STARTER DISTILLATE #86

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STARTER DISTILLATE

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SUMMARY

Chemical Information

Starter distillate is a mixture of substances, not all of which have been identified. No objective definition was found in the official compendia nor in suppliers' manuals. The following provisional definition was proposed by the writer of this monograph and has been agreed to in writing by three major suppliers (0320, 0328, 1335) for the purposes of this monograph:

> "Starter distillate is a steam distillate of starter resulting from the culture of any or all of five species of bacteria together with the medium in which they are cultured, namely, milk or fractions of milk. The five species are *Streptococcus lactis*, *S. cremoris*, *S. diacetilactis*, *Leuconostoc citrovorum*, and *L. dextranicum*." (1778).

Starter distillate is used as a flavor enhancer for cultured dairy products, but it does not enhance the acuity of the chemical senses. Conditions of culture can be varied within limits so as to modify the resulting flavor of the distillate, which is standardized by blending and by organoleptic tests; standards and conditions vary and are proprietary to each supplier. Flavor mixtures simulating starter distillate (1335) are outside the scope of this monograph.

These difficulties in defining the monograph subject appeared to result from the history of starter distillate (0768), its development (1189), and early attempts by the Food and Drug Administration (FDA) to regulate its sale (1169,1815,2090). Diacetyl is the main (about 90%) component of starter distillate.

Long Term Studies

None were found, either on starter distillate or on diacetyl.

Special Studies

The radiomimetic effects on root-tip meristems of a large number of compounds were tested by Loveless (1208) and diacetyl was among the substances found to be inert. However, another study on root-tip meristems (0137) indicated that diacetyl at 0.08-0.10 M/liter delayed metaphase and increased the anaphase count in root-tip meristems. In a study on B. megaterium, O_2 increased radiation-sensitivity, and diacetyl acted similarly with up to 40% of the effect of O_2, so long as the diacetyl concentration was less than 6×10^{-3} M; these findings were not explained (1985).

Mitochondrial swelling in rat liver, an early manifestation of carcinogenicity of azo dyes, was inhibited by 10^{-2} M diacetyl (0077,0078).

A number of pathogenic organisms have been reported as killed or inhibited by diacetyl (1174, 1399, etc.), including some viruses (0413,1379).

Breakdown

Both the formation and the breakdown of diacetyl in foods have been studied and attributed to bacterial action (0250, etc.). The major enzymatic pathways have been worked out (0250); pyruvate is converted to acetolactate and CO_2; acetolactate is converted to AMC and CO_2; AMC and diacetyl are interconvertible, and AMC is reversibly converted to 2,3-butanediol. Four enzymes are reported as involved, and questions of specificity remain to be fully determined (0250).

Absorption and Distribution

No reports of detailed studies were found.

Metabolism and Excretion

In a study using rat liver slices and homogenates, the slices converted diacetyl to AMC which was further metabolized, whereas homogenates did not further metabolize the AMC (0934). In another study, using rat liver mince, high concentrations of AMC were metabolized to 2,3-butanediol with very little CO_2 formation. Three of the enzymes concerned were studied intensively (0606). The metabolism of diacetyl has also been studied in bacteria cultured so that 2,3-butanediol was the sole source of carbon in the medium (0872).

Urinary excretion of diacetyl and AMC were studied in rats and humans, and were increased in both species in the presence of diabetes (0993).

Effects on Enzymes and Other Biochemical Parameters

In a study on the effects of spray-drying on milk-components, diacetyl was found to have greater affinity for proteins than for milk sugars, attributed partly to the conditions of the study (1672). Diacetyl production in cultures has been found to be inhibited by iodophor detergents (2181). Milk from cows with hoof-and-mouth disease contained reduced amounts of diacetyl (1566).

In flavor studies, taste panel preferences for diacetyl or starter distillate flavor against control samples have been inconclusive (1002,1695). Diacetyl perception thresholds have been reported as ranging from 0.01 ppm to 0.2 ppm, according to pH, and the authors suggested that the presence of fat might influence the acid effect on taste perception (0172). Although diacetyl had the lowest taste threshold among 31 volatiles tested in another study (1874), interactions abounded when more than one compound was tested at a time.

Two methods of discriminant analysis have been claimed to correlate organoleptic and gas-chromatographic evaluations of beverages containing diacetyl (2263).

Pyruvate decarboxylase of yeast was partly inhibited by diacetyl *in vitro* (0610), and in diabetic rats and humans, liver xanthine oxidase activity was diminished (0993).

Drug Interactions

Carotene was found to act as an antioxidant when added to butter, and this effect was enhanced by various substances including diacetyl (1819).

Consumer Exposure

Diacetyl occurs widely in natural and processed foods, and no estimates were found of exposures to this source of diacetyl.

Starter distillate and/or diacetyl are added to certain dairy products for flavoring, and estimates of exposure are tabulated in the monograph.

In 1968 a WHO/FAO Committee (0581) included diacetyl among compounds for which exposure recommendations could not be made owing to lack of data.

In 1961 diacetyl was classed as GRAS (0057).

In 1967 diacetyl and starter distillate were allowed to be added to creamed cottage cheeses (0060).

In 1973 both substances were allowed to be added as flavoring agents to oleomargarine in amounts sufficient for purpose (0066).

CHEMICAL INFORMATION

I. Nomenclature

Starter Distillate

A. Common name: Starter distillate

B. Chemical name: None for the complete product, which is a mixture.

C. Trade name: Starter distillate

D. Chemical Abstracts Services Unique Registry Number: None listed.

Diacetyl

A. Common name: Diacetyl. Synonyms: Biacetyl, 2,3-butanedione, butanedione, dimethyl ketone, demethyl diketone, dimethylglyoxal, 2,3-diketobutane.

B. Chemical name: 2,3-Butanedione (8th C.I. Nomenclature)

C. Trade name: None listed.

D. Chemical Abstracts Services Unique Registry Number: 431038

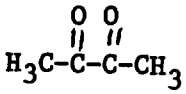
II. Empirical formula:

Starter Distillate is a mixture, of which only the principal component, diacetyl, is treated in this monograph.

Diacetyl: $C_4H_6O_2$, or $CH_3COCOCH_3$

III. Structural formula

Diacetyl:



IV. Molecular Weight:

Diacetyl: 86.09

V. Specifications

A. Chemical

1. Starter Distillate

No mention was found in the official compendia (0034, 0353, 0605, 1495, 2071). No specifications were found in the literature that could constitute an objective standard of identity for starter distillate.

One supplier (0321) defined starter distillate as "... 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). 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."

Because starter distillate is an end-product of natural fermentation and steam distillation, it is a mixture of volatiles, not all of which have been identified (1191) (Table 1). Its history (see section on Description) contraindicated the use of subjective, e.g., organoleptic, specifications as part of a general standard of identity; nevertheless a general standard of identity was required for the purposes of this monograph.

Lindsay (1967) (1188) had listed five species of bacteria as used in the specific culture: lactic acid producers, *Streptococcus lactis* and *S. cremoris*; citric acid producers, *Leuconostoc citrovorum* and *L. dextranicum*; and the aroma producer, *S. diacetilactis* which generates mainly diacetyl. Informal inquiries disclosed that the currently acceptable conditions of culture (among suppliers and their consultants, notably F.J. Babel, Professor of Dairy Microbiology, Purdue University) included a temperature range of 21-25°C with 21°C considered optimal for balance between lactic and citric acids produced and 25°C considered optimal for activity of *S. diacetilactis*; a pH range of 4.1-4.8; and a duration of culture of 24-48 hours with the optimal considered to be approximately 44 hours. The culture is then steam distilled, and the distillate is standardized by blending; in practice the standards are levels (a) of diacetyl and (b) of total volatile fatty acids (VFA) based on analyzed values for acetic and propionic acids, considered representative. The values of the standards used are proprietary and are determined organoleptically.

