AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented May 14, 1965

Title Flavor Chemistry of Butter Culture

Abstract approved _____
(Major Professor)

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

Volatile flavor components of high quality butter culture and control heated milk were isolated from intact samples by means of a specially designed low-temperature, reduced-pressure steam distillation apparatus. Most of the flavor compounds present in the resulting distillate fractions were tentatively identified by gas chromatographic relative retention time data. Flavor concentrates obtained by ethyl ether extractions of aqueous distillates were also separated

by temperature-programmed, capillary column gas chromatography, and the effluent from the capillary column was analyzed by a fast-scan mass spectrometer. Many of the flavor compounds in the flavor concentrates were positively identified by correlation of mass spectral and gas chromatographic data. In addition, supporting evidence for the identification of some flavor components was obtained through the use of qualitative functional group reagents, derivatives and headspace gas chromatography.

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

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

A modified 3-methyl-2-benzothiazolone hydrazone spectrophotometric procedure was adapted for the determination of acetaldehyde produced in lactic starter cultures. The procedure was applied in conjunction with diacetyl measurements in studying single- and mixed-strain lactic cultures. The diacetyl to acetaldehyde ratio was found to be approximately 4: 1 in desirably flavored mixed-strain

butter cultures. When the ratio of the two compounds was lower than 3: 1 a green flavor was observed. Acetaldehyde utilization at 21° C by Leuconostoc citrovorum 91404 was very rapid in both acidified (pH 4. 5) and non-acidified (pH 6. 5) milk cultures. The addition of five p. p. m. of acetaldehyde to non-acidified milk media prior to inoculation greatly enhanced growth of L. citrovorum 91404 during incubation at 21° C. Combinations of single-strain organisms demonstrated that the green flavor defect can result from excess numbers of Streptococcus lactis or Streptococcus diacetilactis in relation to the L. citrovorum population.

Diacetyl, dimethyl sulfide, acetaldehyde, acetic acid and carbon dioxide were found to be "key" compounds in natural butter culture flavor. Optimum levels of these compounds in butter culture were ascertained by chemical or flavor panel evaluations. On the basis of these determinations, a synthetic butter culture prepared with heated whole milk and delta-gluconolactone (final pH 4. 65) was flavored with 2. 0 p. p. m. of diacetyl, 0. 5 p. p. m. of acetaldehyde, 1250 p. p. m. of acetic acid, 25. 0 p. p. b. of dimethyl sulfide and a small amount of sodium bicarbonate for production of carbon dioxide, The resulting synthetic butter culture exhibited the typical aroma, flavor and body characteristics found in natural high quality butter cultures, except that the delta-gluconolactone was found to contribute an astringent flavor.

FLAVOR CHEMISTRY OF BUTTER CULTURE

by

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A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1965

APPROVED:

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Date thesis is presented May 14, 1965

Typed by Marcia Ten Eyck

ACKNOWLEDGEMENT

I would like to thank Dr. E. A. Day for his inspiration, guidance and always willing assistance throughout the course of this investigation. In addition, I would like to express my sincere appreciation to the faculty and graduate students in the Department of Food Science and Technology and the Department of Microbiology for their cooperation.

I gratefully acknowledge the invaluable assistance of Dr. W. H. McFadden, Western Regional Research Laboratory, Agricultural Research Service, U. S. D. A., Albany, California, in obtaining and interpreting the mass spectra.

Thanks is due the American Dairy Association for providing financial support for the research.

A special expression of thanks is extended to my wife, Glenda, and daughter, Alison, for their patience and continuous encouragement.

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INTRODUCTION

High quality cultured cream butter is not generally available in the domestic market. The problems encountered in its manufacture and distribution are responsible for its limited availability. As a result much of the butter available today is inferior with regard to its flavor and sales appeal.

In recent years the majority of high quality butter manufactured in the United States has been made from pasteurized sweet cream. While this product possesses the unique and desirable characteristics peculiar to milk fat, it has a bland flavor which has been described by many as being flat and lacking in flavor. Conversely, the lower grades of butter generally have an abundance of flavor, but the flavor is usually not of the most desirable nature. In attempts to produce a product which has a degree of cultured flavor, some butter manufacturers culture cream with commercially available cultures which are often unsuitable for desirable flavor production. Other practices include the addition of artificial flavor concentrates and commercially prepared starter culture distillates. However, such practices do not always yield highly desirable products. As a result of the aforementioned problems along with a price differential, much of the butter market has given way to butter substitutes.

The major factors which have caused the decline in the manufacture of high quality cultured cream butter in the United States are (1) the difficulty of propagating suitable lactic cultures for maintenance of uniform flavor, (2) the danger of increasing oxidative deterioration of butter where cultured cream is used and (3) the added manufacturing costs coupled with the lack of a price advantage for cultured cream butter. The manufacture and availability of uniform butter possessing the distinctive, pleasing and well balanced flavor and aroma of high quality cultured cream butter would undoubtedly stimulate sales of butter and ultimately aid in the utilization of surplus milk fat.

Although numerous investigations have been made on the contribution of butter cultures to the flavor of cultured cream butter, the production of a uniform desirable cultured butter flavor has not been possible in industry. The primary objective of this investigation was to extend the knowledge about the qualitative and quantitative chemistry of the flavor of high quality butter culture. Certain aspects of cultured cream butter flavor also were investigated. A series of bacteriological studies were conducted using mixed-strain butter cultures and single-strain cultures of organisms normally associated with the mixed-strain cultures. This work was carried out to further elucidate some of the biochemical processes, which are important from a flavor point of view, that are functioning during the

compatible associative growth of lactic acid bacteria and aroma bacteria.

The information derived from these investigations may enable the preparation of a commercially feasible flavor concentrate which would allow a more precise control of the intensity and uniformity of cultured cream butter flavor. In addition to the application for the standardization of butter flavor, the information should also be useful for the standardization of the flavor of cultured buttermilk, cultured sour cream and creamed cottage cheese.

REVIEW OF LITERATURE

Sources of Flavor Compounds in Butter Cultures

Butter cultures normally contain a rather complex mixture of species and strains of lactic streptococci and aroma bacteria. The cultures are propagated in milk media containing varying amounts of milk fat, ranging from only a trace in skimmilk up to approximately four percent in whole milk. In addition, the milk medium is subjected to some degree of heat treatment varying from normal pasteurization to sterilization by autoclaving. The aroma and flavor compounds ultimately present in high quality ripened butter cultures can therefore originate from several sources, namely, normal milk including milk fat, the degradation of normal milk constituents by heat and the degradation of milk medium constituents through metabolic processes of culture organisms.

In view of the potential sources of flavor in butter cultures, the scope of the literature review has been expanded to include investigations which are not directly concerned with the flavor of butter cultures, but which may give an insight into the nature of the processes that contribute to the final flavor of butter cultures.

Flavor of Butter Cultures

The bacterial species (and strains) that may be incorporated in

butter cultures can be placed into three general categories (127): (1) the lactic acid producing streptococci, Streptococcus lactis and Streptococcus cremoris; (2) the citrate fermenting aroma bacteria, Leuconostoc citrovorum and Leuconostoc dextranicum; and (3) the dual purpose lactic acid and aroma producing strains of Streptococcus diacetilactis. It is well known that metabolic products which occur as a result of the associative growth in milk media of various combinations of strains of the above named bacteria give rise to the typical flavor and aroma of mixed-strain butter cultures. The aroma and flavor properties of single-strain cultures of the Leuconostocs and S. diacetilactis have received some attention with regard to butter cultures. Information concerning the aroma and flavor properties of single-strain cultures of lactic streptococci may be equally important in determining the overall contributions made by culture organisms to the aroma and flavor of butter cultures.

In 1943 Hammer and Babel (55) published a comprehensive review of the literature on the bacteriology of butter cultures. Their extensive review has adequately surveyed the numerous pioneering research efforts that have led to the identification of certain compounds classically associated with the flavor of butter cultures.

The early work was concerned primarily with organic acid production by mixed-strain butter cultures. Lactic acid has long been recognized as the main metabolic end-product of homofermentative

lactic streptococci. Pure lactic acid is non-volatile and odorless; therefore, it does not contribute to the odor, but is considered as the compound largely responsible for the acid taste in butter cultures (55).

The volatile acids of butter cultures have been studied by numerous investigators. Although these acids have been reported as total volatile acids by the majority of investigators (55), it has been found that acetic acid comprises the major portion of the volatile acid fraction (57, p. 1-15). Hammer and Sherwood (57, p. 1-15) and Knusden (82) have reported that small quantities of propionic acid were formed in good butter cultures. Recently, Chou (18, p. 126) reported the tentative identification of propionic, butyric and valeric acids in cultured buttermilks.

Formic acid has been reported to be an end product of lactose and glucose metabolism in non-milk media by S. cremoris, S. lactis and L. dextranicum (41, 124, 144). The amounts of formic acid produced were reported to be very small when compared to the amounts of lactic and acetic acid produced. Data on formic acid production in milk media, either by single-strain or mixed-strain cultures, appear to be lacking.

The volatile acids, and in particular acetic acid, are considered to contribute significantly to the mildly acid aroma of butter cultures. They are also considered important in the taste of butter cultures (55).

The recognition by van Niel et al. (152) that diacetyl was a principle component of butter culture flavor has since led to numerous investigations on its production in butter cultures. Hammer and Babel (55) state that in low concentrations it has a pleasing aroma but in higher concentrations the odor is pungent and objectionable. These authors cite works which indicate that concentrations from 1.5 to 2.5 p. p. m. are desirable in high quality butter cultures.

The partially reduced analogs of diacetyl, acetoin and 2, 3-butanediol, have also received considerable attention. According to Hammer and Babel (55) these compounds possess no odor and are probably never present in concentrations sufficient to affect the taste of butter cultures.

Carbon dioxide has been implicated in the flavor of butter cultures in a manner similar to that exhibited in various carbonated beverages. This effect has been considered desirable by several workers (2, 55, 132).

The production of ethanol by single-strain cultures of S. lactis, S. cremoris and L. dextranicum in non-milk media has been demonstrated by Friedman (41) and Platt and Foster (124). It has also been isolated from mixed-strain butter cultures grown in milk media by Badings and Galesloot (6), Day et al. (26) and Chou (18, p. 126).

In the investigation by Platt and Foster (124), glycerol was isolated from a single strain culture of S. cremoris when the pH of

the medium was maintained at seven during a 12 to 16 hr incubation period. However, it was not found in a normally growing culture of the same organism. These workers used glucose as the primary energy source and were able to account for 90 percent of the fermented glucose carbon in the end products produced by the normally growing culture of S. cremoris. The end products found were lactic, acetic and formic acids, ethanol and carbon dioxide.

The production of acetaldehyde in milk cultures by single strains of S. lactis, S. cremoris and S. diacetilactis has been reported by Harvey (59). This worker also found that some strains of S. cremoris and S. lactis produced acetone in milk cultures. The isolation of acetaldehyde and acetone from mixed-strain butter cultures has been reported by Day et al. (26), Chou (18, p. 126) identified both acetaldehyde and acetone in cultured buttermilks.

Jennings (70) identified methyl acetate, ethanol, diacetyl and acetic acid in a flavor concentrate obtained from a commercial starter distillate. More recently, Day et al. (26) tentatively identified a number of compounds isolated from butter cultures by low-temperature, reduced-pressure steam distillation techniques. The compounds tentatively identified by gas chromatography were acetaldehyde, propanal, n-butanal, 2-methylbutanal, n-pentanal, acetone, butanone, diacetyl, acetoin, ethanol, n-butanol, ethyl acetate, dimethyl sulfide and acetic acid.

