usually pasteurized at 163 F for 15 sec and cooled to 90 F before inoculation with the *Salmonella* test organisms. Two vats were made with raw milk as the variable.

Both mixed and single strain cultures of *Streptococcus lactis* and *Streptococcus cremoris* were compared. Inoculum size was varied from 0.1 to 3%. In an attempt to maintain comparable pH values and TA in the cheese during manufacture and curing, the make time-schedule and other variables had to be adjusted. For example, the ripening time for the milk with 3% starter was reduced from 1 hr to 5 min, the time between cooking and draining was shortened to 15 min, cheddaring time was reduced to 30 min, the pressing temperature was lowered to 40 F rather than 70 F and the curing temperature during the first month was 32 F rather than 40 F.

In an attempt to pinpoint the role of pH, various methods for controlling acid were utilized. These included variations in schedule to mill at TA from 0.25 to 0.55%, buffering the cheese milk with phosphates, the use of age-attenuated lactic starters and direct acidification of cold cheese milk with edible acids.

The role of salt in the finished cheeses was studied by varying the salt content from 1.4 to 2.7%. The curd was usually dry salted, but in two experiments salt was added directly to the cheese milk at the rate of 1 and 1.5% w/v.

Make procedures were altered to control moisture levels within a range of 35 to 48%. Other variables included the use of inhibiting agents such as diacetyl, potassium sorbate, nisin, and sodium lactate.

Goepfert et al. (2) reported evidence that the production of volatile fatty acids during curing, especially acetic, may be a major factor in elimination of salmonellae from cheese. To explore this theory, cheeses were made by direct acidification using lactic and acetic acids. Sufficient acid was added to the cold cheese milk (40 F) to lower the pH to 5.35. Rennet and 0.1% starter were also added while cold. The temperature was then slowly raised to 80 F without stirring by circulating 100 F water through the jacket of the vats. After coagulation, in about 0.5 hr, the coagulum was cut and cooked to 100 F. The curd was then drained, cheddared for 1 hr, and salted.

Most cheeses were hooped and pressed at 75 F for 21 hr and were sealed by dipping in melted cheese wax. Most were stored at 40 F, but some were divided so that portions could be stored at 32, 40, and 50 F.

The pH of the cheese was determined after 21 hr in the press, after two weeks curing, and at monthly intervals thereafter.

RESULTS AND DISCUSSION

A typical pattern of salmonellae growth during manufacture is shown in Table 1. There was very little growth up to the time of cutting. Then a thirtyfold increase occurred during the 2-hr interval between cutting and draining. Since one would expect an approximate tenfold increase from cell concentration in the curd, the difference indicates a moderate growth rate of about two generations during the 2-hr period. Multiplication continued, resulting in a sevenfold increase (approximately three generations) during the 3-hr interval between draining and hooping. Following hooping, active growth stopped sometime during the pressing operation as evidenced by an 80% decline between 6-1/2 and 21 hr. These data are in good agreement with those of Goepfert et al. (2) who reported approximately 4.5 generations between cutting and hooping, followed by a 75% decline during pressing.

An assumption that salt initiated the rapid initial decline between hooping and 21 hr was generally disapproved by the fact that subsequent vats of cheese having salt contents from 1.4 to 2.7% showed no difference in decline during the same period. Additional vats of cheese were made in which 1.5% salt was added to the cheese milk. Resulting inhibition of the lactic starter necessitated increasing the inoculum to 3% or lengthening the cheddaring time by about 15% before milling. No inhibition of salmonellae was observed. Additional salt was then added to the curd to raise the final level to normal values. Figure 1 compares salmonellae survival during curing as affected by differences in salt content. Here again, no differences were observed. It can be concluded that salt plays little or no part in hastening the death of salmonellae during cheese manufacturing and curing.

Survival of salmonellae during curing varied from 2 to 9 months with an average of 6.5 months for all vats. The variables significantly affecting survival were pH and type and amount of starter. These data are presented in the accompanying figures.

Figure 2 shows the effect of cheese pH on survival of salmonellae throughout curing. Zero time represents the salmonellae count of the cheese after 21 hr in the press. The pH range given indicates the pH of the cheese from 21 hr to final sampling. Cheeses with an abnormally high pH, caused by starter failure (dead vats) obviously have little or no inhibitory effect on the salmonellae. The most rapid decline was associated with pH 5.0. Such cheese made from pasteurized milk would probably be criticized for acidity and bitterness. However, raw milk cheeses with pH values from 5.0 to 5.1 are common and are usually acceptable in flavor and acidity.