Table 1

Summary of Volatile Compounds Identified in Butter Culture and Control Heated Milk Distillates* (1191)

Compound	GLC packed column				GLC capillary		Mass spectra	
	DEGS		Apiezon M					
	Culture	Control	Culture	Control	Culture	Control	Culture	Control
Methane	–	–	–	–	–	–	+	–
Methyl chloride	–	–	–	–	–	–	+	–
Hydrogen sulfide	–	–	+	+	–	–	–	–
Methyl mercaptan	–	–	+	–	–	–	–	–
Dimethyl sulfide	–	–	+	+	–	+	–	–
2-Mercaptoethanol	–	–	+	+	–	–	–	–
Acetaldehyde	–	–	+	+	+	+	+	–
n-Butanal	–	–	+	+	–	+	–	–
Methylpropanal	–	–	+	–	–	–	–	–
n-Pentanal		–	+	–	+	–	–	–
2-Methylbutanal			–	–	–	–	–	–
3-Methylbutanal			–	–	–	–	±	–
n-Octanal			–	–	+	–	–	–
2-Furfural		–	–	–	+	+	+	–
Acetone	–	–	–	+	+	+	+	–
Diacetyl	–	–	+	–	+	–	+	
Acetoin	–	–	–	–	+	–	+	–
Butanone	–	–	+	–	+	+	±	±
2-Pentanone	–	–	+	–	+	+	–	–
2-Hexanone	–	–	–	–	–	+	–	±
2-Heptanone	–	–	+	+	+	+	+	–
2-Nonanone	–	–	–	–	+	+	+	–
2-Undecanone	–	–	–	–	+	+	+	–
2-Tridecanone	–	–	–	+	+	+	–	–
Methanol	–	–	+	+	–	–	+	–
Ethanol	–	–	+	–	+	–	+	–
n-Butanol	–	–	+	+	–	+	–	–
2-Butanol	–	+	–	–	+	–	+	–
n-Pentanol			–	–	+	–	–	–
2-Furfurol		–	–	–	+	+	+	–
Methyl acetate	–	–	+	+	+	–	+	±
Methyl butyrate	–		+	–	+	+	+	–
Methyl hexanoate			–	–	–	–	+	–
Methyl heptanoate	–		–	–	+	+	–	–
Methyl octanoate	–		–	–	+	–	+	–
Methyl nonanoate		–	–	–	–	–	–	–
Methyl decanoate			–	–	–	+	–	–
Methyl dodecanoate			–	–	+	–	–	–
Methyl benzoate		–	–	–	–	–	±	–
Ethyl formate	–	–	–	+	+	–	+	–
Ethyl acetate	–	–	+	+	–	–	–	–
Ethyl butyrate		–	–	–	–	–	–	–
Ethyl hexanoate					–	–	–	–
Ethyl octanoate					–	–	–	–
Ethyl nonanoate					–	–	–	–
Ethyl decanoate					–	–	–	–
Ethyl dodecanoate					–	–	–	–
n-Butyl formate					–	–	–	–
n-Butyl acetate					–	–	–	
2-Furfuryl acetate					–	–		
γ-Octalactone								–
γ-Decalactone					–	–	±	
Acetic acid						–		

*Includes both tentative and positive identifications.

Accordingly a proposal was circulated to suppliers of "butter flavor intensifiers" listed in a trade list (0065) that, for the purposes of this monograph starter distillate should be identified as -

> "a steam distillate of starter resulting from the culture of any or all of five species of bacteria together with the medium in which they are cultured, namely, milk or fractions of milk. The five species are Streptococcus lactis, S. cremoris, S. diacetilactis, Leuconostoc citrovorum, and L. dextranicum." (1778).

Three of the suppliers replied that they offered starter distillate, and all agreed with this provisional specification as an acceptable standard of identity (0320,0328,1335). In addition, one supplier (1335) stated that "Also a product simply termed starter flavor is developed which may be made entirely from a blend of chemical compounds or in conjunction with a pure starter distillate and then standardized to exact diacetyl levels. Generally the amount of material used in flavoring food products is such as to give a level of not more than 2 to 4 ppm diacetyl in the finished product." It was not intended that this "starter flavor" should come within the specification of starter distillate (1335), and it is accordingly considered to be outside the scope of this monograph, except in regard to its diacetyl content as a source of diacetyl (see section on Occurrence).

2. Diacetyl

Diacetyl is listed in the United States Pharmacopoiea (2071) and Food Chemicals Codex (0353) but not in other standard sources searched. It is specified (0353, 2071) to contain not less than 97% $CH_3COCOCH_3$, and not more than 3 ppm arsenic as As, 40 ppm heavy metals measured as lead, or 10 ppm actual lead as Pb (0353).

B. Food Grade

1. Starter Distillate

This is the only grade, since starter distillate has been reported only as a Flavoring Agent (0353) for dairy products. It is often added to enhance culture flavor in dairy products that have already been cultured with the specified organisms (1694). However, starter distillate is not a flavor enhancer of the type that enhances acuity of the chemical senses.

2. Diacetyl

Food grade diacetyl (0353), also classed as a flavoring agent, has similar specifications (those of medicinal grade diacetyl (2071), i.e., contains not less than 97% $CH_3COCOCH_3$ and not more than 3 ppm arsenic as As, 40 ppm heavy metals measured as Pb, or 10 ppm actual Pb.

C. Official Compendia

As noted above, no mention was found for Starter Distillate, but specifications were listed for Diacetyl per se.

VI. Description

A. Starter Distillate

The history of starter in buttermaking was summarized in 1934 by Hart and Lepper of the Food and Drug Agency (0768) because existing FDA standards of identity for butter were threatened with obsolescence. (Another summary is found on pages 326-328 of (0878). In 1933 Hammer, at the University of Iowa, had developed a cultured starter that seemed likely to alter the then accepted taste and analysis of butter. This development had climaxed more than a century of research during which, first, buttermilk or naturally soured cream had been inoculated into fresh cream about to be churned, and second, the streptococci producing lactic and citric acids had been isolated and cultured. Finally in 1929, in Holland, diacetyl had been identified as the primary odorant characteristic of butter, after which Hammer's 1933 culture had established its bacterial origin and also a production technique. Claims had been made that butter churned from cream inoculated with such cultures had superior keeping qualities; however, verification was needed and, for the FDA, a control over new procedures was needed in order to protect the identity and purity of butter offered for sale in interstate commerce.

One such new procedure that was being debated early in 1936 was the addition of diacetyl or a steam distillate of Hammer's cultured starter to butter as a flavoring agent (1169,2089,2090). This was opposed by the FDA as "not a step in normal butter production" and as "an entering wedge toward recognition of the use of commercial flavors...a deviation from general trade practice" at a time when "because of almost universal butter production on the farm, the large proportion of the population knows how butter is made"

(1169). On this basis the FDA stated (2090) that "the addition of flavoring materials of whatever character produces an article that is no longer butter within the meaning of the statute" and that regulatory action would be taken "against the flavored article wherever we find it in interstate commerce, and without reference to whether the product is sold as butter or as a butter containing artificial flavoring" as soon as means for detection had been worked out. Similar action was taken in other countries (1700) Ritter, W., Schweiz. Milchztg. 60: 567, 1934; no copy available).

By 1940 Hunziker (0878) was advocating Ruehe's method of adding flavors to butter, and by 1961 the whole situation had altered. Butter had become mainly a factory product, diacetyl and starter distillate had become "allowed for use in margarine" (1815), and no analysis had been discovered that would distinguish added flavors from those produced by the permitted starter itself. Thus in 1961 Chumlea's Laboratory, Inc. (0327) claimed that 90% of manufacturers were using the Ruehe techniques, and in the same year Riel and Gibson (1694) reported that keeping quality was unaffected by the added flavors and also that a taste panel had largely approved the flavored butters although official graders had preferred the control samples. In 1962 FEMA issued specifications for starter distillate (0575).

In 1965 Hall and Oser (0734) listed diacetyl but not starter distillate in the FEMA Table of GRAS substances, and Lindsay (1188) identified many of the trace volatiles present in steam distillates of starters. In 1967 Lindsay et al. (1192) reviewed findings to date, and Nielsen (1450) reviewed alterations in butter composition that might result from carelessness with the new techniques. The composition of cultured dairy products was again reviewed by Lindsay in 1967 (1189) and in the same year the addition of starter distillate or diacetyl to creamed cottage cheese was permitted by the FDA (0060).

The present situation has been described in the section on Specifications.

B. Diacetyl

The Merck Index (1926) describes diacetyl as follows: a yellowish green liquid, with quinone odor; the vapor having a chlorine-like odor. Melting point -2.4°C; boiling point 88°C. Specific gravity 0.990; refractive index 1.3933. Soluble in about 4 parts water; miscible with alcohol or ether. Use: as carrier of aroma in butter, vinegar, coffee, and other foods.

On the question of stability, Murdock (1392) stated that diacetyl was elaborated by bacterial spoilage organisms and evaporated except when headspace was restricted; thus determinations of diacetyl content of citrus concentrates and other food products were used as a quality control tools.

Diacetyl was classed as GRAS in 1961 (0057).