Chou (18, p. 126-127) has reported that formaldehyde (or dimethyl sulfide), acetaldehyde, propanal, acetone, acetoin, diacetyl, n-butanal, butanone, 2-pentanone, n- or iso-pentanal, ethyl acetate, ethanol and acetic acid were tentatively identified by gas chromatography as flavor components of cultured buttermilk. Paper chromatographic methods were used to confirm the presence of acetaldehyde, acetone, pentanal, diacetyl and acetic acid in buttermilk. In addition, propionic, butyric and valeric acids, and isovaleraldehyde (3-methylbutanal) were tenatively identified by visual analysis of infrared spectra. Isovaleraldehyde was reported to be present in certain "unclean" flavored buttermilks. The presence of n-pentanal was not detected in fresh buttermilk, but was present in samples stored for three days. Single-strain cultures of S. lactis and S. cremoris grown in a milk media were evaluated for their ability to produce various neutral carbonyl compounds. All strains studied were found to produce acetaldehyde. Some of the single-strain cultures were reported to form acetone, n-butanal, propanal, 2-pentanone, and n-pentanal.

Green Flavor in Butter Cultures: Hoecker and Hammer (64, p. 320-345) noted that the aroma of fully ripened pure S. diacetilactis cultures resembled that of green butter cultures. The aroma of the cultures was not considered disagreeable by these workers, but lacked the suggestion of diacetyl found in good butter cultures.

Chemical analysis of the cultures for diacetyl revealed that from 0.16 to 1.65 p.p.m. of diacetyl were, nevertheless, present. Lundstedt and Fogg (89) also observed the same green culture aroma in S. diacetilactis cultures grown in citrated cottage cheese whey. Mather and Babel (95) and Lundstedt and Fogg (89) did not observe a green culture odor for pure cultures of L. citrovorum.

Badings and Galesloot (6, vol. B, p. 199-208) reported results of studies on the flavor of mixed-strain butter cultures. They found that butter cultures containing L. citrovorum and cultures containing L. citrovorum and S. diacetilactis as the aroma bacteria possessed a flavor in common which was different from cultures containing only S. diacetilactis as the aroma bacterium. Gas chromatographic analyses of butter culture and cultured butter volatiles collected by a nitrogen purging system in a liquid nitrogen trap along with organoleptic comparisons with yogurt demonstrated that acetaldehyde was primarily responsible for the green flavor defect (syn. yogurt flavor defect) in mixed-strain butter cultures. Yogurt was used for comparative purposes because acetaldehyde has been shown to be responsible for the characteristic flavor of yogurt (122). It was pointed out that not all S. diacetilactis cultured butters were down-graded. It was suggested that conditions of culture propagation along with manufacturing procedures influence the final flavor of the butter, and that the defect is criticized only when the influence of acetaldehyde on

flavor becomes too pronounced. In this regard, Seitz (137, p. 60) has reported the manufacture of cultured butters using single-strain S. diacetilactis cultures which possessed a desirable flavor and contained from 1.0 to 1.5 p.p.m. of diacetyl.

Badings and Galesloot (6, vol. B, p. 199-208) found that mixed-strain butter cultures containing L. citrovorum gave negative Schulz and Hingst reactions (135) for acetaldehyde. Positive reactions for acetaldehyde were given by mixed-strain butter cultures containing only S. diacetilactis as the aroma bacterium and by the ordinary non-citrate fermenting lactic streptococci. A 1:1 mixture of a mixed-strain butter culture containing L. citrovorum as the aroma bacterium and a mixed-strain butter culture containing S. diacetilactis as the aroma bacterium gave a positive acetaldehyde reaction immediately after mixing, but after incubation of the mixture for 24 hr at 20° C the reaction was negative. From this the authors concluded that L. citrovorum transformed the acetaldehyde present in the culture.

Leesment (85, vol. B, p. 209-216) has stated that S. diacetilactis strains were responsible for the production of malty flavor in butter cultures. However, malty flavor producing organisms isolated from raw milk were reported to be varieties of S. lactis. Reiter and Møller-Madsen (126) have cited work by Jensen and Møller-Madsen which has shown that the growth of L. citrovorum in a malty culture

produced substances which reduce the aldehydes causing malty flavor. In view of the report by Virtanen and Nikkilä (156), in which they attributed malty flavor to acetaldehyde, and the work by Badings and Galesloot (6, vol. B, p. 199-208), some confusion as to the definition of green and malty flavors may still exist.

Harvey (60) observed that all S. lactis, S. cremoris and S. diacetilactis cultures studied produced significant quantities of acetaldehyde in autoclaved skimmilk (115° C for 20 min). The amount of acetaldehyde in the S. lactis cultures varied from 0.4 to 4.5 mg/l; S. cremoris cultures contained from 0.5 to 9.0 mg/l; a culture of S. diacetilactis contained from 11.0 to 13.0 mg/l. Taste thresholds of acetaldehyde in skimmilk were determined to be from 0.5 to 1.0 p.p.m. for the least concentration difference detectable. The author stated that acetaldehyde production could have a significant effect on the flavor and aroma of milk cultures.

Malty Flavor in Cultures: Early work by Hammer and Cordes (56) showed that Streptococcus lactis var. maltigenes was the organism most commonly associated with the malty flavor defect in lactic cultures. The organism appeared only to differ from S. lactis in its ability to produce a malt-like aroma in milk.

Virtanen and Nikkilä (156) isolated a similar type cocci from malty butter cultures that differed from S. lactis var. maltigenes in

its biochemical reactions. The organism liberated considerable quantities of acetaldehyde in milk. A simulation of the malty defect in normal butter cultures by the addition of acetaldehyde led these workers to the conclusion that acetaldehyde was the compound responsible for malty flavor in milk.

Later work by Zuraw and Morgan (168) demonstrated that acetaldehyde was liberated in milk cultures of S. lactis var. maltigenes, but that in most cases non-malty strains of S. lactis produced more acetaldehyde than malty strains. These workers were not able to simulate the malty flavor in milk and normal lactic cultures by the addition of acetaldehyde and concluded that the liberation of acetaldehyde was not responsible for the characteristic malty flavor.

In 1954, Jackson and Morgan (68) reported that the characteristic malty aroma of S. lactis var. maltigenes had been conclusively shown to be due principally to 3-methylbutanal. The organism was shown to possess enzyme systems for conversion of leucine and isoleucine to 3-methylbutanal and 2-methylbutanal, respectively. Organoleptic comparisons of malty cultures with normal pasteurized milk containing added 3-methylbutanal revealed that a concentration of 0.5 p.p.m. of 3-methylbutanal gave the most typical malty aroma. Attempts to modify the aroma produced by 3-methylbutanal by addition of various concentrations of 2-methylbutanal, acetaldehyde and acetone (neutral carbonyl compounds also isolated from malty cultures)

resulted in no improvement of the malty aroma. No attempts to simulate the malty aroma in acidified culture medium (fresh skim-milk heated in flowing steam for 45 min) were reported.

Subsequent work by MacLeod and Morgan (90, 91) revealed a transaminase enzyme system in S. lactis and S. lactis var. maltigenes which effects the transfer of the amino group of leucine to alpha-ketoglutaric acid, resulting in the formation of glutamic acid and alpha-ketoisocaproic acid. S. lactis var. maltigenes possessed an additional enzyme which decarboxylated the ketoacid to 3-methylbutanal. S. lactis cells apparently lacked the decarboxylase enzyme.

Further work by MacLeod and Morgan (92) demonstrated that resting cells of S. lactis var. maltigenes were capable of similar enzymatic degradations of isoleucine, valine, methionine and phenylalanine. Incubation of washed cells suspended in phosphate buffer in the presence of the amino acids under investigation resulted in the production of the corresponding aldehydes (2-methylbutanal, methylpropanal, 3-methylthiopropanal and phenylacetaldehyde). Of the strains of S. lactis and S. cremoris studied, only one strain of S. lactis appeared to slightly degrade the amino acids to aldehydes. However, the authors did not believe these aldehydes could be involved extensively in the typical malty aroma because previous work (68) had shown the aroma could be simulated by simple addition of 3-methylbutanal.

Flavor of Cultured Cream Butter

Many of the flavor compounds which may be implicated in the flavor of butter culture and butter are discussed in more detail in other sections of the literature review. In view of the close relationship between the flavor of butter cultures and cultured butter, it appears appropriate to include results of investigations on the nature of butter flavor *per se*.

In a review by Babel and Hammer (5), it was stated that the very early workers believed the flavor of butter to be due to certain volatile fatty acids formed during the ripening of cream. The acids that were considered important in flavor were butyric, caproic, caprylic and capric.

The work of van Niel *et al.* (152) in 1929 established the importance of diacetyl as a flavor compound in cultured cream butter. Many research efforts have since substantiated its importance and it is now considered as the main keynote of cultured cream butter flavor. The review by Babel and Hammer (5) summarizes a majority of the work concerning the significance of diacetyl in cultured butter flavor. The presence of acetoin and 2, 3-butanediol are also discussed in detail.

There are discrepancies in the literature as to the optimum concentrations of diacetyl in butter necessary for producing a

desirable cultured flavor. For example, Davies (23, vol. 2, p. 76-78) has reported diacetyl contents of mild flavored butters to range from 0. 2 to 0. 6 p. p. m. ; butter with a full flavor contained from 0. 7 to 1. 5 p. p. m. , while higher concentrations gave a strong repulsive flavor. Swartling and Johannson (145) have reported the preparation of high flavored butters containing as much as 2. 1 p. p. m. of diacetyl. The lack of agreement may be attributed in part to the particular type of butter to which different individuals are accustomed. However, the balance of various flavor compounds which modify the overall effect exerted by diacetyl may be of equal or greater importance in the flavor of cultured butter. This has been indicated in recent work by Riel and Gibson (127) where it was found that butters flavored with starter distillate had superior flavor properties to those flavored with synthetic diacetyl. It has also been observed by Day et al. (29) that 40 p. p. b. of added dimethyl sulfide, a flavor compound isolated from butter, had the capacity to smooth out the harsh flavor of a synthetically flavored sweet cream butter containing 2. 5 p. p. m. of diacetyl, 30 p. p. m. of acetic acid and 500 p. p. m. of lactic acid.

Hammer (54, p. 440) states that the volatile acids, and especially acetic acid, give cultured butter a pleasing slightly acid aroma. Lactic acid contributes to the flavor, but is non-volatile. This author also states that the addition of acetic acid in the proper amount to sweet cream that is churned into butter without use of a culture

gives a pleasing flavor to the product. It is pointed out, however, that the flavor imparted is not a typical culture flavor.

The organic acids of butter, both volatile and non-volatile, have received considerable attention, but the data are limited on amounts of individual free acids found in butter. Most of the work has been concerned with the determination of total acidity and total volatile acidity of butters. The review of Babel and Hammer (5) adequately summarizes these data; increases in acidity are directly related to butter quality deterioration.

Hillig and Ahlmann (62) found that a sample of sweet cream butter contained 0.006 gm of acetic acid per kg of butter; butyric and lactic acids were not found. They also analyzed two commercial samples of butter and found the following amounts of acids per kg of butter: 0.138 and 0.192 gm of acetic acid, 0.621 and 1.56 gm of lactic acid, and 0.026 and 0.184 gm of butyric acid, respectively. Khatri and Day (80) and Bills et al. (13) have quantitatively determined the free fatty acids ($C_{4:0}$ through $C_{18:3}$) in milk fat isolated from sweet cream and ripened cream butters using gas chromatographic procedures. Significant quantities of free acids were found in the samples of milk fat and values for ripened cream butters were comparable to those found for sweet cream butters (80).

Steuart (141) reported evidence for the presence of butyric acid and a neutral compound which gave butyric acid upon saponification

(presumably ethyl butyrate) in the steam distillate of a full flavored butter. Babel and Hammer (5) cite work by Davies in which he suggested that the harsh flavor imparted to butter by the addition of synthetic diacetyl was due to the lack of other volatile flavoring compounds, such as esters, which were normally produced in the cream during the ripening process. This worker believed that milk fat, curd, salt, diacetyl and traces of esters such as ethyl butyrate, ethyl caproate and ethyl lactate were responsible for the desirable flavor of cultured cream butter.