Figure 3 shows dramatic differences in effects of amount of starter on survival during storage. Zero time in the figure represents the salmonellae count

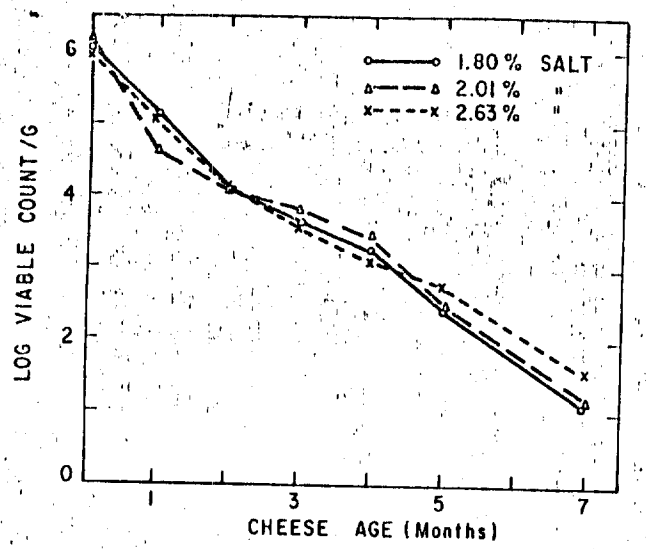

Figure 1. Effect of salt on *Salmonella* survival in Colby cheese.

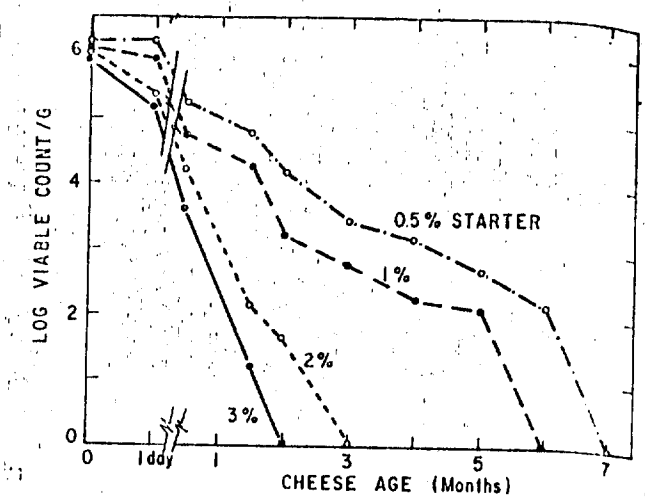

Figure 3. Effect of lactic starter inoculum on *Salmonella* survival in Cheddar cheese.

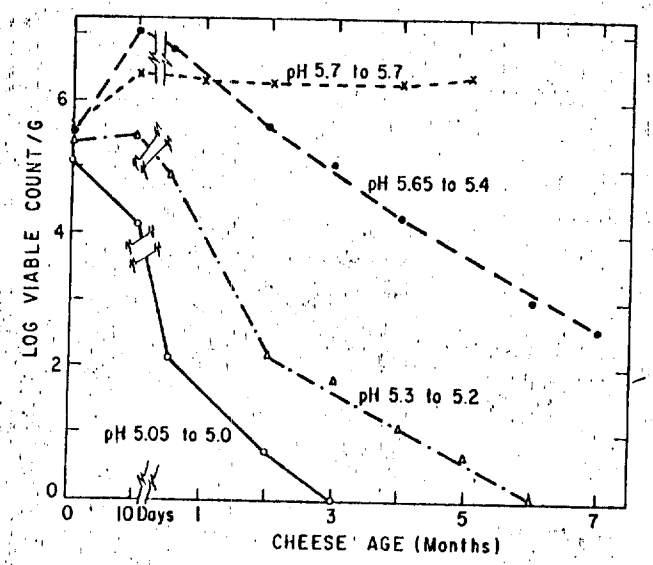

Figure 2. Effect of pH on *Salmonella* survival in Cheddar cheese.