VII. Analytical Methods

A. Extraction and Separation

1. Volatilization - Because diacetyl is a volatile compound, extraction from samples is often carried out by steam distillation. In the method of Prill and Hammer (1626), recoveries of diacetyl by steam distillation were "practically quantitative" when 0.2 mg was added to 50 ml of fresh skim milk. The basic disadvantage of steam distillation is that any air allowed to enter will oxidize acetoin (acetylmethylcarbinol; AMC) to diacetyl.

Other workers have avoided the oxidation problem by purging the sample with a stream of N_2 or CO_2 (0477,1511). No difference was found between the gases (1511), although N_2 was preferred because it was less expensive.

2. Salting-out chromatography. - Speckman and Collins (1914) have separated diacetyl, acetoin, and 2,3-butylene glycol by salting-out chromatography. Columns were prepared with anion exchange resin converted to the sulfate form (when elution salts were sulfates) or the chloride form (when elution salts were chlorides). The four salts tested, ammonium sulfate, ammonium chloride, sodium sulfate, and sodium chloride were all capable of separating diacetyl and acetoin, but only ammonium sulfate effectively separated acetoin and 2,3-butylene glycol. The compounds always emerged from the column in order of decreasing polarity: 2,3-butylene glycol, acetoin, and diacetyl.

The method was found to be capable of separating the compounds when 25-100 ug of each was present in 1 ml of sample solution to be tested. Absorption to the column was not a problem as recoveries of the three compounds in over 100 tests ranged from 95-104%.

3. Time Considerations - As noted above, satisfactory separation of diacetyl, acetoin, and 2,3-butylene glycol were obtained by Speckman and Collins (1914) using salting-out chromatography with two different size columns. Using a 1.9 x 27 cm column eluted with 0.5 M ammonium sulfate at

0.5 ml/min, diacetyl was collected between ca. 75 and 112 ml effluent, or about 3.8 hours column time. When the column used was 3.5 x 54 cm eluted with 0.5 M sodium sulfate at 1.5 ml/min, diacetyl was collected between ca. 450 and 600 ml effluent, corresponding to about 6.7 hours of column time.

The Prill and Hammer steam distillation apparatus (1626) will extract diacetyl from 50 to 100 g of sample in 30 minutes of distillation time.

Drews, et al. (0477) using a gas purge of N_2 or CO_2 extracted diacetyl from 30 g of beer in 2 hours. Pack, et al. (1511), also using N_2 or CO_2, were able to extract diacetyl from 20 ml of Starter in 1.5 hours and, by using a manifold, were capable of purging and analyzing twelve 20 ml samples simultaneously.

B. Detection and Estimation

1. Voges-Proskauer Reaction - In 1946, Westerfield (2175) realized the need for a sensitive method for the detection and estimation of diacetyl and acetoin (specifically in blood). His method is a modification of other methods based on the observations of Voges and Proskauer that in the presence of alkali, diacetyl will form a colored product with a guanido group. The method is commonly referred to as that of Voges-Proskauer even though the original Voges-Proskauer method was not quantitative.

The reagents are added to the solution containing diacetyl and acetoin in the order of creatine (the guanido compound, first used by R.A.Q. O'Meara, (1472) followed by fresh alpha-naphthol in sodium hydroxide (first used by M. M. Barrit, (0136). The color is developed for 10 minutes at room temperature and read at 540 nm for diacetyl. Maximum color for diacetyl is obtained 5-10 minutes after the alpha-naphthol addition, and fades to a 10% error in 30 minutes. Blood acetoin is determined by oxidation to diacetyl using ferric chloride and ferric sulfate and subsequent analysis of "total" diacetyl. Standard curves showed a linear relationship for diacetyl at amounts less than 5-6 μg while the relationship departed slightly at amounts above this.

Subjecting the blood to acid hydrolysis prior to the oxidation of acetoin led to the production of excess diacetyl, probably from glucose. Oxidation of acetoin also led to small amounts of extraneous diacetyl, probably arising from pyruvate and lactate.

Owades, et al. (1504) further modified the Voges-Proskauer Method, which they called the VDK or vicinal diketone method. In addition to diacetyl, the method also measures 2,3-pentanedione and 1-phenyl-1,2-propanedione. The major deviations from the Westerfield modifications are that the alpha-naphthol is prepared in isopropanol and the creatine is prepared in a potassium hydroxide solution. Negative results were observed for:

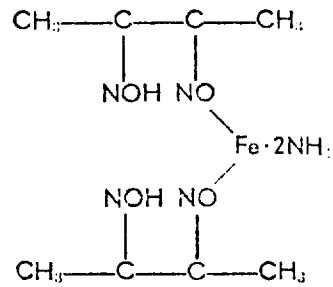
acetone
glyoxal
acetylacetone
acetaldehyde
butyraldehyde
1,2-cyclohexanedione
methyl hydroxy butanone

Based on a study of four available methods, the above procedure was recommended for diacetyl determinations in beer for amounts up to approximately 1.5 ppm. The recommendation and study was submitted by an appointed subcommittee to the Institute of Brewing Analysis Committee and published (0101).

Although the Voges-Proskauer reaction was almost the universal basis for diacetyl estimation in the citrus concentrate industry through the mid-1960's there was no standard method or procedure in use. It was this realization which led D. I. Murdock to study the optimum conditions for the Voges-Proskauer method of diacetyl determination in citrus juice products and to propose a method based on his findings (1389). The method outlined standards for both preparation of reagents and procedure of the determination. Murdock noted the importance of reading the color at a specific time after adding the reagents (alpha-naphthol and creatine in KOH). He suggested that the optical density be read 60 seconds after adding the second reagent and further noted that the order of adding the reagents was not critical.

2. Prill and Hammer Method - Prill and Hammer (1626) presented a method for the determination of diacetyl (in milk) which is used widely. Diacetyl is extracted by distillation and reacted with hydroxylamine hydrochloride in a solution of sodium acetate to form dimethglyoxime. The excess hydroxylamine is removed and the solution buffered to near neutrality with dipotassium phosphate. The dimethylglyoxime is reacted with ammonium hydroxide in a saturated tartrate solution, and then with ferrous sulfate.

The red complex which is formed is probably the ammono-ferrous-dimethylglyoximate (1626) with the formula (0226):



The complex is determined colorimetrically at 530 nm.

The method as described by Prill and Hammer is able to detect a difference between 0.001 mg and no diacetyl in 5 ml of water. The range for determination is 0.01 to 0.5 mg diacetyl in a volume of 10 ml. The data showed good agreement with a gravimetric method (precipitation of the dimethylglyoximate with Ni^{2+} in chloroform).

Glyoxal, methyl glyoxal, and acetylpropionyl all showed interfering red colors. Pyruvic acid developed a yellow color. Acetoin does not interfere, but may be measured by oxidizing it to diacetyl.

Arthur Brandon (0226) has presented an automated method for diacetyl determination in beer which is based on the Prill and Hammer method. The automated procedure showed good agreement with a manual procedure, with a reproducibility of $\pm$ 0.0055 at the 0.1 ppm level.

Pack, et al. (1511) have used the Prill and Hammer colorimetric method for the determination of diacetyl in mixed-strain starters. Diacetyl is purged by passing N_2 (or CO_2) through a tube containing 20 nl of sample. The volatilized diacetyl passes into a tube containing the hydroxylamine reagent. The tube with the hydroxylamine and diacetyl (now dimethylglyoximate) is treated with ferrous sulfate and the optical density determined at 530 nm. By using a manifold, several samples may be purged and reacted with hydroxylamine simultaneously. The method was sensitive to 3.0 ppm diacetyl and recovery was 92%

3. Other Colormimetric Methods - Englis, et al. (0521) have shown that dimethylglyoxime absorbs strongly at 226 nm, and therefore diacetyl converted to the dioxime can be measured directly. Good agreement was found

between this method and the Prill and Hammer method. The method is sensitive and may be used to measure quantities of diacetyl between 0 and 10 ppm. No interference was noted from acetoin.

Siest and Landry (1875) studied the color reaction of diacetyl with urea (in the presence of acid and phenazone) as a method of diacetyl estimation. Two different acid systems were examined, one using arsenic-sulfuric acid solution and the other $FeCl_3$-phosphoric acid solution.

In both systems, optical density is measured at 460 nm and both are equally sensitive; 10 nmoles gave an OD reading of 0.07. Each system has a unique extinction coefficient. The reaction in arsenic-sulfuric acid is complete in 15 minutes, while the ferric chloride-phosphoric acid system requires 30 minutes. In the arsenic-sulfuric solution, acetoin was only one-tenth as effective as diacetyl in producing the color. In the ferric chloride-phosphoric acid solution, acetoin is oxidized to diacetyl and measured simultaneously.