The recent development of highly refined isolation and analytical techniques has resulted in the identification of a considerable number of trace constituents of butter which previously had escaped detection. The isolation of dimethyl sulfide and its relation to butter flavor (29) has been mentioned previously. The work by Boldingh and Taylor (14) on the trace constituents of butterfat has demonstrated the presence of several compounds which may be of significance in butter flavor. A summary of the work by the (Netherlands) Unilever Research group on the flavor of butter has been given by Taylor in 1962 (140). These investigators believe, on the basis of their work, that the flavoring constituents of butter serum are diacetyl, lactic acid and its salts, the lower fatty acids (predominately acetic) and their salts and sodium chloride. This group of workers propose that the most important class of compounds, which they have isolated and identified as

anilides from milk fat, are the C_8, C_{10}, C_{12}, C_{14}, C_{16} and possibly traces of C_{11} aliphatic delta-lactones. The concentrations of C_{10} and C_{12} delta-lactones were found to be about five and ten p.p.m., respectively, in "normal temperature" butterfat. Other compounds believed to be contributors to butter flavor were methyl ketones (resulting from the breakdown of beta-ketoacids bound as glycerides in milk fat), aldehydes (probably from decarboxylation of alpha-ketoacids), indole, skatole and dimethyl sulfide. Taylor (140) stated that indole, skatole and dimethyl sulfide appeared to be individual metabolites. In this regard, he suggested that the flavor components of butter fell into three classes: (1) those that were concomitant with physiological function, giving rise to homologous series of compounds; (2) those that were metabolites of ingested food or directly transmitted constituents; (3) and those that were produced by the butter making process.

Day et al. (26) using low-temperature, reduced-pressure steam distillation techniques isolated a variety of volatile flavor compounds from cultured cream butter. The compounds tentatively identified by gas chromatographic procedures included acetaldehyde, propanal, n-butanal, 3-methylbutanal, diacetyl, acetoin, ethanol, n-butanol, ethyl formate, ethyl acetate, ethyl butyrate, dimethyl sulfide and acetic acid.

Recently, Winter et al. (161) conducted a rather intensive study

of the volatile carbonyl compounds found in a "fresh dairy butter". The butter was made from unpasteurized cream in France. The reported diacetyl and acetoin content of the butter was 4.50 and 18.82 p.p.m., respectively, indicating that the cream was allowed to "ripen" to some degree before churning. Vacuum steam distillation was used to separate the volatile constituents from the butter. The carbonyl compounds of the aqueous distillate were converted to their 2,4-dinitrophenylhydrazones and separated by fractional recrystallization and column and paper chromatography. The compounds identified by comparison with authentic derivatives were formaldehyde, acetaldehyde, 2-methylpropanal, 3-methylbutanal, n-hexanal, n-nonanal, phenylacetaldehyde, acetone, 2-heptanone, 2-nonanone, diacetyl and (-)-acetoin. These workers believed that formaldehyde, acetaldehyde, 2-methylpropanal, 3-methylbutanal and phenylacetaldehyde were formed by enzyme catalyzed oxidative decarboxylations of glycine, alanine, valine, leucine and phenylalanine, respectively. The presence of n-nonanal and n-hexanal were attributed to the oxidative degradation of oleic and linoleic acids, respectively. These investigators also reported that the non-carbonyl part of the butter distillate revealed none of the characteristic butter notes, and especially, no lactone odor could be detected.

Begemann and Koster (11) have recently reported the isolation and characterization of 4-cis-heptenal from fresh butter and autoxidized milk fat. According to these workers, the compound possessed a definite "cream-flavor". Its concentration was estimated to be about 1.5 p.p.b. in fresh butter. Evidence was given which indicated that 4-cis-heptenal originated through the autoxidation of milk lipid fractions. Previously, Wong and Patton (165) had reported the isolation of a volatile fraction from cream which possessed an odor described as waxy or nut-like. The fraction had an odor highly characteristic of cream and appeared to have an extreme odor potency. The odor could be detected easily at a gas chromatograph exit when only a very small peak was apparent on the chromatogram.

Artificial Butter Flavor Concentrates

The chemical nature of butter flavor has intrigued chemists and flavorists for many years. Numerous patents have been issued for various synthetic or artificial butter flavor concentrates. The flavor concentrates that have been developed for butter, and for products intended to have the flavor of butter contain primarily diacetyl although many other flavor materials have been included. The labels on synthetic butter flavor concentrates reveal that ketones, acetals, esters and organic acids are common classes of compounds incorporated into the mixtures.

Apparently, many of the flavor concentrates available are not the result of chemical analysis, but rather are formulated by imaginative flavorists. Of importance is the fact that while an artificial flavor mixture may not simulate the natural flavor of butter, it may impart a desirable flavor in the product for which it is intended. An example is the use of artificial butter flavor concentrates in sweetened bakery goods.

In the review by Babel and Hammer (5) several works are cited where synthetic diacetyl was added to butter. The general conclusions were that added diacetyl imparted a definite enriched butter aroma and flavor, but at the same time gave a somewhat undesirable harsh flavor. These observations have been substantiated in the recent reports of Riel and Gibson (127) and Day et al. (29).

Butter culture distillates (often called starter distillates) have been employed for producing a "cultured" flavor in butter, sour cream and cottage cheese. The flavor concentrates are prepared by growing butter cultures in such a manner as to produce increased amounts of flavoring constituents. The cultures are then steam distilled to recover volatile flavoring compounds (5). These distillates often impart desirable flavor properties to butter, but do not duplicate the very delicate flavor of high quality cultured cream butter.

Babel and Hammer (5) have listed many compounds which have been recommended for the production of aroma in butter. A few of

the compounds mentioned were tributyrin, triketopentone, acetyl-propionyl, dipropionyl, benzaldehyde, coumarin, vanillin, ethyl butyrate, ethyl propionate and isopropyl butyrate. Many other compounds were mentioned, and it was noted that none of the compounds, either alone or in combination, duplicated the odor of butter.

The use of certain aliphatic lactones in preparing artificial flavor concentrates for margarine (and butter) has been discussed in a recent review by Pardun (109). The work by Boldingh and Taylor (14) on the isolation of various lactones from heated milk fat has revived interest in this class of compounds as flavoring agents in butter and margarine. Pardun (109) cites examples of recently patented artificial flavor mixtures for margarine which include significant quantities of short chain aliphatic acids, diacetyl and various gamma-, delta- and epsilon-aliphatic lactones. It is stated that the use of such mixtures results in an improved butter-like aroma and flavor.

Stoll et al. (142) have recently synthesized alpha-carboxy-delta-(or gamma-) lactones for use as lactone precursors in artificial flavoring mixtures. The alpha-carboxylactones are reportedly well crystallized, odorless and quite stable at room temperature. When suitable isomers were incorporated into butter or margarine, they were easily decarboxylated to yield simple odorous lactones upon heating at 80°-120° C, and gave rise to a characteristic lactone flavor exactly as did natural butter.

Flavor of Fresh Milk and Milk Fat

Patton et al. (119) first isolated methyl sulfide from milk and found that near its flavor threshold (12 p. p. b.) it exhibited a typical milk odor. Day et al. (27) found trace amounts of acetaldehyde and from one to ten p. p. m. of acetone in skimmilk. Jenness and Patton (69, p. 370) have stated that trace quantities of the above mentioned compounds along with butyric acid may contribute to the slight olefactory character of fresh milk. Fresh milk has a slightly sweet character due to its lactose content and a slightly salty character because of chloride salts. The milk proteins contribute to the tactual properties of milk flavor.

Recently Wong and Patton (165) have reported the results of a study on the volatile compounds isolated from fresh milk and cream by low-temperature, vacuum distillation techniques. Their data established the presence of formaldehyde, acetaldehyde, acetone, butanone, 2-pentanone and 2-hexanone in milk and cream. Tentative evidence also was obtained for 2-heptanone. In addition, gas chromatographic retention times and mass spectral data established the identity of ethyl ether, methyl sulfide, ethanol, chloroform, acetonitrile and ethylene chloride in the volatile fractions of milk and cream. A subsequent investigation by Wong (164) has also demonstrated the presence of 3-methylbutanal (isovaleraldehyde) in fresh cream.

Ammonia, n-propylamine, n-hexylamine and non-condensable gases have been listed as volatile compounds isolated from milk in a review by Day (24).

The lipid portion of milk contributes to the rich pleasing flavor of milk; it also tends to concentrate organic compounds which are soluble in the fat phase. Milk fat possesses several unique characteristics which distinguishes it from other edible fats and oils and some of these properties are believed to be related to its characteristic flavor. The glycerides of milk fat contain significant quantities of caproic, caprylic and capric acids. Another flavorful acid, butyric acid, is present to the extent of approximately ten mole percent (75). Magidman et al. (93) have shown the presence of at least 60 different fatty acids in the lipids of cows' milk. The significance of the majority of these acids to the flavor of fresh dairy products has not been determined. Work by Harper et al. (58) and Khatri and Day (80) has demonstrated that milk contains significant levels of unesterified fatty acids (straight chain, C_{18} or less) which are normally esterified in milk fat glycerides. At optimum levels these fatty acids are believed to contribute favorably to the flavor of milk and milk fat.

Boldingh and Taylor (14) have reported that trace amounts of delta-decalactone and delta-dodecalactone were found in fresh non-pasteurized cream. These compounds may also be significant in the flavor of milk and milk fat.

It was first shown by Duin (35) that long chain aldehydes (isolated from butter) exist as normal components of milk phospholipids. Subsequently, Schogt et al. (133) demonstrated the presence of significant amounts (as much as 50 mg/kg) of bound aldehydes in the triglycerides of milk. Parks et al. (112) studied the bound aldehydes originating in the phospholipid and triglyceride fractions of milk fat. They found straight-chain aldehydes from C_9 to C_{18}, branched-chain aldehydes from C_{11} to C_{18} (except C_{12}), C_{12}, C_{18} and C_{20} monounsaturated aldehydes and traces of di-unsaturated aldehydes. The aldehydes are apparently attached to the glyceride molecule through the enol-ether linkage (35, 133). The bonding is reported to be labile to acid hydrolysis and possibly enzymes and metal complexes.

Heat Induced Changes in Milk

Cooked Flavor: When milk is subjected to heat treatment, the type of flavor alteration is dependent upon the extent of heat treatment involved. Jenness and Patton (69, p. 384) state that a distinct cooked flavor commences to develop with momentary heating near 74° C and intensifies as the heat treatment is extended. According to Hutton and Patton (67) sulfhydryl groups are activated by heat denaturation of beta-lactoglobulin and the proteins of the fat globule membrane. The flavor has been attributed to volatile sulfides and hydrogen sulfide arising from the heat denatured proteins. Yoshino et al.(167) have

shown that there is a decrease in sulfhydryl activity and also an increase in disulfide activity in milks heated for various times at temperatures in excess of that required to produce a cooked flavor. When milk is subjected to heat treatments which are prolonged or extended to higher temperatures, the cooked flavor gives way to a variety of undefined flavors that are culminated in the production of a caramelized flavor. This is accompanied to some degree by simultaneous development of brown (melanoidin) pigments (117).

Browning and Associated Changes: In 1955 Patton (117) extensively reviewed the literature concerning browning and changes in milk associated with browning. The knowledge of the chemistry involved in browning of milk is far from complete, but the primary mechanism is believed to be that of the amino-sugar or Maillard-type browning. Hodge (63) has reviewed the literature concerning the mechanisms involved in this type of browning.

In the review by Patton (117) several points are discussed which are pertinent to this investigation. The mechanism of formation and the compounds responsible for the caramelized flavor in heated milk are not known. Casein appears to be involved because heated fluid whey does not develop a caramelized flavor. In general, the degree of caramelized flavor is correlated positively with the degree of browning and associated changes.

Patton (117) further points out that the two principle reactants in the browning of milk are lactose and casein as neither will brown readily when heated alone. On the basis of the fact that amino compounds catalyze dehydration, fragmentation and condensation of sugars, it has been suggested that the epsilon-amino groups of lysine in casein are involved in catalytic decomposition of lactose.