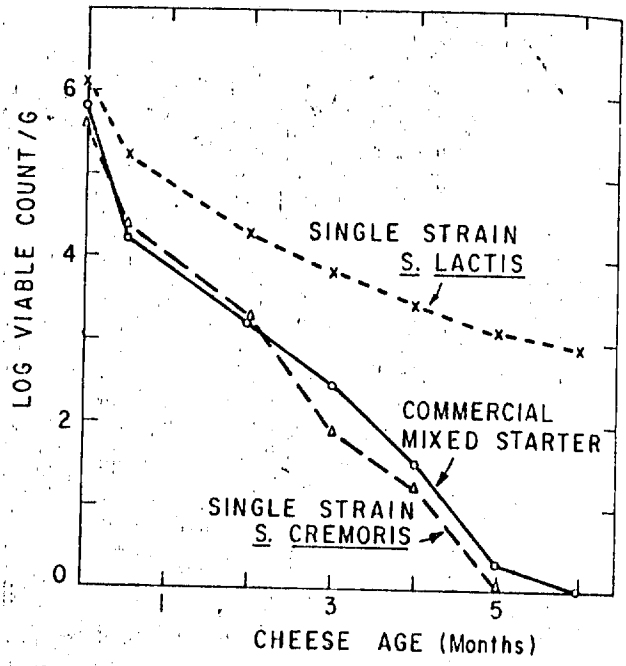

Figure 4. Comparison of cheese starter on *Salmonella* survival in Cheddar cheese.

of the cheese curd just prior to pressing. The count of the cheese made with 0.5% starter showed no decline during the first day of storage. In contrast, an initial reduction of about 80% occurred in the cheese made with 3% starter. All of this decline was not caused by the acid. The various control techniques mentioned earlier succeeded in maintaining essentially identical pH development through pressing. At milling, the TA of all four lots was 0.53%, and the 21-hr pH values were 5.55, 5.55, 5.45, and 5.45, respectively, for 0.5, 1, 2, and 3% starter. The differences at two months were greater, varying from pH 5.31 (0.5% starter) to 5.00 (3% starter). Thus the control of acid development until the cheeses with less starter could "catch up" to that with 3% starter indicates that the lactic starters were somewhat inhibitory.

In order to study possible differences in natural inhibitory effects of different species of lactic starter, several species of *S. cremoris* and of *S. lactis* were compared to commercial multiple-strain cultures. Efforts were made to develop comparable acidities and to produce cheese of similar pH and composition throughout manufacture and curing, thus eliminating variables other than starter. The pH of these cheeses was within the range of 5.18 to 5.29 after one day and 5.19 to 5.25 after two months of curing. Figure 4 is representative of the results obtained. The species of lactic starter used in cheesemaking appears

TABLE 1. SURVIVAL OF SALMONELLAE IN CHEDDAR CHEESE MANUFACTURE[1]

Manufacturing operation	Time hr	Salmonellae No/ml - g
Starters added	0	1,400
Milk at setting	1	1,500
Curd + whey at cutting	1 1/2	1,560
Whey at cooking	2	230
Whey at draining	3 1/2	410
Curd at draining	3 1/2	47,100
Curd at salting	5 3/4	310,000
Curd at hooping	6 1/2	318,000
Cheese after pressing	21	60,000

[1]0.75% lactic starter; milling TA 0.40%; 21 hr pH 5.4.

TABLE 2. EFFECT OF TEMPERATURE ON SURVIVAL OF SALMONELLAE DURING STORAGE

Cheese age months	32 F		40 F		50 F	
	No./g	pH	No./g	pH	No./g	pH
0	61×10^4	5.52	61×10^4	5.52	61×10^4	5.52
1	18×10^3	5.38	11×10^3	5.38	16×10^3	5.30
2	39×10^2	5.31	32×10^2	5.23	20×10^2	5.16
3	65×10^1	5.30	60×10^1	5.22	6×10^1	5.15
4	60	5.25	18	5.18	0	5.10
5	30	5.21	2	5.15	0	5.10
6	10	5.22	0	5.15	0	---

to have a marked effect on survival, probably because of the production of an unknown inhibitory factor. Species of *S. cremoris* were much more inhibitory than *S. lactic* species. These results were confirmed by comparing lactic streptococci grown on agar plates seeded with salmonellae. *S. cremoris* strains produced greater zones of inhibition than either *S. lactis* or *S. thermophilus*. The antibiotic "nisin" and nisin-producing strains of *S. lactis* had no effect on salmonellae. Supplemental starter microorganisms, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Pseudomonas fragi*, and *Micrococcus cascolyticus* had no apparent effect on salmonellae when added to the cheese milk. In contrast, large numbers of *Leuconostoc* bacteria and *Propionibacterium shermanii* added as starters resulted in the survival of salmonellae at higher levels and for longer periods of time than in controls. The pH of these cheeses was slightly higher which may account for this effect.