Lees and Jago (1158) found that mixing 1 ml of 1mM diacetyl with 1 ml of 6.7 nM semicarbazide resulted in a product which had a strong absorption maximum at 272 nm. Acetoin and acetaldehyde do not interfere, since their semicarbazones absorb at lower wavelengths. The relationship for diacetyl at 272 and 224 nm was linear for concentrations up to 500 µg/ml. The ratio of absorbance at 272 nm to absorbance at 224 nm for diacetyl was always greater than 2, which is useful in determining the presence or absence of acetoin and acetaldehyde.

The semicarbazone of methylglyoxal also shows an absorption maximum at 272 nm. Glyoxal semicarbazone absorbs strongly at 281 nm.

4. Gas Chromatography - Drews et al. (0477) have presented a chromatography method for separation and quantitative determination of diacetyl and 2,3-pentanedione (in beer). The diketones are removed from the beer by volatilization in a gas stream and are trapped in carbonyl-free 96% alcohol in an ice-bath. 1-2 µl of this solution are chromatographed. The best separations were obtained on a glass or stainless steel column 4.2 meters long, packed with 5% Igepal (nonylphenoxypolyethyleneoxyethanol). Copper columns could not be used. Column temperature was 70°C and an electron capture detector was employed.

Quantitative evaluations were made by comparing the sample peaks to peaks obtained from a series of standards run under identical conditions. Detector readings were linear and reproducible with maximum deviations of $\pm$ 5%.

Scanlan and Lindsay (1784) have determined diacetyl quantitatively in the ppb range by a gas entrainment-on column trapping procedure using gas chromatography and an electron capture detection system. A curve was obtained using a series of standards and was shown to be linear (based on peak height) through 160 ppb. Milk containing as little as 2-3 ppb was successfully analyzed by this method.

5. Polarographic Method - Ferren et al. (0557) have presented a polarographic method for the determination of diacetyl which offers three distinct advantages:

a) no involved pre-treatment of sample is necessary,

b) N_2 blanket eliminates auto-oxidation of acetoin to diacetyl,

c) measurements are made in situ, and not on the sample distillates.

The half-wave (which was found to be pH dependent) for diacetyl is -0.76 volts. At -1.7 volts, the second carbonyl group is reduced to a hydroxyl group and acetoin will interfere. No interference is observed from acetoin at -0.76 volts. Diacetyl content can be determined down to the 3 ppm range using ordinary equipment. By adding an auxiliary micro-extender unit, 1 ppm can be measured.

6. Micro-detection Method - Markovic (1262) has presented a rapid and simple method for the detection of micro-quantities of diacetyl. The sample containing the volatile ketone is placed in a beaker which is covered with a specimen holder carrying a "hanging drop" of 15% aldehyde-free acetic acid, into which a few crystals of p-nitrophenylhydrazine are dissolved. After setting at room temperature for some time, the drop can be observed microscopically for formation of the hydrazone reaction product. When sufficient product is formed, the drop is removed and to it is added a drop of acetone-water (4:1). When all the crystals are dissolved in the acetone mixture, the excess acetone is evaporated, leaving the precipitated hydrazones of the ketone being sought and the acetone. (A second evaporation may be performed to insure the conversion of all the residual hydrazine compound to the acetone hydrazone.) After the crystals are collected and dried, the eutectic temperature of the mixed hydrazone is determined.

Markovic has found the method to be sensitive to 10 μg per ml of water. The sensitivity was determined for a reaction time of 10-15 minutes and, as the author suggested, could possibly be increased by increasing the reaction time or decreasing the size of the reagent drop.

The eutectic temperature for the mixed diacetyl-acetone hydrazones is listed as 143°; temperatures for other mixed hydrazones are given, as well as for ternary systems with acetone. No ternary systems involving diacetyl are given. No mention is made of acetaldehyde or acetoin interference.

VIII. Occurrence

Starter distillate occurs only as already described (see section on Description), as a synthetic product of microbial action. The major component of starter distillate, diacetyl, occurs naturally in many plant and animal sources (Table 2). In most cases its presence is due to microbial action (see below) and is considered desirable or undesirable according to circumstances. Diacetyl occurs naturally in angelica root oil, cheese, cocoa, coffee, coffee extract, cooked chicken, pears, raspberries, and strawberries (0585). It is used in the following flavors: almond, blueberry, butter, buttermilk, butterscotch, caramel, cheese, cherry, chocolate, coffee, cream soda, fruit, ginger-ale, liquor, nut, raspberry, rum, spice, strawberry, vanilla, and wine (0585).

Many instances of the way that diacetyl is formed in natural or processed foods have been documented in the literature. Hammer (0741) showed in 1933 that the diketone associated with butter flavor in butter cultures was diacetyl rather than some homolog of diacetyl.

In 1936 Virtanen and Tarnan (2126) found that diacetyl was formed in butter only in the presence of oxygen, and they recommended maximal aeration during manufacture.

Michaelian and Hammer (1937) (1324) studied the oxidation of acetoin to diacetyl in butter cultures using *Streptococcus lactis* as the inoculum, and concluded that oxidation resulted from the citric-acid-fermenting activity of *S. lactis*, and not endogenously to the culture medium.

Table 2. Occurrence and Levels of Diacetyl

Source	Level	Reference
Animal Sources		
Butter	2.83-5.04 mg/kg	1618
Butter	2.5-4.0 ppm	0878
Butter-oil	$1/10^{8}$-10^{11}	0589
Butter, unsalted	0.16-1.46 ppm	0788
Butter, sweet cream	0.19-3.86 ppm	0788
Butter, sour cream	0.37-2.57 ppm	0788
Cream, raw	0.61-1.11 mg/kg	1627
Cheese, Cheddar	0.16-3.35 mg/kg	0279
Cheese, cottage	0.6-1.6 ppm	1276
Egg white	present	1780
Ham	present	1483
Chicken, raw muscle, stored	present	0676
Plant sources		
Apple juice	present	0833
Beer	0.13-0.25 mg/liter	2167
Beer	0.42-1.51 ppm	1614,1615
Beer	0.04-0.90 ppm	2215
Beer, wort	0.17-0.37 ppm	2215
Beer, wort, fermented	0.43-0.95 ppm	1505
Bread	present	2185
Bread	0.4-10.1 ppm	1740
Broccoli, frozen	0-1.5 mg/100g	0253
Celery	present	0659
Chicken, cooked	191 mg/5.6 kg	1511
Cocoa beans, roasted	2.8 mole %	0115
Oat flakes, toasted	present	0851
Oranges, Florida, segments, juice, peel juice	present	1973
Orange concentrate, sterile with 2.7 x 10^{6} microbe-count	0 ppm 14.6 ppm	0813 0813
Orange juice, frozen conc.	0.04-0.35 ppm normal over 0.6 ppm unsanitary	1387 1387

Table 2. (Cont'd)

Pears, Bartlett, canned	0-6.2 mg/kg	1215
Peas	0.29-0.61 mg/kg	1652
Peas, fresh	4.25 mg/kg	1394
Pineapple	present	0354
Potato chips	present	0472
Smoke, wood	present	0459
Soybean oil	present	1828
Strawberry essence (puree)	0.51-1.88 ppm	0448
Tea, Ceylon	2-22 μg/100g	2186
Tobacco smoke	157.3 mg/kg	1800
Vinegar	present	0102
Wheat, milled	present	1307
Wine, Australian red	0.1-7.5 ppm (av. 2.4 ppm)	1655
Wine, sherry	1.8-160 ppm	0372
<u>Synthetic natural sources</u>		
Amino acid & sugar mixtures heated	present	0508
Glucose, heated	present	2154
Starter flavor	2-4 ppm	1335

McDowall (1959) (1298) studied the distribution of diacetyl and related intermediates among milk fractions at various temperatures up to 190°F. The higher the temperature, the greater the proportion of diacetyl (and also of acetoin) that was found in the butter fat, and the smaller the proportions in skim-milk or lactose solution.

A classic method of standardizing biacetyl content was reported by Mather and Babel in 1959 (1276). In brief, diacetyl was not increased by addition of citric acid 0.15% or S. citrovorus to cottage cheese at 45°F, but was increased by both additions, especially if the mixture was adjusted to pH 4.2 with citrate, at 70°F. The authors recommended a working procedure to utilize these findings.

In 1962 Lightbody (1184) found that S. diacetilactis produced diacetyl rapidly in cream at 13-18° for 16-24 hours and then reduced most of it. Peak production was at pH 5.6 and was enhanced by addition of sodium citrate, which did not inhibit the reduction of diacetyl.