Many simple organic compounds appear to be directly or indirectly involved in browning. Some of the compounds have been isolated from milk which has been subjected to heat treatments that greatly exceed those employed in normal product manufacture. However, the compounds may be formed in much lower concentrations in less severely heat treated milk and thus have escaped detection. Compounds associated with browning which have been reported to be formed in heated milk include 5-hydroxymethyl-2-furfural (116), 2-furfuryl alcohol (120), maltol (115), acetol (74), methyl glyoxal (74) and 2-furfural (101). These compounds, along with formic acid (117), are believed to be formed either substantially or completely from lactose. Honer and Tuckey (66) observed that when lactose is destroyed in milk, galactose, but not glucose, accumulates. Work by Patton and Filpse (118) in which they used labeled lactose, has shown that the carbon atom "number 1" of lactose is the principle source of formic acid; evidence is also given which indicates that the carbon skeletons of 2-furfuryl alcohol and maltol from heated milk are

derived from the two to six and one to six carbon atoms, respectively, of the glucose moiety of lactose. Morgan et al. (101) have suggested that 2-furfural is an intermediate in the formation of 2-furfuryl alcohol.

Morgan et al. (101) also isolated acetaldehyde from heated skim-milk. In a series of experiments using uniformly C^{14} labeled lactose and 2-C^{14} labeled alanine, Dutra et al. (36) found that both lactose degradation and Strecker degradation of alanine were sources of acetaldehyde in evaporated milk.

Other simple organic compounds have been isolated from browning systems. Aldehydes, such as those reported by Nawar et al. (103), are usually attributed to incidental oxidation of milk lipids. However, aldehydes may be produced as intermediates or products in the browning of amino-reducing sugar systems by the Strecker degradation reaction. Schönberg et al. (134) have reviewed the mechanisms involved. Patton (117) has stated that the necessary reactants are present in milk; these include free amino acids, dehydroascorbic acid, 2, 3-diketogulonic acid and other dicarbonyls resulting from lactose and glucose degradations by heat. The Strecker degradation has been considered to be a principle route of carbon dioxide production during browning (63).

Short Chain Acids: The formation of short chain organic acids during the course of somewhat prolonged heat treatments of milk has been the subject of numerous investigations. The earlier work on acids in heated milk has been reviewed by Gould (51). The lack of suitable methods for adequate separation, identification and quantitation of these compounds has restricted the value of many of these investigations.

More recent work by Morr et al. (102), utilizing a lyophilization concentration procedure and a silica gel column chromatographic separation technique, has given quantitative and qualitative information on the formation of various organic acids in normal skimmilk and skimmilk containing 0.5 percent added disodium phosphate and heated at 100° C up to six hours. Their work revealed the presence of butyric, propionic, acetic, pyruvic, lactic and formic acids in heated skimmilk. The acids were reported to have been positively identified by crystallography or by paper chromatography. In addition, four unidentified acids were also reported to be present. Acetic, formic, lactic and one of the unidentified acids accounted for approximately 98 percent of the heat produced acids. The amounts of butyric, propionic, pyruvic and the other three unidentified acids were not increased appreciably as a result of the heat treatments. The normal skimmilk heated for one hour at 100° C contained an average of:

formic acid	1, 470 microequivalents/l (0. 068 g/kg)
acetic acid	690 microequivalents/l (0. 041 g/kg)
lactic acid	746 microequivalents/l (0. 067 g/kg)

The skimmilk containing 0. 5 percent added disodium phospate heated for one hour at 100° C contained an average of:

formic acid	2, 190 microequivalents/l (0. 100 g/kg)
acetic acid	846 microequivalents/l (0. 050 g/kg)
lactic acid	884 microequivalents/l (0. 080 g/kg)
propionic acid	48 microequivalents/l (0. 004 g/kg)

The formic acid content of the phosphate treated skimmilk heated for 1. 5 hours at 100° C increased sharply to 4150 microequivalents/l (0. 191 g/kg). The authors concluded that the large amounts of formic acid encountered substantiated the theory that the lactose molecule is cleaved at the one-two position during heating. The simultaneous production of other acids (two or more carbon atoms) suggested the possibility that additional cleavages of the lactose molecule are involved.

Lactone Formation: Keeney and Patton (76, 77) first isolated delta-decalactone from heated milk fat and demonstrated its presence in evaporated milk, dried cream and dried whole milk. Later, Tharp and Patton (147) isolated delta-dodecalactone from steam distillates

of milk fat. Recently, Boldingh and Taylor (14) have reported the isolation and identification of the anilides of several optically active lactones from heated milk fat. The delta-lactones with 8, 10, 12, 14 and 16 carbon atoms were found to comprise the major portion of the lactone fraction. They also isolated and identified the anilides of delta-undecalactone, delta-dodecene-9-lactone and gamma-dodecene-6-lactone. Another unique gamma-lactone, the enol lactone of 2, 3-dimethyl-4-keto-nonene-2-oic acid, was also identified; it possessed an odor similar to celery and was thought to originate in the feed. These workers, therefore, have obtained indirect evidence that hydroxy-acid precursors of lactones are normal constituents of milk fat. They postulate, as a result of experiments with model systems, that heat effects the hydrolysis of the hydroxy-acids from the glycerides and subsequent ring closure forms the corresponding lactones.

Cobb (19, p. 69) reported that small amounts of free fatty acids were found in raw milk. Heating was observed to cause the production of additional amounts of free acids presumably through glyceride hydrolysis. The longer chain acids were not considered to be of great significance in flavor, but the shorter chain acids, especially butyric acid, were believed to contribute to the flavor of heated milks.

Methyl Ketone Formation: In unheated milk fat, acetone has been found to be the predominant methyl ketone (27), but traces of other

methyl ketones with odd numbered carbon atoms have been reported (164). When milk fat is heated in the presence of water an homologous series of methyl ketones with odd-numbers of carbon atoms is produced (121, 166). Recently, van der Ven et al. (151) have reported the isolation and identification of an homologous series of pyrazolones from milk fat corresponding to the appropriate postulated precursor beta-ketoacids. Parks et al. (110) have described the direct isolation of the methyl ketone precursors from milk fat. They have demonstrated that the C_5 through C_{15} odd numbered methyl ketones are derived from beta-ketoacids esterified to triglycerides of normal milk fat. Their evidence indicates that one beta-keto acid and two fatty acid moieties comprise the triglyceride in question. Heating milk fat in the presence of water effects hydrolysis of the beta-ketoacids and subsequent heating decarboxylates the acids to form methyl ketones.

The increases in acetone concentrations in heated milk fat (83, 103) cannot as yet be explained by the same reactions involved in the formation of other odd numbered methyl ketones because beta-ketobutyric acid has not been shown to be a moiety of the triglycerides of milk fat.

Some even numbered methyl ketones, butanone (83, 103), 2-hexanone and 2-octanone (103), have been isolated from heated milk fat. The origin of these compounds remains undefined at the present

time.

Langler and Day (83) have determined the average flavor threshold (AFT) of a mixture of methyl ketones, prepared in the ratios found in heated milk excepting acetone and 2-pentadecanone, to be 1.55 p.p.m. in four percent homogenized milk. They also reported the concentrations of each ketone present in the AFT mixture and the potential concentrations of each ketone in a four percent milk assuming maximum methyl ketone production. Their data are summarized as follows:

Ketone	Concentration at AFT of Mixture (p.p.m.)	Maximum Concentration from 4% Milk (p.p.m.)
butanone	0.13	0.52
2-pentanone	0.20	0.80
2-heptanone	0.38	1.52
2-nonanone	0.18	0.72
2-undecanone	0.20	0.80
2-tridecanone	0.46	1.84
total	1.55	6.20

From these data it was concluded that milk fat contains sufficient ketone precursor to produce detectable flavors in beverage milk.

Miscellaneous Changes: Jenness and Patton (69, p. 322-346) have discussed some of the other effects of heat on milk. Heating to pasteurization temperatures causes destruction of enzymes in milk; a notable example is the inactivation of milk lipase. It also destroys all pathogenic organisms and the bulk of the non-pathogenic organisms. Heat treatment of milk removes carbon dioxide and oxygen and the net result is no change in pH because of a shift in the salt balance to compensate for carbon dioxide loss, but there is a lowering of the Eh. Heat treatments as extreme as autoclaving apparently neither destroys citrate in milk nor affects the amount of dissolved citrate in milk.

Jenness and Patton (69, p. 354) state that in addition to hydrogen sulfide, ammonia is produced as a result of heat degradation of proteins, and that possibly urea may be a source of ammonia in heated milk.

Weurman and De Rooij (158) have reported the presence of methylamine, ethylamine, butylamine and dimethylamine in the steam distillates of fresh milk. They also reported tentative evidence for pyrrolidine in the milk distillates. The steam distillations were carried out at atmospheric pressure which was shown to degrade certain phosphotides.

Cobb et al. (20) identified vanillin in commercial evaporated milk. These workers suggested that lignin derived precursors from

the feed may be passed through the metabolic processes of the cow into the milk. If such were the case, they theorized that heating could then possibly give rise to vanillin in evaporated milk.

Other somewhat unusual compounds have been found in heated milk products. Patel et al. (114) have presented evidence for dimethyl sulfide, a pentyl acetate and other compounds, previously mentioned, in sterilized milk. Parks and Patton (113) have reported the isolation and identification of benzaldehyde from stored dry whole milk. Recently, Parks et al. (111) isolated and identified o-aminoacetophenone from stale dry whole milk.

Bacteriology and Biochemistry of Butter Cultures

Taxonomy and Classification of Butter Culture Bacteria: The species of bacteria which may be included in butter cultures have been mentioned briefly in the section of the literature review pertaining to the flavor of butter cultures. The bacteria incorporated into butter cultures are also commonly found in commercially formulated cultures used for the manufacture of cheddar cheese, buttermilk, cottage cheese and sour cream. In the manufacture of such products, cultures are added for one or both of two purposes: to produce lactic acid or to produce desired aroma.

The reviews of Hammer and Babel (55), Galesloot (43, vol. D, p. 143-158), Collins (21) and Reiter and Møller-Madsen (126) discuss

in detail the evolution of the taxonomy of lactic starter bacteria. The classification and nomenclature of some of the organisms involved has been, and in some cases still is, a controversial topic.

Three species of streptococci belonging to Lancefield's Serological Group N are now generally recognized as the lactic acid producing bacteria which may be present in mixed-strain butter cultures. These are Streptococcus cremoris, Streptococcus lactis, and Streptococcus diacetilactis (126). Many criteria, such as growth in individual sugar media, tolerance to high levels of sodium chloride, carbon dioxide production and ability to grow at various temperatures and pH values, have been useful in classifying these bacteria. However, these reactions are often variable within a species and yield results which are difficult to interpret.

Sandine et al. (128) have used rather simple and well-known methods for characterizing starter bacteria. The production of sufficient lactic acid in 48 hours at 30° C to coagulate litmus milk, along with the reduction of the litmus, served to differentiate the lactic streptococci from the Leuconostoc organisms. S. diacetilactis and S. lactis were separated from S. cremoris on the basis of the Niven et al. arginine hydrolysis test (104); the former give positive tests and the latter give negative tests. S. diacetilactis was separated from S. lactis on the basis of the ability of S. diacetilactis to produce diacetyl and acetoin; these compounds were detected by the

modified creatine test of King (81). High carbon dioxide production from citrate by S. diacetilactis also was used as a criterion for characterization of S. diacetilactis. S. lactis and S. cremoris do not ferment citrate.

Galesloot (43, vol. D, p. 144) has expressed some doubt as to the validity of the creatine test for differentiation between S. diacetilactis and S. lactis. He cites instances where non-citrate fermenting strains of S. lactis and S. cremoris give a slightly positive creatine test. However, Sandine et al. (128) favor the classification of these organisms as S. lactis var. aromaticus and S. cremoris var. aromaticus. Galesloot (43, vol. D, p. 144) also points out work by Collins and Harvey (22) which has demonstrated that S. diacetilactis organisms may lose their ability to produce the enzyme, citrate permease, and thereby give negative creatine tests. The intracellular citritase enzyme has been found to be constitutive in S. diacetilactis (60), and cell free extracts still possess the ability to produce acetoin and carbon dioxide from citrate.

The associative citrate fermenting aroma bacteria found in butter cultures, as previously mentioned, can be differentiated from the lactic streptococci by their lack of acid production in litmus milk at 21° and 30° C (128). L. citrovorum can be differentiated from L. dextranicum by virtue of the latter's ability to produce dextran (large slimy colonies) from sucrose when streaked on a sucrose

enriched agar medium (128).