None of the additional variables tested were of value as a means of salmonellae control. No apparent differences were detected when raw milk cheese was compared with pasteurized milk cheese. Moistures had no direct influence. Potassium sorbate and sodium lactate had no inhibitory effects while high levels of diacetyl slowed the growth of both salmonellae and lactic starter.

Results of storage at 32, 40, and 50 F, shown in Table 2 revealed slight differences with a more rapid decline at higher temperatures. These variations were not considered significant and appeared to be related more to greater acid development at the higher temperatures.

The results obtained from the cheeses acidified with lactic and acetic acids confirm the roles of both pH and starter. Both cheeses were inoculated with only 0.1% starter, much less than would normally be used. The starter organisms grew slowly in the lactic acid cheese to a maximum of 2,000,000/g. This cheese became gassy in three months with a coliform count of 180,000/g. The salmonellae declined slowly during storage but the rate of decline was about one-half that of cheeses with a comparable pH developed by normal amounts of starter. The cheeses acidified with acetic acid gave the same pattern for salmonellae; the decline was about half as fast as expected. This cheese, however, indicated inhibition of lactic starter and coliforms. The starter grew to a maximum of 16,000/g while no coliforms were evident. It appears that a pH of 5.2 to 5.3 was sufficient to cause death of salmonellae during storage, but the lower rate of decline in the absence of normal amounts and development of starter offers further evidence of the natural inhibitory nature of starter organisms. While acetic acid appears to be inhibitory to some organisms, it had no apparent effect on the salmonellae.

The results of this study revealed no variables that could be used to insure freedom of Cheddar or Colby cheese as a source of salmonellosis should these organisms enter cheese milk as post pasteurization contaminants. Only pH and starter significantly influenced salmonellae survival. All other variables had indirect effects; that is, their role was obvious only to the degree that they influenced the development of acid. Thus, factors which contributed to subnormal acid production enhanced growth and survival of salmonellae and those that contributed to normal or excessive acid production were detrimental. It should be pointed out that the degree of salmonellae contamination used in these studies far exceeded that which would likely occur from post-pasteurization contamination. However, it does reflect the need for quality control.

Although the incidence of salmonellosis is apparently increasing, the dairy industry has maintained a good record. United States Public Health officials have found no salmonellae in their survey of Cheddar cheese. In addition, no outbreak of salmonellosis has been traced to Cheddar type cheese

since 1945. This paper, by pointing out the possibility of salmonellae survival for long periods of time, reemphasizes the need to maintain strict adherence to proper and sanitary procedures.

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Effect of Diacetyl on Microorganisms.* (21054)

LOYD W. HEDGECOCK AND DAVID V. COHN. (Introduced by Tom Hamilton.)

From the Research Laboratory and Radioisotope Unit, Veterans Administration Hospital, Kansas City, Mo., and the Departments of Medical Microbiology and Pharmacology, University of Kansas Medical Center, Kansas City, Kans.

It is not clear whether diacetyl enjoys a major or relatively minor role in the metabolism of plants and animals. This compound occurs in a large variety of natural products such as tobacco, butter, coffee, cocoa, beer, and honey. The citric acid-fermenting streptococci produce diacetyl and cultures of these organisms are widely used for the production of desirable flavors in dairy products(1). This compound is metabolized by bacteria, bacterial extracts and mammalian tissue through

* A portion of this work was done in the Department of Bacteriology, St. Louis University School of Medicine, St. Louis, Mo.

TABLE I. Inhibitory Concentration of Diacetyl for Microorganisms.