Crowell and Guymon (1963) (0372) studied the formation of diacetyl and acetylmethylcarbinol during grape fermentation for sherry production. During anaerobic fermentation 0.5-1.5 ppm diacetyl resulted from addition of grape pulp to the mixture, and 0.5-1.0 ppm from addition of grape skins. However, during aerobic fermentation the pulp elicited 65-94 ppm diacetyl, and the skins 14-350 ppm diacetyl and up to 2050 ppm acetylmethylcarbinol. The values in various sherries were 1.8-160 ppm diacetyl and 4.3-450 ppm acetylmethylcarbinol.

Holck and Fields (1965) (0833) reported that ethanol, acetoin, and diacetyl in stored apple juice were valid indices of microbial quality as of the time of manufacture. The first two remained constant, and although the third declined with time of storage, its presence was evidence of poor sanitation, because it was produced by yeasts.

Great variations in diacetyl production and diacetyl/acetaldehyde ratio (considered important for flavor of dairy products) were reported by Bottazzi and Dellaglio (1967) (0217) among many strains of lactic streptococci tested.

In 1966 Habaj et al. (0718) reported that the diacetyl content of starter cultures tended to rise and fall with their content of citric acid.

Pack et al. (1967) (1510) found that temperature and timing both influenced the production of diacetyl by single or mixed strains of bacteria in lactic cultures, and the authors proposed a method for regulating and stabilizing diacetyl production by careful manipulations of the times and temperatures of incubations.

Scanlan et al. (1968) (1785) found that a level of 5 ppb of diacetyl in raw milk rose to 38 ppb after the milk was heated; since the latter was far above the taste threshold, the authors suggested that diacetyl contributed to the taste of heated milk.

Keenan et al. (1968) (1001) found that Pediococcus cerevisiae cultures in milk could produce small amounts of diacetyl, and similar organisms isolated from Cheddar cheeses also produced only small amounts of diacetyl.

Chuang and Collins (1968) (0326) studied the pathways by which bacteria and yeasts produce diacetyl, and concluded that acetoin was not a precursor, and that pyruvate was, but needed acetyl-CoA. Different species had different limiting or inhibiting characteristics in terms of added chemicals, and the mechanisms were considered obscure. The authors appeared to favor the aceto-lactate pathway, and a more recent report by others (1963) supported that view.

In 1969 Inoue et al. (0888) investigated the formation of diacetyl in beer. They concluded that the precursor was not AMC but α-acetolactate secreted by yeast cells into fermenting wort and converted to diacetyl by oxidative decarboxylation in the presence of sufficient redox potential.

Hargrove et al. (1969) (0759) studied the survival of salmonellae in 72 lots of experimentally manufactured Cheddar or Colby cheese. They found no influence of previous pasteurization, or of salt, moisture, or chemical additives. They found that the amount and rate of acidity, and the amount and type of starter influenced survival, which was promoted by the presence of large numbers of Propionibacteria and Leuconostoc (spp); however, lactobacilli and enterococci were found to have no influence.

In 1970 Collins and Bruhn (0351) found that S. diacetilactis required acetate as a precursor for acetyl-CoA, needed for production of both diacetyl and ATP; acetate was produced endogenously from 73% of the pyruvate available.

Garibaldi and Bayne (1970) (0616) tested 42 Salmonella spp. for ability to produce acetoin and diacetyl, and found them all to be abundant producers when cultured on a minimal medium containing 1% glucose.

Many reports show that the occurrence of diacetyl in orange juice is prima facie evidence of spoilage due to microbial action (0563,1391, 1473,2216), especially by lactic acid producing bacilli (0323).

BIOLOGICAL DATA

I. Acute Toxicity

A. Starter Distillate

No mention was found in the Toxic Substances List (0322) in preparation for 1974, and no acute toxicity studies were found elsewhere.

B. Diacetyl

Two reports of acute toxicity studies were found:

In the first study (0942) groups of young Osborne-Mendel rats evenly divided for sex were fasted for approximately 18 hours with water available at all times. Doses of diacetyl were intubated without dilution, and the animals were then fed immediately. They were watched closely until they either died or recovered normal health and weight gain; the usual period was two weeks. LD_{50}'s and slope functions, 95% confidence limits for each, and clinical data were reported.

Table 3. Acute Toxicity Table

Ref.	Animals	Dose mg/kg	95% Confidence Range	Slope Function	Route	Dilution	Pre-treatment
0942	Rats	1580	1310-1920	1-5(1.2-1.9)	tube	nil	18 hr fast
0942	Guinea pigs	990	728-1350	2-4(1.2-4.6)	tube	nil	18 hr fast
0349	Male Rats	3400	3180-3640		tube	20%	18 hr fast
0349	Fem. Rats	3000	2420-3720		tube	20%	18 hr fast
0349	Male Rats	400	340-480		i.p.	20%	18 hr fast
0349	Fem. Rats	640	570-720		i.p.	20%	18 hr fast

Deaths occurred between a few minutes and two hours after the dose, preceded by depression, and then convulsions 10-15 minutes after the dose. In guinea pigs grouped and treated similarly, death (a few minutes to 4 days later) was preceded by ataxia, gasping, and coma.

The second study (0349) involved both acute and short-term observations in both sexes of SPF-derived CFE rats. The acute studies were designed similarly to the foregoing (0942) except that the doses were intubated as 20% solutions in water and some groups were treated intraperitoneally (i.p.). (Table 3).

The authors (0349), commenting on their higher LD_{50} findings, noted that their doses were diluted whereas the previous (0942) doses were not. However, they did not comment on the fact that they had used a different breed of rats, nor on the sex differences in response to oral doses, that were reversed in the responses to i.p. doses.

II. Short Term Studies

A. Starter Distillate.

No reports of short term studies were found.

B. Diacetyl

Colley *et al*. (0349) conducted short term studies with groups of 15 male and 15 female SPF-derived CFE rats housed 5/cage. For 90 days each rat received daily, by intubation, 5 ml/kg of a solution of water containing zero, 0.2, 0.6, 1.8 or 10.8% diacetyl (0, 30, 90, or 540 mg/kg). Food and water were given *ad libitum*, and *in vivo* studies of blood, urine, and liver and kidney function were performed. All rats were killed and autopsied at 90 days, and laboratory studies followed.

The *in vivo* period of the study disclosed no significant differences between any groups, except for slower growth and greater water consumption in the highest dose group (540 mg/kg) especially the males.

The terminal investigations disclosed few significant inter-group differences, relative to the number of parameters. Rats given 540 mg/kg/day had less Hb, fewer packed cells, more reticulocytes, and more leucocytes (mainly neutrophils) than the rest. Their organ weights relative to body weight increased, and in some cases increased absolutely although body weights diminished 25%; this was especially so with the adrenals. The gastric squamous epithelium of these animals had sloughed, and ulcers were seen microscopically. These, according to the authors, accounted for the blood differences, and also for the differences of organ weights.

The non-significant differences found led the authors to state that the no-effect level of diacetyl given orally to rats was 90 mg/kg/day. From survey data they guessed that human intakes would approximate to 10 mg/day/person, and concluded that human maximum intakes on a body-weight basis would be approximately 500 times less than the no-effect (90 mg/kg) intake level for rats. The authors did not compare the intakes on a skin surface area basis.

III. Long Term Studies

No reports of long term studies were found, either on starter distillate or on diacetyl.

IV. Special Studies

A. Mutagenicity

Loveless (1951) (1208) tested the radiomimetic, specifically mutagenic, properties of a large number of compounds on root-tip meristems of *Vicia faba*, and found only those compounds capable of reacting by a carbonium ion mechanism to have such properties. Diacetyl was among the substances tested that were found to be inert.

Barthelmess and Elkabarity (1962) (0137) tested a number of substances for influence on genetic expression, using root meristems. Diacetyl diluted to 0.08-0.10 mol/liter was found to delay metaphase and consequently to increase the frequency of observed anaphase.

B. Teratogenicity

The final report to FDA by the Food and Drug Laboratories Inc. (0583) in August 1973 concluded that administration of up to 1600 mg/kg BW of starter distillate to pregnant mice and rats for ten consecutive days, to pregnant hamsters for five consecutive days, and to pregnant rabbits for 13 consecutive days, had no evident effect on births or on maternal or fetal survivals. The report also stated that the number of abnormalities of soft or skeletal tissues did not differ from the number occurring spontaneously in controls.