Sandine et al. (128) state that considerable confusion has arisen regarding the taxonomy of the Leuconostoc genus. These workers recommend the designation of Leuconostoc mesenteroides for those organisms which do not associatively produce diacetyl and acetoin in nonfat milk but do elaborate dextran on sucrose medium. So-called Leuconostoc organisms having neither of the properties would be placed in the Pediococcus genus. Galesloot (43, vol. D, p. 145) has stated that no reliable evidence for the presence of pediococci in starter cultures has been reported. L. mesenteroides have been found only infrequently in starters.

Bacterial Composition of Mixed-Strain Butter Cultures: Some dairy plants apparently use mixed-strain cultures of S. lactis and S. cremoris for the production of cultured cream butter. This practice appears to be used for one or both of two reasons: first, the maintenance of high quality butter cultures is often difficult and, second, some plant operators do not fully realize the importance of aroma bacteria in cultured butter flavor. The use of "non-aromatic" lactic cultures formulated for acid production in cottage and cheddar cheese manufacture results in poorly flavored cultured butters (64).

Galesloot (43, vol. D, p. 145) has classified mixed-strain butter cultures according to the types of aroma bacteria present (assuming

that ordinary lactic streptococci are also included in the cultures). These are: cultures with only Leuconostoc sp. as aroma bacteria; cultures with only S. diacetilactis as aroma bacterium; and cultures containing both Leuconostoc sp. and S. diacetilactis.

Hammer and Babel (55) have summarized the earlier work on the bacterial composition of butter cultures. These authors stated that satisfactory butter cultures normally contain from one million to ten million aroma bacteria per ml. They cited work in which it was reported that a culture which failed to produce its normal amounts of diacetyl, acetoin and acetic acid and lacked flavor contained less than 1,000 aroma bacteria per ml, and the lactic acid organisms were present in numbers in excess of 100 million per ml. Other works were cited which indicated that the aroma bacteria disappeared from cultures with successive transfers and a harsh acid or malt flavor became apparent.

Glenn and Prouty (46) studied the progressive changes in the numbers of L. citrovorum and S. cremoris in a culture during incubation at 22° C. These workers found that approximately five percent of the total microbial population was L. citrovorum through the first ten hr of incubation. After 16 hr the L. citrovorum organisms decreased to about one percent; this was accompanied by a decrease in the pH of the milk medium to a value of 4.85. After 25 hr of incubation the L. citrovorum organisms accounted for about four

percent of the total population.

Recently, Overcast and Skean (105) examined 72 lactic cultures used for buttermilk, cottage and cheddar cheese and sour cream manufacture for citrate fermenting organisms. They reported that the citrate fermenting bacteria per ml, expressed as percent of each total count, had a median of 13 percent. The cultures examined ranged from two days to over ten years in age and the numbers of citrate fermenting bacteria varied from fewer than 10, 000 to 2. 1 billion per ml. The culture containing less than 10, 000 citrate fermenting organisms per ml also contained 1. 3 billion lactic acid bacteria per ml. On the basis of a statistical analysis of their data, the authors concluded that the mean percentages of citrate fermenting bacteria in cultures were not significantly different according to their original source, time mantained in the dairy plant or particular use. They further stated that their data did not support the idea that citrate fermenting organisms tend to disappear with successive transfers.

According to Galesloot (43, vol. D, p. 146), the percentage of inoculum and the incubation temperature affect the composition of butter cultures. A small percentage of inoculum and high incubation temperatures increased the proportion of S. diacetilactis in cultures containing only these organisms for aroma production. The reverse appeared to be the case for cultures depending on Leuconostoc organisms for aroma production. Apparently, cultures containing both

S. diacetilactis and Leuconostoc sp. are affected even more by the method of culture propagation. Galesloot (43, vol. D, p. 146) cited work where it was shown that a small percentage of inoculum, an increased incubation temperature and a transfer at low acidity shifted the aroma bacteria population in the direction of S. diacetilactis. The opposite method of propagation reportedly increased the numbers of Leuconostoc organisms.

Galesloot (43, vol. D, p. 143) has stated that the rate of acid production of S. diacetilactis is usually somewhat slower than that of S. lactis and S. cremoris strains. Anderson and Leesment (85, vol. vol. B, p. 217-224) have reported an incidence of slow acid production in two-strain cultures containing a strain of S. diacetilactis as the aroma bacterium. It was found that the S. diacetilactis strain had supplemented the S. cremoris or S. lactis strains to such an extent that the rate of acid production was decreased. Recently, Vedamuthu et al. (155) observed that the addition of as little as 0.2 percent citrate to milk media caused a dominance of a strain of S. diacetilactis over strains of either S. lactis or S. cremoris in two-strain mixtures. It was believed that the ability of S. diacetilactis to ferment citrate accounted for the strain dominance.

DeMann and Galesloot (34) noted seasonal variations in the numbers of Leuconostoc organisms in butter cultures which could be prevented by the addition of 0.25 p. p. m. of manganese. They attributed

the fluctuations in numbers of Leuconostoc sp., along with the ease of contamination of cultures with S. diacetilactis during periods of low Leuconostoc numbers, to seasonal variations in the manganese content of milk. Reiter and Møller-Madsen (126) have cited work which demonstrated very wide fluctuations in the manganese content of milk. The spring months apparently yield milk deficient in manganese and the fall months yield milk with the highest levels of manganese. The work of DeMann and Galesloot (34) showed that the addition of manganese stimulated the Leuconostoc organisms only when the cells were actively growing. Resting cell suspensions in phosphate buffer plus manganese, calcium, dextrose, lactose and citrate did not respond. The addition of manganese had no effect on the ordinary lactic streptococci or S. diacetilactis.

Certain Aspects of Butter Culture Metabolism: The metabolism of mixed-strain cultures is complex, in that it encompasses not only the production of lactic acid and flavor or aroma substances, but includes production of other compounds from carbohydrates, utilization of nitrogenous compounds, vitamins, salts and fats as well as the development of antibiotic-like substances. The general nutritional requirements and factors affecting the activity of bacteria comprising the flora of butter cultures have been recently reviewed (43, vol. D, p. 147-158; 126, 146).

The mechanisms involved in the associative growth of the organisms in butter cultures are often difficult to study in a complex biological fluid such as milk. As a result, many investigators have resorted to the use of synthetic or semi-synthetic media for their studies. In such cases, the enzyme constitution of a cell may be altered and care must be taken in relating results obtained in these media to those observed in milk. However, the combination of the two approaches of study has contributed greatly to the knowledge and understanding of the growth and activities of culture organisms in milk and other dairy products. For the scope of this investigation, a discussion of sugar and citrate fermentations is pertinent.

Carbohydrate Utilization by Homofermentative Lactic Streptococci: The lactic streptococci are called "homofermentative" because lactic acid is the principal metabolic end-product from glucose. A distinguishing characteristic of these organisms from heterofermentative lactic bacteria is the production of L (+)- lactic acid (43, vol. D, p. 144). Lactose, the principal carbohydrate in milk must be converted into some form of glucose before it can be metabolized by the lactic streptococci. The hydrolysis of lactose yields glucose and galactose; glucose can immediately enter the well known Embden-Meyerhof metabolic pathway. According to Kandler (73), galactose is transformed into a utilizable form by the following scheme:

(1) Galactose + ATP $\xrightarrow{\text{Galactokinase}}$ Galactose - 1-PO_4 + ADP

(2) Galactose-1-PO_4 + UDP-Glucose $\xrightarrow{\text{Galactose-1-PO}_4 \text{ Transurydilase}}$ UDP-Galactose + Glucose-1-PO_4

(3) UDP-Galactose $\xrightarrow{\text{UDP-Galactose-4-Epimerase}}$ UDP-Glucose

The Glucose-1-PO_4 produced in reaction (2) then enters the Embden-Meyerhof pathway.

Van Slyke and Bosworth (154) found that S. lactis grown in pasteurized and separated fresh milk at 32.2° C fermented only 20 percent of the original lactose in 96 hr. The percentage of fermented lactose that was converted to lactic acid reportedly varied from 70 to 93 percent. Homofermentative bacteria, however, apparently possess the enzymes necessary for direct oxidation and decarboxylation of glucose-6-PO_4 to ribulose-5-PO_4 and, hence, could carry out heterofermentative reactions (73). This pathway may account for the formation of products other than lactic acid by homofermentative organisms.

Some interesting observations have been made concerning the metabolism of lactic streptococci which are not normally mentioned in metabolic schemes. Palladina (108) observed that S. lactis decomposed ethanol while S. cremoris did not attack it. Harvey (59) reported that lactic streptococci produce acetaldehyde and some strains of S. cremoris and S. lactis produce acetone. However,

the strain of S. diacetilactis studied did not produce acetone, but rather reduced the acetone concentration in milk to zero, thus indicating that S. diacetilactis may in some way utilize acetone. Harvey (59) proposed that the accumulation of acetaldehyde by lactic streptococci was the result of the decarboxylation of pyruvate.

Carbohydrate Utilization by Heterofermentative Lactic Bacteria: The bacteria of the Leuconostoc genus derive the name "heterofermentative", because they normally produce several metabolic end-products from glucose. They are capable of producing limited amounts of D (-)-lactic acid which is a distinguishing characteristic for these organisms when compared to the lactic streptococci (43, vol. D, p. 144). The metabolic pathway for the utilization of hexose sugar by heterofermentative bacteria has been described by Kandler (73) and is shown in Figure 1. This pathway is commonly called the hexose monophosphate shunt and the heterofermentative organisms utilize this pathway rather than the Embden-Meyerhof pathway because they lack the enzyme aldolase, which mediates the conversion of fructose-1, 6-diphosphate to dihydroxyacetone phosphate and 3-phosphoglyceraldehyde (126).

Galesloot (43, vol. D, p. 149) has pointed out that the reduction of acetylphosphate to ethanol is a waste of considerable energy, but the Leuconostoc organisms are compelled to do this in order to

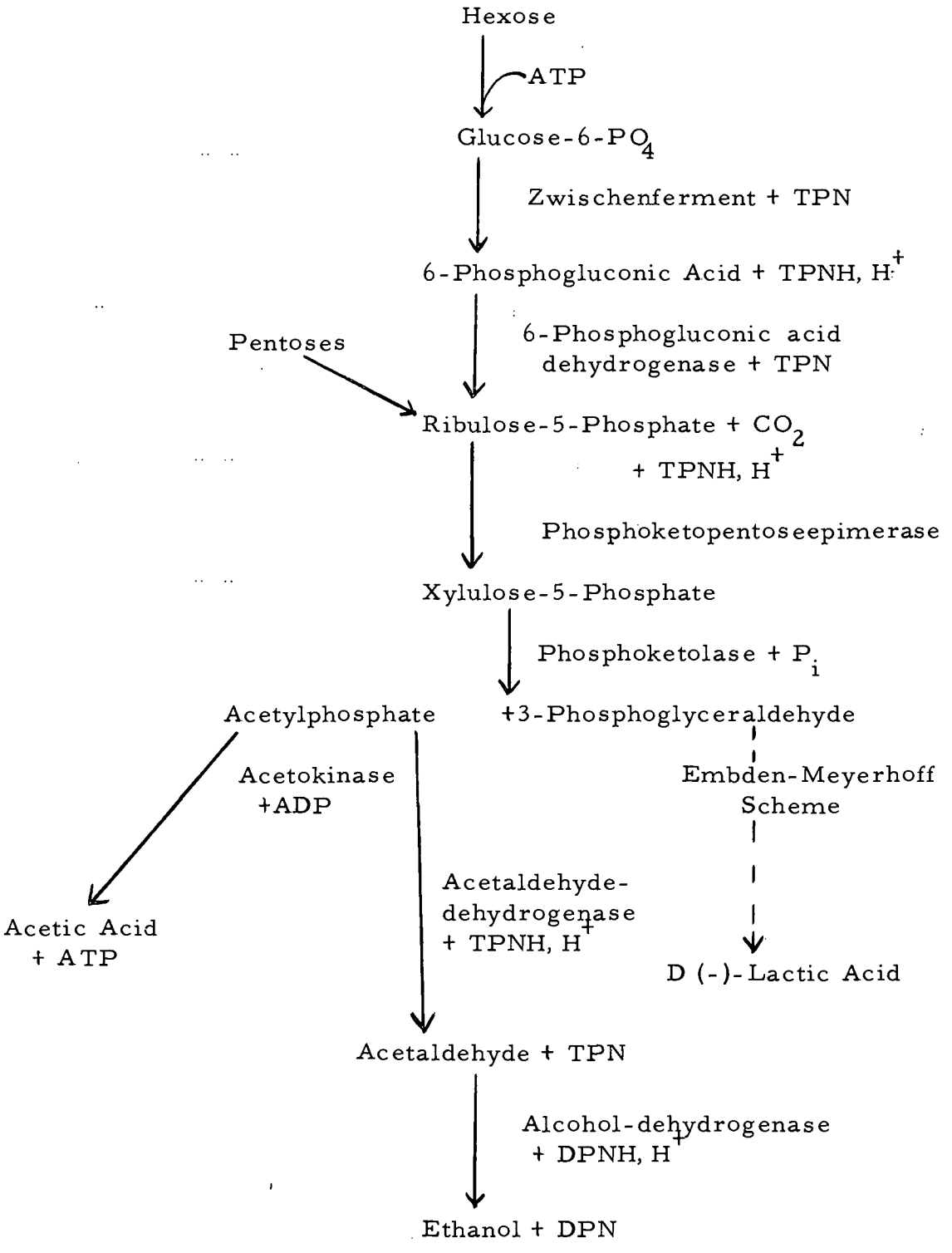


Figure 1. Carbohydrate metabolism of heterofermentative lactic acid bacteria as described by Kandler (73, p. 524).