Organism	Diacetyl (μg/ml)	Organism	Diacetyl (μg/ml)
E. floccosum	200	*A. aerogenes*	400
A. niger	"	*P. vulgaris*	"
S. carlsbergensis	"	*B. mycoides*	"
S. griseus	"	*B. subtilis*	"
E. coli	300	*B. anthracis*	"
P. pyocyaneus	"	*Cl. tetani*	"
S. paradysenteriae	"	*S. viridans*	"
S. schottmuelleri	"	*M. pyogenes*	60
S. typhosa	"	*S. hemolyticus*	80
M. tuberculosis (607)	"	*S. fecalis*	"
M. pulei	"	*S. lactis* (8043)	120
N. catarrhalis	"	*L. casei* (7469)	160

Laboratory strains except where indicated.

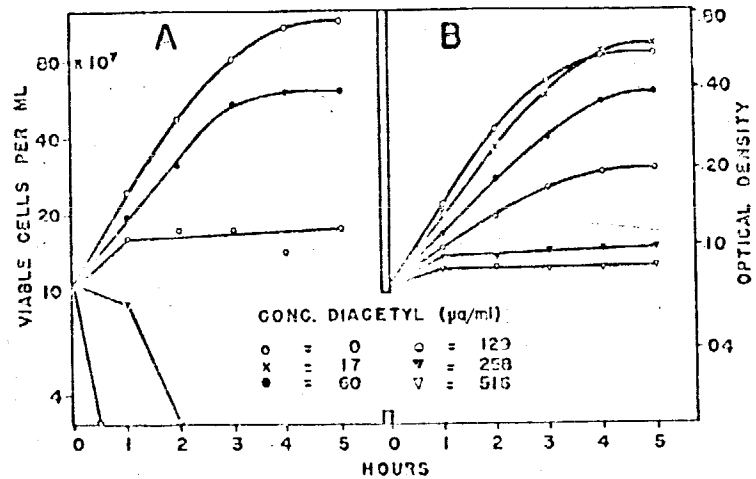

FIG. 1. Effect of diacetyl on microorganisms.

reactions in which diphosphothiamine is involved(2,3). In view of this information it seems surprising that little attention has been directed toward the antimicrobial properties of diacetyl which were first reported by O'Meara(4) and Jolander(5). The present paper describes the effect of diacetyl on the growth of a variety of microorganisms as well as a quantitative evaluation of its antibacterial action.

Experimental. The concentrations of diacetyl necessary to prevent growth of various microorganisms in Trypticase Soy broth for a 24-hour period are listed in Table I. The growth of all organisms tested was inhibited by diacetyl. Relatively small differences existed between the susceptibility of the fungi and many of the bacteria. However, the streptococci and lactobacilli, certain of which are known to produce substantial amounts of diacetyl, were the most resistant to its action. The values are maximal since an indefinite amount of diacetyl escaped from the unsealed tubes through volatilization and small amounts may have been inactivated by components of the medium. To evaluate the antibacterial action of diacetyl, *Escherichia coli* was chosen as the test organism because of its relatively simple growth requirements, its intermediate location in the diacetyl-sensitivity spectrum, and the availability of information concerning its metabolism. The medium used in this study was composed of the following ingredients per liter of solution: sodium phosphate, dibasic, 3 g; potassium phosphate, monobasic, 2 g; ammonium sulfate, 1 g; potassium nitrate, 1 g; dextrose,† 0.25 g; salts B[1](6), 2.5 ml. An inoculum consisting of 6 x 10⁶ organisms in the logarithmic growth phase was transferred to screw cap test tubes containing 6 ml of sterile media. Growth was followed turbidimetrically, and aliquots were plated for determination of viable cells.

Results. The rate of growth and viability of *E. coli* in the presence of varying amounts of diacetyl are shown in Fig. 1A and B. In the *logarithmic phase* the rate of growth of *E. coli* decreased progressively with increasing amounts of diacetyl. There was close agreement between viable cell counts and turbidity measurements when the concentration of diacetyl was 60 μg/ml or less. With an increase in the amount of diacetyl to 129 μg/ml majority of the cells reproduced during the first hour only; however, a gradual increase in turbidity occurred throughout the 5-hour experiment. This increase may have been due to either slow reproduction of some cells balanced by death of others or may be explained by changes in the size of inhibited cells. A pronounced lethal action of diacetyl was effected in concentrations exceeding 12

† Solutions [illegible]

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TABLE II. Effect of Concentration of Medium on Action of Diacetyl.