C. Effects on radiation sensitivity

Tallentire *et al*. (1968) (1985) studied the effects of diacetyl on the sensitivity of spores of the anerobic *Bacillus megaterium* to X-rays. The basis of the study was the high affinity of diacetyl for free electrons,

by which it stabilizes electrons produced by high-energy radiations in aqueous solutions. The authors found that diacetyl increased radiation-sensitivity of the spores in the absence of oxygen as concentration increased up to 4 x 10^{-3} M and the sensitivity increase was 40% of the full increase produced by oxygen (the mechanism of the oxygen effect was stated as "unknown"). However, diacetyl did not increase radiation sensitivity in the presence of oxygen, and the authors inferred that the oxygen effect probably included a mechanism similar to that of diacetyl (e.g. electron stabilization). Diacetyl above 6 x 10^{-3} M became less effective and at 6 x 10^{-2} M failed to sensitize the spores to radiation. The authors offered no explanation for this.

D. Carcinogenicity

In 1959 Arcos et al. (0077) studied rat-liver mitochondrial swelling as an early manifestation of the carcinogenic activity of aminoazo dyes and found that the swelling was inhibited by 10^{-2} M diacetyl or alloxan but not by other carbonyl compounds lacking a vicinal pair of carbonyl groups. The same authors in a 1960 report (0078) discussing mainly the same data but extended to microsomal swelling, concluded that these were generalized effects on membranes in which permeability could be influenced by sulfhydryl groups and by diketones.

E. Effects on microorganisms

In 1936 Levy-Bruhl and Cado (1174) found that Staphylococcus aureus was killed by 0.1% diacetyl but not by 0.04%, and that Streptococcus, Pneumococcus, Gonococcus, typhoid, paratyphoid, and coliform species of bacilli were killed by 0.04% diacetyl but not by 0.02%.

In 1951 Schales (1788) found that diacetyl and glyoxal were the most effective among 32 diketones and 4 related compounds tested for activity against Micrococcus pyogenes var. aureus (ATCC 6538), Escherichia coli (ATCC 9637) and Streptococcus Lancefield H69-D5.

In 1954 Myrvik and Volk (1399) tested the activity of seven reductogenic compounds against tubercle bacilli using culture and incubation techniques, and found that diacetyl was considerably the most potent, by factors ranging from 10 to nearly 100. The spectra of activity were similar for all seven, and the authors concluded that the presence of, or the capability to form, diketones was the basis of the activity.

DeBock et al. (1957) (0413) tested the antiviral activity of 34 dicarbonyl and related compounds using hemagglutination techniques, and found that compound no. 12, diacetyl, had moderate antiviral activity.

Hedgecock and Cohn (1959) (0787) found that diacetyl at 0.2-1.6 mg/ml prevented growth of a variety of organisms, and was lethal to a strain of Escherichia coli at concentrations as low as 0.129 mg/ml. The authors found that diacetyl was metabolized and utilized by this strain of E. coli, and hypothesized that utilization must interfere with some other essential metabolic process.

Sprössig and Mücke (1963) (1917) found that diacetyl was strongly active against influenza virus strain FM1 by standard hemagglutination procedures. The same authors in 1967 reported chemical studies and tests in mice that supported their earlier data (1379).

BIOCHEMICAL ASPECTS

I. Breakdown

In 1943 Elliker and Horrall (0513) reported that *Pseudomonas putrefaciens* destroyed more than half the diacetyl in butter stored for 7 days at 21°C, when sterile butter maintained its dicetyl content. Butter prepared with starter was found to remain relatively stable with little diacetyl destruction.

In 1946 Suomalainen and Jannes (1960) reported that diacetyl could be an intermediate in the formation of aroma substances from pyruvic acid.

In 1949 in an early report, Calbert and Price (0278) found that diacetyl was produced in Cheddar cheese at most stages of manufacture, and was increased by agitation or increased acidity; they concluded that the process of diacetyl production was similar to that in starter cultures. When decreases were noted, the authors considered them probably due to volatilization and by reduction to acetylmethylcarbinol and 2,3-butylene glycol.

Strecker and Harary (1954) (1955) partly purified the enzyme butyleneglycol dehydrogenase which catalyzes the reversible oxidation of butyleneglycol to acetoin, and the enzyme diacetyl reductase which catalyzes the reduction of diacetyl to acetoin. Both of these enzymes had been isolated from *Aerobacter aerogenes* and *Staphylococcus aureus*. The enzyme for reversible oxidation was inactive in the forward direction with the following substrates: ethanol, isopropanol, isoamyl alcohol, isobutanol, ethyleneglycol, glycerol, glucose, malic or tartaric acids. Its equilibrium constant (K) was determined in a number of experiments and was found to average 2.74×10^{-10} M/liters.

Juni and Heym (1956) (0971) analyzed cell-free extracts from bacteria grown in a medium supplying carbon and energy only from diacetyl and acetylmethylcarbinol (AMC). They found and purified an enzyme semispecific for conversion of diacetyl to diacetylmethylcarbinol and acetic acid. The enzyme reacted other α diketones but not pyruvic acid.

In 1956 Petkevich (1566) reported a "drastic" reduction in the diacetyl and AMC content of milk from cows with hoof-and-mouth diseases; however, the quality of the butter remained high.

In 1957 Wales and Harmon (2146) inoculated 30 samples of fresh creamed cottage cheese with one or another of 12 bacteria, yeasts or molds known to cause spoilage. They analyzed for diacetyl and acetylmethylcarbinol and tested the samples for subjective quality. The diacetyl content of these samples was not found to be related to subjective quality, nor were diacetyl and acetylmethylcarbinol contents related to pH of the inoculated samples.

In 1958 Burger et al. (0262) reported that yeast present in beer removed diacetyl in the presence of other fermentable matter if the yeast was prevented from flocculating, if there was enough yeast, and without undesirable autolysis of the yeast if the beer was stored at 32°F under carbonation.

In 1963 Seitz et al. (1832) studied the distribution of diacetyl reductase activity among species and strains of bacteria. Strains of Streptococcus diacetilactis were the most active; strains of S lactis, S. cremoris, and Leuconostoc citrovorum were less active; most but not all strains of Escherichia coli, Alcaligenes spp., Pseudomonas spp., coliforms, and Aerobacter aerogenes were more active. These results were interpreted in terms of the need both to avoid specific contaminations of foods, and to remove diacetyl from beverages.

In 1964 Lindsay et al. (1193) studied earlier findings that had suggested 2,4-dinitrophenylhydrazine as a metabolite of diacetyl, acetolactate, and acetoin (acetylmethylcarbinol). Further in vitro studies showed that this metabolite should be correctly identified as 2,4-dinitroaniline, which also had been identified in samples of autoxidizing fat.

In 1966 Lacrosse (1125) found that concentrations of approximately 1 ppm diacetyl in butter were decreased in storage when the butter was also contaminated with yeast cells; the additional presence of Klebsiella aerogenes did not increase the rate of diacetyl breakdown.

In 1968 Pack et al. (1509) studied the formation and breakdown of diacetyl by Streptococcus diacetilactis alone and in company with S. cremoris and Leuconostoc citrovorum. They found that treatment of the cultures with H_2O_2 and catalase increased the production of diacetyl and inhibited its breakdown, but were unable to select which of several possible mechanisms was involved.

In 1968 Keenan and Lindsay (1000) studied the production and utilization of diacetyl by Lactobacillus casei, brevis, and plantarum cultured in milk or fractions of milk. They found that both L. brevis and L. lactis utilized

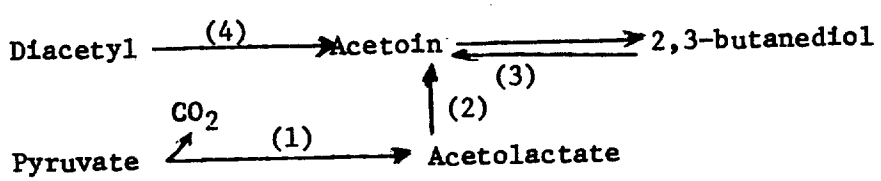
diacetyl, and that L. plantarum produced it. Species that utilized diacetyl possessed NADH-dependent diacetyl reductase activity, and those that did not utilize diacetyl lacked this enzyme activity. The authors concluded that Lactobacillus spp. contributed to both formation and breakdown of diacetyl in Cheddar cheese during curing.

In 1970 Thompson et al. (2023) studied the use of NADH-dependent diacetyl reductase, part-purified from Aerobacter aerogenes, to remove diacetyl from beer. The enzyme activity deteriorated at the natural pH (4.1) of beer unless protected in 10% gelatin; yeast cells included in the gelatin regenerated the NADH. This combination, at 0.5 ppm in beer at 5°C, reduced the diacetyl level to under 0.12 ppm in 48 hours. It also reduced the diacetyl level in orange juice at pH 3.8 to about 0.2 ppm. It was thought to have reduced the diacetyl levels in high-proof distillers' products, but the analyses were ineffective, and the authors commented on the need for further research.