consume the hydrogen generated by the conversion of hexose to 6-phosphogluconic acid and by that of the latter compound to carbon dioxide and ribulose-5-phosphate. When the circumstances permit, the Leuconostoc bacteria do not reduce acetylphosphate to ethanol, but convert the former compound to acetic acid.

Citric Acid Fermentation and the Production of Diacetyl: This feature of butter cultures has been for many years the subject of much research and confusion. Recently, the use of radioactive isotopes and more elegant biochemical techniques has led to a more complete understanding of this process. The reviews by Galesloot (43, vol. D, p. 150-153), Marth (94) and Reiter and Møller-Madsen (126) cover in detail the work which has led to the current theories.

The pathways for enzymatic conversion of citric acid by S. diacetilactis have been summarized by Seitz (137, p. 91-96) and are shown in Figure 2. According to Galesloot (43, vol. D, p. 153), the scheme for citrate metabolism by other butter culture aroma bacteria appears to be the same as that given in Figure 2. The generally accepted facts concerning diacetyl and acetoin production are that no aroma is produced from sugar alone, but is produced from citrate alone and from a mixture of sugar and citrate. Harvey and Collins (60) have discussed the mechanisms and source of diacetyl and acetoin in S. diacetilactis and L. citrovorum. Pyruvic acid is the

Citric acid $\xrightarrow{1^a}$ Oxaloacetic acid + Acetic acid

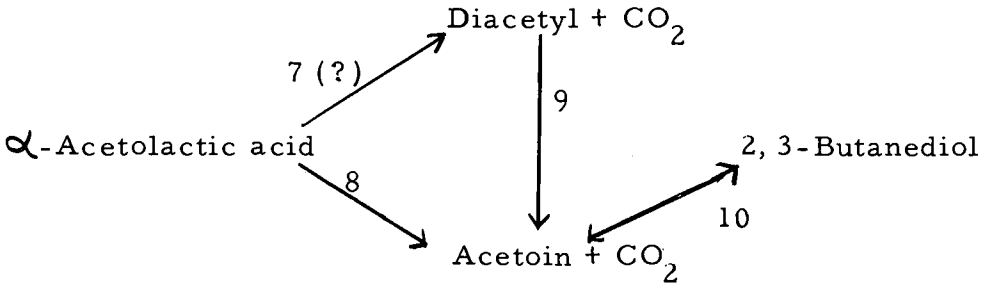
Oxaloacetic acid $\xrightarrow{2}$ Pyruvic acid + CO_2

2 Pyruvic acid + 2 TPP[b] $\xrightarrow{3}$ 2-Acetaldehyde · TPP + $2CO_2$

Acetaldehyde · TPP $\xrightarrow{4}$ Acetaldehyde + TPP

Acetaldehyde · TPP + Acetaldehyde $\xrightarrow{5}$ Acetoin + TPP

Acetaldehyde · TPP + Pyruvic acid $\xrightarrow{6}$ α-Acetolactic acid + TPP



[a]Enzymes catalyzing each reaction:

1. Citritase
2. Oxaloacetate decarboxylase
3. Pyruvate decarboxylase
4. Non-enzymatic
5. Acetoin synthetase
6. α-Acetolactate synthetase
7. α-Acetolactate oxidase
8. α-Acetolactate decarboxylase
9. Diacetyl reductase
10. 2, 3-butanediol dehydrogenase

[b]Thiamine pyrophospate

Figure 2. Pathways for enzymatic conversion of citric acid by S. diacetilactis as described by Seitz (137, p. 95).

starting point for the synthesis and is formed from both sugar and citrate fermentations. These organisms are essentially anaerobic in metabolism and the sugar fermentation yields reduced pyridine nucleotides simultaneously with pyruvic acid. The citrate fermentation yields pyruvic acid without the presence of reduced pyridine nucleotides. In the case of sugar, pyruvic acid must be reduced to lactic acid in order to reoxidize the reduced pyridine nucleotides and permit the sugar fermentation to proceed. Thus, there is no pyruvic acid remaining for alpha-acetolactic acid synthesis. When the substrate is a mixture of sugar and citrate, as in milk, more pyruvic acid is present than can be reduced to lactic acid. When the pH is low enough the alpha-acetolactic acid synthesis starts by utilizing the pyruvic acid stored in the organism. At lower pH values (near pH 5.0), the sugar fermentation is slowing down and when aroma production starts the pyruvic acid pool is fed mainly by the citrate fermentation (43, vol. D, p. 152-153).

DeMann and Galesloot (34) have reported that very active Leuconostoc starters (containing associative lactic streptococci) produce very little diacetyl aroma. It appears that the Leuconostoc organisms tend to avoid the reduction of acetylphosphate to ethanol and prefer to convert the former into acetic acid with the production of one ATP (see Figure 1). In such case the hydrogen formed during the sugar fermentation appears to be used in the reduction of diacetyl

to acetoin and 2, 3-butanediol. Galesloot (43, vol. D, p. 153) had suggested that when the Leuconostoc organisms cannot finish their metabolic processes because of low numbers or low pH, diacetyl accumulates instead of being reduced to acetoin and 2, 3-butanediol.

Effect of Heating Milk on Lactic Culture Activity: The phenomenon of microbial response to the "heat history" of milk has been extensively investigated. The literature on this subject is contradictory in many cases. Greene and Jezeski (52) have adequately reviewed the earlier literature and cite instances where heat treatment generally improved lactic culture development; in other instances the reverse was true. Considering the discrepancies in the literature, Greene and Jezeski (52, 53) carried out a series of well controlled experiments using lactic streptococci in an attempt to resolve these contradictions. These workers found that gradual increases in severity of heat exposure resulted in an initial stimulation, followed by a zone of inhibition, followed by another zone of stimulation, and terminating in a second area of heat induced inhibition.

Greene and Jezeski (52, 53) were able to simulate the initial stimulation zone (62° - 72° C for 40 min.) using various methods to lower the Eh, and by adding cysteine or milk protein hydrolysate to raw or normally pasteurized milk. They concluded that the

initial stimulation was due to lowering the Eh, denaturing serum protein, partially hydrolyzing protein and destroying heat-labile inhibitors.

The first zone of heat-induced inhibition (72° C for 45 min through 90° C for 45 min) was attributed to an excess of cysteine and volatile sulfides in milk. The condition could be simulated by adding cysteine to milk exhibiting the characteristics of the initial stimulation zone or by bubbling hydrogen sulfide through milk.

The second stimulation zone (90° C for 60 to 180 min or 120° C for 15 to 30 min) was reported to be due to a heat-induced diminution in toxic sulfides. The final zone of inhibition occurred after autoclaving (120° C) for longer than 30 min and was accompanied by severe browning.

The effect of heat treatment on culture activity has been studied primarily by using cell counts or acid production as indicies of activity. Seitz (137, p. 83) studied the ability of S. diacetilactis and Leuconostoc sp. to produce diacetyl in milks given various heat treatments. Under the conditions of his investigation, this worker found that pasteurized milk was least satisfactory, and that milk heated for 60 min at 100° C or autoclaved (121° C for 12 min) was best suited for diacetyl production by the organisms studied. The Leuconostoc organisms were most affected by heat treatment of the medium. In pasteurized milk they produced little, if any, diacetyl

while in milk heated for 60 min at 100° C as much as seven p. p. m. of diacetyl were produced. It was suggested that heat liberated growth stimulants were important in insuring maximum flavor and aroma production by Leuconostoc organisms.

Flavor Research Methods

Strong (143) has outlined the objectives of chemical research into the flavors of foods. The prime consideration is to positively identify each substance that contributes significantly to the flavor of a food. The chemical compounds that are involved in a food flavor must be known before efforts to establish quantitative methods for specific flavor contributors can be initiated. After specific compounds have been identified and quantitative methods have been developed, optimum levels of flavor components must be ascertained. In order to achieve this goal, flavor evaluations utilizing subjective flavor panel methods are necessary to corroborate data revealed by chemical analyses.

The ultimate goal in flavor research is to exactly duplicate natural flavors and use this information to improve the quality and uniformity of food products. In this respect a natural flavor does not always imply a desirable flavor. In some instances where the exact cause of a flavor defect is known, measures can be taken to prevent its formation. In other instances special procedures can be used to

remove the chemical compounds responsible for the flavor defect. In many food products flavor may be lacking or difficult to maintain; in such cases flavor concentrates and flavor stabilizers can be added to yield a product with a desirable natural flavor.

According to Jenness and Patton (69, p. 361) flavor has three basic components, olefactory, gustatory and tactual which are concerned, respectively, with the odor, taste and feel of a flavor stimulus. Variations in odor are almost limitless, but taste is limited to the detection of sweetness, sourness, saltiness and bitterness. Tactual responses in flavor refer to the way the substance feels in the mouth. Smoothness, graininess and astringency are examples of tactual properties of foods.

An investigation into the chemistry of the flavor of butter cultures necessarily involves some contact with all three basic components of flavor. An example is the organic acids present in ripened butter cultures. They are responsible for the definite sour taste. Their presence lowers the pH and causes coagulation of milk proteins, thereby influencing the tactual properties. The volatile acids contribute directly to the odor, and are both directly and indirectly involved in the formation of other volatile flavor compounds.

Separation, Identification and Quantitation of Flavor Compounds: Some flavor components are present in sufficient quantities and are of such a nature that concentration procedures are not necessary for

their evaluation. However, in most cases they must be isolated from the intact sample before quantitation is possible. An example of a class of compounds which lend themselves to this type of analyses is the short chain organic acids found in butter cultures. Many methods have been described for separating these acids by column chromatographic procedures (12, p. 12-18). The column procedure of Wiseman and Irvin (162) has been modified so that intact culture samples can be directly added to the column in the form of an acidified silicic acid cap (30). Elution with increasingly polar solvent mixtures separates propionic, acetic, formic and lactic acids. The amounts of acids present are determined by titrating the column fractions with standard base. The quantitation of the longer chain free fatty acids (13) and other short chain acids present in much lower concentrations require concentration steps prior to analysis (102).

In general, the flavor compounds which impart aroma to foods are present in very small quantities and do not lend themselves to simple analytical procedures. Methods for isolation and concentration of volatile flavor compounds include solvent extraction (20, 76), atmospheric steam distillation (84, 158), reduced-pressure steam distillation (25, 26, 28, 161), molecular distillation (86) and closed system gas cycling procedures (143). The distillates obtained by the various modified distillation procedures described above may then be extracted with solvents for further concentration or they may

be reacted with specific reagents to form derivatives for subsequent separation and identification. Strong (143) has suggested that many procedures used in isolating flavoring materials distort the quantitative and even the qualitative composition of the original flavor mixture.