Conc. of medium	Optical density (× 10³) Log phase (2 hr) Diacetyl μg/ml 0	17	60	Stationary phase (5 hr) 0	17	60	Final growth (22 hr) 0	17	60
1X	213	215	165	677	550	290	820	770	460
2X	266	215	195	685	660	420	770	770	630
1X	207	231	191	495	450	415	530	695	509
½X	215	209	179	345	595	345	390	540	425

μg/ml, although the increase in turbidity during the first hour indicated that initial reproduction had occurred. Thus, diacetyl appeared to exert an inhibitory action at lower and a lethal action at higher concentrations. To test the possibility that these effects might be influenced by the initial number of cells, the amount of inoculum was varied in 5 steps from 9 x 10⁵ to 12 x 10⁶ cells. The rate of cellular division was constant regardless of the size of inoculum. Restoration of viability of cells(7) which had been exposed to lethal concentrations of diacetyl for 22 hours could not be demonstrated following incubation of the cells for 24 hours in buffered solutions of a variety of metabolites.

As the *stationary phase* of growth was approached the effect of diacetyl at a concentration of 17 μg/ml differed from that of higher concentrations in that turbidity finally exceeded that of the controls. This observation suggested that the normal medium was deficient in amounts of components necessary for maximal growth, and that diacetyl was serving as a substrate for growth. To test this possibility the action of diacetyl was determined in ½, 1, 2, and 4 fold (X) concentrations of the medium used previously. The results of this experiment are summarized in Table II. At concentrations of both 17 and 60 μg/ml diacetyl enhanced final growth in the most dilute medium although this effect was not observed in the more concentrated media. That a deficiency in buffering capacity and dextrose content actually existed in the 1X medium was demonstrated in experiments in which the amounts of individual components of the normal medium were increased.

The postulate that diacetyl served as a substrate for growth was confirmed by a demonstration of its disappearance from the medium during growth (Table III). Furthermore, when the *utilization per unit turbidity* (K) was calculated this was found to be greatest in deficient media. For example, in dilute medium (½X) K equaled 52 after 24 hours growth, whereas, in a more concentrated medium (2X), K equaled only 38.

Discussion. The manner in which diacetyl influences growth of *E. coli* is not understood. It has been established that the inhibitory effects of this compound remained unaltered throughout an 8-fold range of medium-concentration. Hence, inhibition may not be explained by a non-reversible combination of diacetyl with an essential substance in the growth medium. Although the possibility exists that diacetyl reacts irreversibly to inactivate essential components of the cell, failure to observe any correlation between inhibition and size of inoculum does not support this concept. The initial reproduction of cells

TABLE III. Utilization of Diacetyl by *E. coli* during Growth in ½X Medium.

Incubation, hr	Exp. conditions	Diacetyl recovered (μg/ml) 0°C	37°C	Δ (0°-37°)	Utilization over control (Δ_E-Δ_C)
5	A. Inoc. M[1]	0	0	—	
	B. Inoc. M + 60 μg/ml D	57	41	16	14
	C. M + 60 μg/ml D. No inoc.	57	55	2	
21	A. Inoc. M	0	0	—	
	B. Inoc. M + 60 μg/ml D	57	24	33	22
	C. M + 60 μg/ml D. No inoc.	61	50	11	

[1] Diacetyl determined by method of White, *et al.*(8). Individual tubes of inoculated and uninoculated ingredients were maintained at 0°C and 37°C to control for loss of diacetyl through volatilization and possible chemical reactions.

[2] Inoc. M = Inoculum M; M = Medium; D = Diacetyl.

It occurred during the first hour in the presence of inhibitory amounts of diacetyl may indicate that in addition to its utilization in a normal metabolic pathway the compound may also interfere with the metabolism of an essential substance. Studies concerning this phase of the problem are now in progress.

Summary. Diacetyl prevented the growth of all of a variety of microorganisms in a range of 200-1600 μg/ml. In quantitative studies with a single strain of *E. coli*, diacetyl exerted an inhibitory action at low concentrations (< 129 μg/ml) and lethal action at higher concentrations (> 129 μg/ml). It was demonstrated that diacetyl was utilized during growth of *E. coli* and enhanced final growth when the medium was deficient in dextrose.

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