In 1970 Tolls et al. (2030) studied the enzymatic removal of diacetyl from beer. They found that ethanol at levels similar to those of beer inhibited diacetyl reductase activity; but other NADH-dependent enzyme activities, cells of Streptococcus diacetilactis, Aerobacter aerogenes, and crude extracts of enzyme thought to contain diacetyl reductase, removed the diacetyl at varying rates. The crude enzyme extracts were found by electrophoresis to contain at least three NADH-dependent enzymes.

In 1970 Brenner (0228) reported evidence that NADH-dependent yeast alcohol dehydrogenase reduced acetaldehyde to alcohol and, at the same time, reduced diacetyl to acetoin and 2,3-butanediol. The latter reaction was about 7000 times slower than the former, and was temperature-dependent; thus the authors concluded that it could be used to advantage in brewing, by suitable manipulation of the conditions of the process.

In 1971 Bryn et al. (0250) studied the enzyme systems catalyzing the formation and breakdown of the intermediate metabolite acetylmethylcarbinol (acetoin), starting with the reactions shown in the flow diagram illustrated.



Enzymes were identified as follows: (1) pH 6 acetolactate-forming enzyme; (2) acetolactate decarboxylase; (3) butyleneglycol dehydrogenase, and (4) diacetyl reductase. The authors provided evidence that (3) and (4) were the same enzyme, which represented 0.8% of the total protein (after 124-fold purification) in A. aerogenes. This enzyme system represented about 2.3% of the total protein after induction either by diacetyl or by acetic acid. The authors suggested that enzyme (3) (4) should be named diacetyl (acetoin) reductase. The authors described reaction (3) as reversible, but it is a matter of opinion whether or not the evidence is satisfactory that both directions of this reaction are catalyzed by one and the same enzyme.

II. Absorption and distribution

No studies on the absorption and distribution of starter distillate or diacetyl were found.

III. Metabolism and excretion

Four *in vitro* studies on metabolism were found, but no studies on excretion.

In 1954 Järnefelt (0934) investigated the metabolism of diacetyl by rat liver slices and homogenates under aerobic conditions,using a method for determining the amounts of diacetyl and acetylmethylcarbinol (acetoin) in the same sample. He found that homogenates converted part of the diacetyl to acetoin but did not metabolize the acetoin. On the other hand,the slices converted all of the diacetyl to acetoin and further metabolized the acetoin. The difference between homogenates and slices is that in the former the cells are broken, and in the latter they are mostly intact.

In 1962 Hullins and Hassall (0872) used *Pseudomonas* spp. to study the operation of the glyoxylate cycle by which butane-2,3-diol was used when it was the sole source of carbon for growth. Extracts of cells grown on butane-2, 3-diol medium catalyzed the conversion of diacetyl to 3-hydroxy-3-methyl-pentane-2,4-dione. Isocitratase activity was as great as when acetate was the growth substrate and 20-fold greater than when succinate was used. In the presence of acetate, diacetyl, 2-hydroxybutan-3-one, and cofactors, the cells transferred labeled carbon from glyoxylate to malate, which showed that the cycle was operating.

Gabriel et al. (1971) (0606) used a "rat liver mince" to verify in mammallian systems the enzyme pathways for interconversion of diacetyl, acetoin, and 2,3-butanediol that have been outlined in the previous Section for bacteria. They incubated 1000-fold physiological concentrations of acetoin which disappeared coincident with formation of 2,3-butanediol but with very little elaboration of CO_2. They isolated two enzymes: acetoin dehydrogenase (EC 1.1.1.5) and 2,3-butanediol dehydrogenase (EC 1.1.1.4) that were NAD^+ - or $NADP^+$-dependent and differed from alcohol dehydrogenase. The activity of EC 1.1.1.4 diminished on dialysis against EDTA and was restored by addition of Co^{++}, Cu^{++}, Zn^{++} and other divalent metal ions. Chromatography and electrophoresis showed rat liver diacetyl reductase to consist of multiple proteins.

Another pathway for diacetyl metabolism was reported in 1956 by Shinagawa et al. (1867). Citrate was formed from diacetyl and oxaloacetate in the presence of 2P-thiamine and acetyl-CoA, with negligible O_2 consumption and unaffected by malonate, NAD, or ATP.

IV. Effects on Enzymes and Other Biochemical Parameters

A. Flavor studies

Petkevich (1566) reported in 1956 that during an outbreak of hoof-and-mouth disease,milk yields fell, butterfat percentages rose, and diacetyl and AMC production in the milk were "drastically reduced". Both butter and skim-milk products were of high quality.

Reineccius and Coulter (1960) (1672) investigated the effects of spray-drying on the flavor of milk components, by adding measured amounts of diacetyl, acetoin, or acetone before drying, and measuring the amounts remaining afterwards. Diacetyl appeared to have a greater affinity for milk proteins than for milk sugars, partly explained by different effects of the proteins and sugars on vapor pressure through the spray nozzle.

Riel and Gibson (1961) (1695) studied the effect of starter distillate additions to Canadian butter on flavor and keeping quality. The flavor panel was divided on preference for butter, flavored either with distillate or with diacetyl, as against control samples. The official graders preferred the control samples. Preference for diacetyl diminished with increasing concentration, with 0.5 oz of distillate/100 lb butter preferred over 02.5

or 1.0 oz/100 lbs. In flavored butters acetoin was present at 0.11-0.27 ppm and diacetyl at 0.24-0.64 ppm. These levels did not change after storage for 1 year at -10°F, nor did peroxide values.

The perception of diacetyl in dairy products was studied by Bennett et al. in 1965 (0172). In skim milk or cream at normal pH the threshold was 0.01 ppm, at pH 4.4 it was 0.05 ppm, and at pH 5.0 it was 0.2 ppm. The threshold was lowered by formic or acetic acids or by acetaldehyde, but not by propionic acid in sour cream, and the authors suggested that the fat content of cream might suppress the acid effect.

In 1967 Whelton and Foley (2181) found that diacetyl production in lactic cultures was inhibited by residues of iodophor detergents used for washing dairy equipment. Part-inhibition was found when 20 ppm of iodine was present in milk, and complete inhibition with 60-80 ppm.

In 1968 Keenan et al. (1002) judged flavor and analyzed for acetaldehyde, diacetyl and microbial contents of buttermilk from ten regional dairies. In 8 samples, the diacetyl/acetaldehyde ratio ranged from 8.6/1 to 33.6/1, far above the 4/1 balance reported to be required for good flavor. In one sample neither substance was present and in one sample the ratio was 1.1/1. The microbial quality was uniformly good, but the flavor score varied from 34-39. Judges agreed that "most of the samples lacked a well-balanced culture flavor."

In 1969 Siek et al. (1874) sought the taste thresholds for 31 volatiles of butter and found that the lowest was that of diacetyl, at 0.055 ppm. There was much synergism among methylketones and free fatty acids, little among aldehydes, and none among lactones. Compounds with taste thresholds below their reported concentrations in butter would, according to the authors, be those responsible for sweet cream butter flavor.

In 1970 Young et al. (2263) reported two methods of discriminant analysis, procedures designed to compare organoleptic evaluation of diacetyl-containing beverages with gas-chromatographic evaluations so as to test for correlations. See the original paper for details.

B. Enzyme studies

In 1961 Gale (0610) studied the inhibition in vitro of yeast pyruvic decarboxylase by pyruvate analogs, and found, among other things, that diacetyl progressively inhibited the enzyme with time.

In 1961 Katsumata and Niki (0993) found, in diabetic rats and humans, that the urinary excretion of diacetyl and AMC was increased and the liver xanthine oxidase activity was diminished.

V. Drug Interactions

No mention of starter distillate or diacetyl was found in the compendium, Drug Interactions (2070). One report was found elsewhere that could possibly be classed as a drug interaction.

Schuller (1957) (1819) reported that autoxidation of butter was delayed by the addition of 8 or 16 ppm of β-carotene, and that this effect was enhanced by various substances including diacetyl. He found that diacetyl at 1 mg/kg of butter enhanced the inhibitory activity of the carotene for 3 days of storage at 14°, after which the carotene again promoted autoxidation. However, the carotene itself was decomposed considerably during the 3 days, and autoxidation of the butter was found to retard the rate of decomposition of the carotene. Although butter would normally contain both carotene and diacetyl, in these studies additional amounts of both compounds were added *in vitro*. The relevance is to the stability of butter enriched with vitmain or provitamin A, or flavored with diacetyl.