Separation of individual compounds (or their derivatives) generally has been achieved by some form of chromatography. The literature concerning the development and application of chromatographic techniques has become voluminous. However, a brief discussion on the use of gas chromatography in conjunction with other modern analytical techniques appears appropriate in view of the objectives of this investigation.

Gas chromatography is a very effective method for the separation and isolation of very small quantities of organic compounds. Bassler and Silverstein (10) have pointed out that it affords little help in identification of compounds when used alone. They have also criticized flavor chemistry studies which have relied on comparison of retention volumes for identification of compounds. It was also suggested that the identification of simple organic compounds such as acetone and acetaldehyde in foods contributes little to the characterization of a flavor. Since the identification of compounds present in very small quantities is seldom possible by conventional organic analytical techniques, Bassler and Silverstein (10) have discussed and

advocated the use of infrared and ultraviolet spectroscopy, mass spectrometry and nuclear magnetic resonance for identification of organic compound samples when only one to two mg of pure material is available.

In many instances it is impossible to obtain pure samples of volatile flavor compounds in amounts as much as one mg. The application of fast-scan mass spectrometry in conjunction with capillary column gas chromatography has recently afforded a means of identification of flavor compounds in complex mixtures which has not been possible by previously existing methods. Work by McFadden et al. (98), Teranishi et al. (148), Buttery et al. (15) and McFadden and Teranishi (99) has adequately demonstrated the usefulness of mass spectrometric monitoring of the effluents from highly efficient capillary columns in the identification of flavor components. Positive identifications can be made using coincidence of gas chromatographic retention times and mass spectral fragmentation patterns.

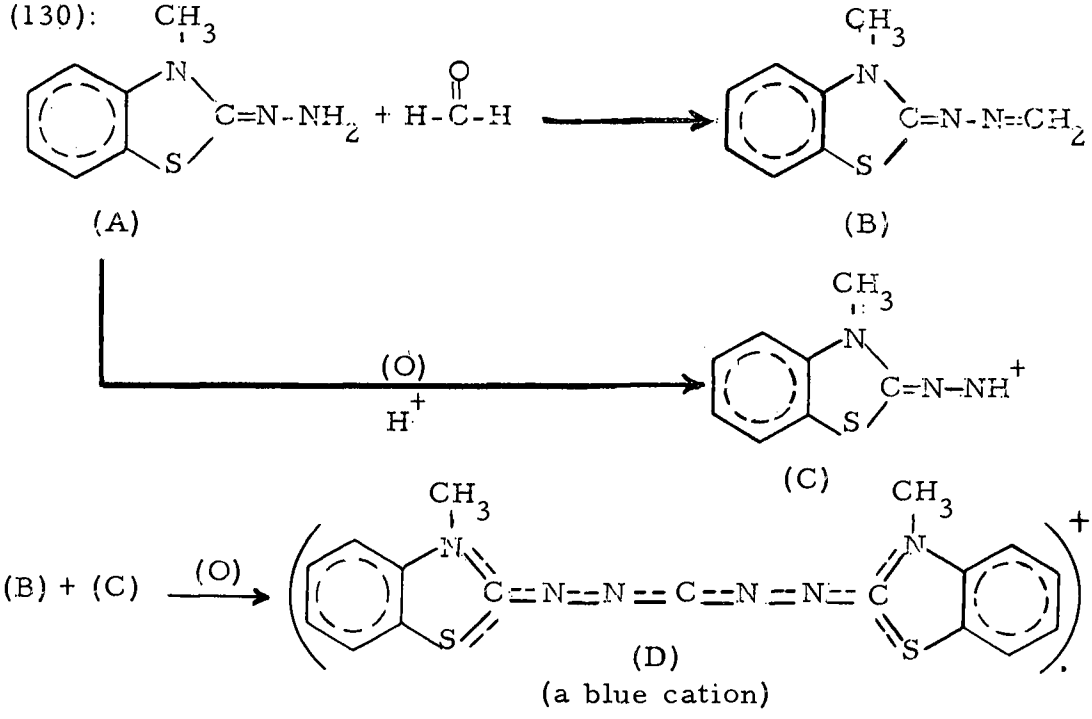
The use of ultra-sensitive gas chromatographs equipped with hydrogen flame ionization detectors has shown promise recently in aroma and flavor chemistry studies. Instruments of this type are capable of detecting as little as 10^{-12} mole of an organic compound and have been used for the analysis of direct vapor samples obtained from foods (8, 16, 17, 71). Again, identifications of organic compounds by retention times are of limited value in these investigations.

Bassette et al. (8, 9) have used techniques which involve the removal of certain classes of compounds by direct addition of selective qualitative organic reagents to food samples prior to chromatographic analysis. Recently, Hoff and Feit (65) have developed techniques for functional group analysis by reacting organic compound vapors with selective reagents in the syringes used for injections. Combinations of these techniques along with retention data apparently yield fairly reliable identifications for some classes of compounds. Buttery and Teranishi (17) and Kepner et al. (79) have developed methods for the quantitative determination of volatile compounds in the vapors above foods by direct vapor injection gas chromatographic techniques.

Certain classes of compounds can be studied effectively by means of derivative formation. The 2, 4-dinitrophenylhydrazones have been particularly useful in studying carbonyl compounds isolated from foods. These derivatives are colored and can be quantitatively determined by spectrophotometric procedures (25, 28). Jones et al. (72) found that the molar absorptivity for a number of these monocarbonyl derivatives was near 22, 000 at their wavelength maxima in neutral polar solvents, such as chloroform.

Recently, Sawicki et al. (130) reported a new method for the selective spectrophotometric determination of water-soluble aliphatic aldehydes. The method was developed for the quantitative determination of formaldehyde, a common air contaminant, but can

be used for other aldehydes. The reaction is carried out in an aqueous system, and 3-methyl-2-benzothiazolone hydrazone reacts with formaldehyde according to the following proposed mechanism (130):



Sawicki et al. (130) found that compound (D) has a molar absorptivity of 65, 000 at its wavelength maxima of 670 mμ. These workers also suggest that the same general mechanism is involved in the reaction of 3-methyl-2-benzothiazalone hydrazone with other aldehydes.

The quantitative determination of diacetyl has received considerable attention because of its importance in the flavor of butter cultures and other foods. Hammer and Babel (55) have reviewed the literature on the many methods used for determining diacetyl. Since acetoin can be oxidized to diacetyl by ferric chloride (and 2, 3-butanediol by bromine), some methods for the quantitative

determination of diacetyl have been modified so that they are applicable in the measurement of acetoin (and 2, 3-butanediol). It should be pointed out that alpha-acetolactic acid, the now accepted precursor of diacetyl, would be decarboxylation and oxidized to diacetyl under the conditions employed for acetoin determinations.

The colorimetric procedure utilizing the reaction of o-diaminobenzidine (3, 4, 3', 4'- tetra-amino-biphenyl) hydrochloride with diacetyl was first described by Pien et al. (123). It has since been modified (55, 37, 30) so that reasonably rapid and accurate diacetyl microdeterminations can be made. However, it has been criticized for giving high diacetyl values for milk cultures because of its reaction and color formation with other alpha-dicarbonyl compounds formed during the steam distillation step (106, 107).

Prill and Hammer (125) developed a colorimetric procedure for the microdetermination of diacetyl based on the formation of an intensely colored ferrous-ammino complex with dimethyl glyoxime. The original procedure has been recently modified by Owades and Jakovac (106) to eliminate the steam distillation step in the analysis of beer. These workers also altered the reagents slightly to stabilize the colored ferrous-ammino complex. The modified procedure (106) utilizes a nitrogen purging system to remove the diacetyl from the sample which is trapped in hydroxylamine. Pack et al. (107) have recently reported the successful adaptation of this procedure for the

analysis of diacetyl in mixed-strain starter cultures.

The hydroxamic acid reaction has been used to estimate the volatile ester content of wine by Mattick and Robinson (96). Bassette and Keeney (7) used a similar method for characterizing milk fat. As a result of an extensive study of the hydroxamic acid reaction, Goddu et al. (47) reported optimum conditions for the spectrophotometric determination of the highly colored hydroxamic acid-ferric ion complexes of numerous esters and anhydrides. However, the molar absorptivity of the short chain acid-hydroxamates measured at their wavelength maxima was found to be only about 1, 000. This places severe limitations on the use of these derivatives for spectrophotometric quantitative microdeterminations.

In this brief summary of flavor research methods, it can be seen that the chemical characterization of a flavor involves the use of many techniques. As new analytical techniques become available, more detailed information about a given food flavor, including its formation and stabilization, will be accumulated and ultimately such information should permit its chemical duplication.

EXPERIMENTAL

Methods for the Preparation of Butter Cultures and Butters

Selection of Mixed-Strain Butter Culture

During the initial phases of this investigation, 30 lactic cultures obtained from domestic and foreign laboratories were evaluated for their ability to produce a good cultured cream butter flavor (30). The culture found most suitable was of commercial origin and was obtained from Molkerei Laboratory, Niebuell, Schleswig, West Germany. Strains of S. lactis, S. cremoris, S. diacetilactis and Leuconostoc sp. comprised the flora of the culture.

Culturing Conditions for Mixed-Strain Butter Cultures

Raw whole milk (approximately 3.8 percent milk fat and 8.5 percent solids-not-fat) obtained from the University dairy herd was used as the culturing medium for all culture propagations. Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) was added to the extent of 0.2 percent (approximately 0.01 M calculated as citric acid) from a stock aqueous solution containing 0.6 g per ml. The milk was then heated in a boiling water bath for one hr and cooled immediately to 5° C.

The commercial cultures were obtained as lyophilized cultures and propagation of mother cultures was accomplished by carrying

duplicate cultures in 500 ml long necked culture flasks containing 300 ml of the whole milk media. The mother cultures were transferred every other day using from 0. 6 to 3. 0 percent of inoculum and varying the incubation time at 21° C so that the titratable acidity of the cooled culture was from 0. 68 to 0. 78 percent acid (10-12 hr). If the acidity exceeded 0. 78 percent the culture usually developed a flavor defect, described as "green", in subsequent transfers even when ripened to higher acidities. The flavor defect could not be eliminated in subsequent transfers and consequently these cultures were discarded.

Bulk cultures prepared in ten gallon lots in stainless steel cans and 300 ml mother cultures for flavor evaluation were cultured under the same conditions except that the titratable acidity was allowed to develop to 0. 88 - 0. 97 percent (12-16 hr). The cultures were then held for 24 hr at 5° C to allow maximum flavor development prior to flavor evaluation, distillation or culturing cream for butter manufacture.

The titratable acidities were determined in the conventional manner for dairy products using nine g samples according to the procedure outlined by Goss (49). All titratable acidities were calculated as percent lactic acid.

Culturing Conditions for S. diacetilactis 18-16 Culture

A pure culture of S. diacetilactis 18-16 was used for the initial studies on flavor volatiles. A method described by Seitz (137, p. 31) was used for preparing ten gallons of S. diacetilactis 18-16 bulk culture. Homogenized milk (3.8 percent milk fat) containing two percent added sodium citrate was inoculated with two percent of an 18-hr, nonfat milk culture of S. diacetilactis 18-16 and incubated for 24 hr at 21° C (final pH 5.25). The milk was then acidified with 25 percent phosphoric acid to pH 4.30 and incubated an additional 24 hr before cooling to 5° C. A concentration of 0.99 p.p.m. diacetyl was present at the time of distillation.

For the remainder of the studies on flavor components the selected commercial mixed-strain culture was used.

Manufacture of Cultured Cream Butters

Several manufacturing procedures were evaluated in preliminary investigations. The procedure utilized for the present investigation was selected on the basis of its yielding excellent highly flavored butters manufactured in the University creamery (30).

Approximately 1,000 pounds of fresh cream (38 percent milk fat) was pasteurized at 71° C for 30 min, cooled to 21° C and inoculated with one percent of bulk culture (0.88-0.97 percent acidity).