VI. Consumer Exposure Information

Diacetyl occurs widely in natural and processed foods (see page 12) largely as a byproduct of microbial metabolism, and no estimates were found of the quantities of exposure to these sources of diacetyl.

Estimates of exposure to diacetyl and to starter distillate added to foods were found in the NAS NRC Comprehensive GRAS Survey (0584) and are shown in Tables 4 through 7. The total 1970 poundate of starter distillate as reported to the National Academy of Science by 6 reporting firms was 1,014,958 pounds (0584, table 11C). The 1970 poundage reported for buffer starter distillate was 26,428 pounds (0584, table 11C).

Another estimate of exposure to diacetyl (0585) is shown in Table 8, but no other estimate of exposure to starter distillate was found. However, an estimate of overall consumption of butter and cheese is given in Table 9.

A number of studies have been conducted on consumer preferences. According to Lindsay et al. in 1967 (1192), the flavor of cultured cream butter was rated highest in samples that contained diacetyl at less than 2.14 ppm or between 2.32 and 2.84 ppm. In another test the same panel also tasted sour cream, cottage cheese, and buttermilks with high or low diacetyl contents. As between high-quality natural products and synthetically flavored products they expressed no clear preference, and among the synthetics they expressed no clear preference between high and low levels of added diacetyl.

The authors (1192) concluded from these findings and others reported by Hempenius et al. in 1965 (0794), that consumers did not detect differences of diacetyl content in creamed cottage cheeses of less than 1.4 ppm, and that large variations did not affect consumer preferences. However, Hempenius et al. (0794) had actually concluded that consumers preferred high-diacetyl cottage cheeses, except for those consumers who disliked all creamed cottage cheeses and who expressed a "slight" preference for low-diacetyl samples.

In 1968 a WHO/FAO Committee (0581) listed diacetyl among compounds for which exposure recommendations could not be made owing to lack of data. They stated that monographs would not be published on these compounds but tentative specifications were available on request. They added that no distinction should be drawn between natural and exactly similar synthetic substances, and recommended that in this case, in the absence of specific information, note should be taken if intakes totaled more than 3.65 mg/year or levels in food exceeded 10 mg/kg.

Diacetyl was classed as GRAS in 1961 (0057) and, together with starter distillate, was allowed to be added to creamed cottage cheese in 1967 (0060).

In 1973 the only other regulation found that pertained to starter distillate and diacetyl (0066) described them as flavoring agents and permitted their addition to oleomargarine in amounts "sufficient for purpose."

Table 4. Usage Levels Reported for Starter Distillate (0584, table 2)

Substance	Food Category	No firms Reporting	Usual use WTD Mean,%	Maximum Use WTD Mean,%
Starter Distillate NAS 0326	04 Fats Oils(R)	*	.07015	.30000
	05 Milk Prods (R)	*	.00930	.00930
	28 Lmit Dairy (R)	*	.00653	.01694

Table 5. Usage levels reported for FEMA questionnaire substances not in NAS Appendix A (Group III) - regular foods only (0584, table 4)

Substance	Food Category	No firms Reporting	Usual use WTD Mean,ppm	Maximum use WTD Mean,ppm
Buffer Starter Distillate FEMA 21730	01 Baked goods	12	302.535	693.902
	04 Fats oils	*	1118.182	1159.091
	07 Frozen dairy	6	445.853	590.560
	16 Soft candy	10	338.539	700.422
	20 Gelatin pud	*	78.609	96.887
	23 Bev type I	4	2.890	6.979
	28 Limit dairy	*	0.30	0.80
	31 Chewing gum	*	28.50	28.50
Diacetyl FEMA 23708	01 Baked goods	32	28.169	49.941
	04 Fats oils	8	6.014	62.175
	05 Milk prods	*	4.70	7.00
	06 Cheese	*	3.679	7.954
	07 Frozen dairy	22	11.172	35.081
	10 Meat prods	*	27.809	27.809
	16 Soft candy	26	17.125	47.318
	20 Gelatin pud	15	13.451	26.580
	22 Snack foods	*	0.381	0.762
	23 Bev type I	24	10.351	21.434
	24 Bev type II	*	6.325	15.275
	27 Gravies	*	7.190	25.041
	28 Limit dairy	*	10.50	25.50
	30 Hard candy	15	10.65	30.08
	31 Chewing gum	5	0.69	9.2
	49 Misc unclas	*	22.4	904.9

Table 7. Possible daily intakes of Diacetyl (0584, table 13B)

Food Category	No. Firms Reporting	Average Intakes Age 0-5 Mos.	Age 6-11 Mos.	Age 12-23 Mos.	Age 2-65+ Yrs.	High Intakes* Age 0-5 Mos.	Age 6-11 Mos.	Age 12-23 Mos.	Age 2-65+ Yrs.
Baked goods	32	0.10	0.72	1.54	3.86	A) 0.13	1.46	2.53	5.74
						B) 0.17	1.27	2.72	6.85
Fats oils	8	0.003	0.02	0.04	0.11	A) 0.003	0.05	0.07	0.19
						B) 0.03	0.17	0.39	1.09
Milk prods	*	0.03	0.29	0.26	0.19	A) 0.02	1.41	0.82	0.57
						B) 0.04	0.44	0.38	0.28
Cheese	*	--	0.01	0.03	0.03	A) .0004	0.04	0.08	0.09
						B) --	0.02	0.06	0.07
Frozen dairy	22	0.01	0.11	0.16	0.29	A) 0.05	0.29	0.38	0.69
						B) 0.04	0.33	0.51	0.90
Meat prods	*	0.03	0.58	0.84	2.18	A) 0.08	1.55	1.44	3.62
						B) 0.03	0.58	0.84	2.18
Soft candy	26	0.003	0.04	0.06	0.10	A) 0.03	0.12	0.16	0.30
						B) 0.01	0.10	0.15	0.25
Gelatin pud	15	0.03	0.17	0.19	0.27	A) 0.04	0.52	0.45	0.71
						B) 0.05	0.34	0.37	0.54
Snack foods	*	--	.0002	.0004	.0005	A) .0003	.0004	0.001	0.001
						B) --	.0003	.0008	0.001
Bev type I	24	0.02	0.23	0.56	1.08	A) 0.04	0.80	1.08	2.87
						B) 0.05	0.49	1.16	2.23
Bev type II	*	--	--	--	0.21	A) --	.0006	0.001	0.60
						B) --	--	--	0.50
Gravies	*	.0007	0.01	0.03	0.06	A) 0.002	0.03	0.07	0.15
						B) 0.003	0.04	0.09	0.21

Tabel 7 (Cont'd)

Category	No. Firms Reporting	Average Intakes: Age 0-5 Mos.	Average Intakes: Age 6-11 Mos.	Average Intakes: Age 12-23 Mos.	Average Intakes: Age 2-65+ Yrs.		High Intakes*: Age 0-5 Mos.	High Intakes*: Age 6-11 Mos.	High Intakes*: Age 12-23 Mos.	High Intakes*: Age 2-65+ Yrs.
Limit dairy	*	--	0.01	0.01	0.01	A)	--	0.02	0.04	0.02
						B)	--	0.04	0.02	0.03
Hard candy	15	--	0.001	0.003	0.006	A)	--	0.003	0.01	0.02
						B)	--	0.003	0.01	0.02
Chewing gum	5	--	.00007	.00007	.0001	A)	--	.00007	.00002	.00003
						B)	--	0.001	.0009	0.002
All	69	0.22	2.19	3.70	8.39	A)	0.39	6.30	7.74	15.56
						B)	0.42	3.81	6.70	15.14

* See explanatory notes for definition of HIGH A and HIGH B

Table 8. Contents of diacetyl in foods listed in Chemicals Used in Food Processing (0585)

Foods in which used	Approximate Average Maximal ppm
Beverages	2.5
Candy	21
Gelatin desserts	19
Shortenings	11
Ice cream, ices	5.9
Baked goods	44
Chewing gum	35

Table 9. United States balance-sheet of butter and cheese 1968 and 1970 in millions of pounds*

	1968		1970	
	Total Butter	Total Cheese	Total Butter	Total Cheese
Production	1514**	1938	1474**	2204
Imports	0.739	170	0.748	161
Sum	1515	2108	1475	2365
Exports	1.55	6.543	0.315	6.734
Exposure	1513	2101	1474	2358

* Data from Agricultural Statistics 1972 (2069). The exposure calculations to the nearest 1 million pounds were made for the purposes of this monograph.

** Factory produced butter totaled 1501 lbs (1968) and 1465 lbs (1970).

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