The cream was incubated at 21° C for one to two hr depending on the activity of the culture and the amount of developed acid desired. Following incubation, the cream was cooled to 5° C and held approximately 14 hr prior to churning. The cream was churned at 10° to 16° C, depending upon the season of the year. The butter granules were not washed, and chilled buttermilk was used to adjust the moisture content during the working process. The salt content of the butters ranged from 0. 7 to 1. 2 percent. The finished butters were tempered at 5° C, printed into one pound blocks and samples were taken for immediate diacetyl and short chain acid analyses. Aliquots were wrapped and stored at -30° C for a maximum of three weeks until used for flavor panel evaluation.

Recovery and Evaluation of Flavor Volatiles

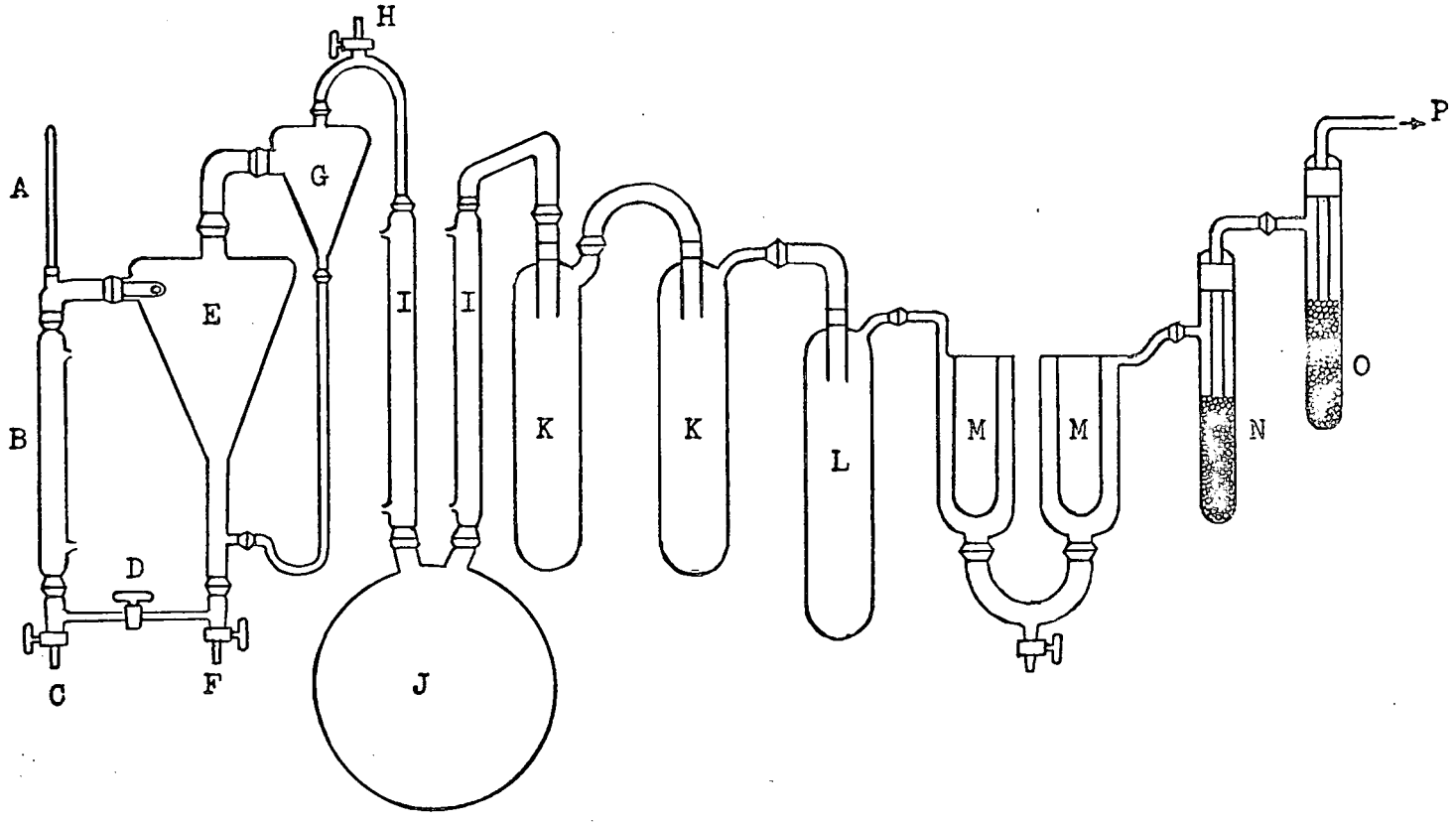
Distillation Apparatus

Butter cultures and heated milk medium were steam distilled in a specially designed low-temperature, reduced-pressure glass apparatus fitted with ground-glass ball or standard taper joints. Culture distillations A and B were limited to a maximum of ten gallons of butter culture due to blockage of certain cold traps in the vacuum system by ice accumulation. After modifications of the distillation apparatus to alleviate the problem, 20 gallon lots of culture

or heated milk were used for subsequent distillations. Approximately 15-20 percent of the volume was removed by the distillation process in all of the trials.

A diagram of the distillation apparatus is shown in Figure 3. The total length of the apparatus was approximately eight feet and the height approximately four feet. Referring to Figure 3, the pressure was measured manometrically near position P and was maintained near three mm of mercury during the course of the distillation. In order to remove the residue from the system a 20 liter Pyrex carboy was connected by pressure tubing to positions H and F, thereby maintaining the reduced pressure of the system. This also allowed gravity flow removal of the residue from position F into the carboy.

The culture sample was introduced slowly through stopcock C and was immediately flash-heated to 37-40° C in the heat exchanger (B) by steam injection into the jacket of a water condenser. The fluid material then surged into the vaporization chamber (E) where, during the course of foaming, the volatile compounds were vaporized and removed. A continuous feed of culture was maintained to the heat exchanger to prevent "cook-on". The stopcocks at C, F and D were adjusted to give a proper intake of new sample, an exit of distilled sample and a recycling of partially distilled sample, respectively. The chamber labelled G was a foam trap to prevent intact sample



A-Thermometer
B-Heat Exchanger
C-Sample Inlet
D-Cycle Control Valve
E-Vaporization Chamber
F-Sample Exit
G-Foam Trap
H-Vacuum to Residue Container
I-Tapwater Cooled Condensers
J-Crushed Dry Ice Trap
K-Ethanol-Dry Ice Traps
L,M,N-Liquid Nitrogen Traps
O-Liquid Nitrogen Safety Trap
P-To Vacuum Pump

Figure 3. Diagram of vacuum steam distillation apparatus for collection of volatile flavor components from butter culture and heated milk.

from contaminating the steam distillate.

The steam distillate was condensed in a series of cold traps. The two water-cooled condensers (I) above trap J condensed the majority of the aqueous steam distillate. Trap J (a 12 liter balloon flask) was cooled by crushed dry-ice and the distillate contained within it remained liquid during the distillation. Traps, designated K, with a capacity of approximately two liters each were cooled with ethanol-dry-ice slush baths. A recent modification of the system has replaced traps K and L with equivalent sized pear-shaped flasks which reduced breakage upon thawing of the distillate. Traps L, M and N were cooled with liquid nitrogen and after distillation contained a limited amount of water. However, considerable quantities of carbon dioxide and highly volatile flavor compounds were trapped in these containers. Trap O served as a liquid nitrogen cooled safety trap to prevent contamination from the vacuum pump.

Diacetyl removal by the distillation procedure was determined by adding diacetyl to milk acidified with 20 percent phosphoric acid to pH 4. 60. The amount of diacetyl in the milk was determined prior to and after distillation to remove 20 percent of the volume. From these data, and correcting for the decrease in volume, it was possible to obtain an estimate of the distillation efficiency of the apparatus.

Distillation Fractions from Butter Cultures and Heated Milk

During the course of this investigation, four complete butter culture distillations and evaluations were carried out; in addition, one control heated milk was similarly evaluated. For convenience and clarity, the culture distillations were designated chronologically as A, B, C and D, respectively.

Culture distillations A and B (a single-strain S. diacetilactis culture and the commercial mixed-strain culture, respectively) were made before refined methods of evaluation of isolated flavor material were available. In these trials the volatile flavor components were all combined into an aqueous distillate fraction which was extracted with purified ethyl ether. Information as to the identity of certain compounds was derived from these trials by combining selective qualitative organic reagents with gas chromatographic effluent trapping procedures.

Culture distillations C and D were made using the selected commercial mixed-strain culture. For culture distillation C, the aqueous distillates from traps J, K, L and M (Figure 3) were combined and extracted with ethyl ether. The highly volatile fractions from traps L, M and N were combined and evaluated by gas chromatography according to the method described by Libbey et al. (86). Since considerable quantities of volatile acids (primarily acetic acid) were

steam distilled and collected in traps L and M, a different series of fractions were made for culture distillation D. In the analysis of the gas chromatographic data no qualitative differences in the compositions of the culture flavor concentrates were apparent. Therefore, the data obtained for culture distillation D and its control heated milk medium distillation were selected for detailed discussion.

For culture distillation D, the aqueous distillate from traps J and K was designated as "acidic fraction" because it contained significant amounts of volatile acids. Approximately one-half of the acidic fraction was made distinctly basic with sodium carbonate after thawing and was designated as "basic fraction". The aqueous distillate obtained from traps L and M contained very little, if any, volatile acids and was designated as "neutral fraction". The aqueous distillate fractions were extracted with ethyl ether. The highly volatile fractions from traps L, M and N were evaluated by the gas chromatographic procedure of Libbey et al. (86). A portion of the acidic fraction was reacted with 2, 4-dinitrophenylhydrazine to form carbonyl derivatives.

A duplicate heated milk medium control for culture distillation D was distilled the following day to ascertain the contribution to butter culture flavor by components present in heated milk. The aqueous distillates from all traps were combined into one fraction and extracted with ethyl ether. The highly volatile fractions from traps L,

M and N were combined and evaluated by the gas chromatographic procedure of Libbey et al. (86).

Extraction and Concentration of Flavor Compounds from Aqueous Distillates

The peroxides were removed from ethyl ether in a manner similar to that described by Valseth (150). This was accomplished by swirling three liters of ethyl ether with 600 ml of ten percent sulfuric acid containing 60 g of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in a six liter Erlenmeyer flask (using a magnetic stirring bar) for one hr.

After peroxide removal the ether was fractionally distilled utilizing a three x 100 cm column (packed with glass helices) equipped with a vacuum-jacketed fractionation head. The rate of distillation was controlled electronically at a reflux ratio of 1:3 (collect to return). The distilled ether was collected in an 8.5 x 40 cm flask cooled by crushed dry-ice. The major portion of the water contained in the ether was filtered off as ice crystals using a glass-wool plugged funnel.

The aqueous distillates of the respective samples were extracted in a conventional continuous liquid-liquid extractor designed for a solvent less dense than water. Approximately two liters of aqueous distillate were saturated with reagent grade sodium chloride and extracted with approximately 300 ml of ethyl ether continuous for 24 hr.

A magnetic stirring bar was used to gently swirl the distillate in the 8.5 x 60 cm extraction chamber.

The ethereal flavor concentrate solution was fractionally distilled to remove excess ethyl ether using a one x 60 cm fractionation column (packed with glass helices) after drying with excess sodium sulfate. The rate of distillation was controlled electronically at a reflux ratio of 1:4 (collect to return). Upon completion of distillation approximately ten ml of residue containing the flavor material remained in the distillation flask. In order to concentrate the flavor components to a greater degree, the residue was transferred to a 20 ml ground-glass stoppered tube, and with the stopper loosened excess ethyl ether was allowed to slowly evaporate at 5° C until a volume of approximately five ml remained. Equivalent volumes of control ethyl ether concentrated in the same manner resulted in a gas chromatographically pure ethyl ether residue.

Gas Chromatography

For culture distillations A and B, the flavor concentrates were analyzed only as liquid samples dissolved in residual ethyl ether. One to five µl of the flavor concentrate were injected into an Aerograph A-100 gas chromatograph equipped with a thermal conductivity detector. The instrument was operated at 110° C with a nine ft. x 1/4 inch OD packed aluminum column containing 21.5 g of 25 percent

diethyleneglycol succinate (DEGS) stationary phase on 80-100 mesh alkali-acid treated Celite 545. Utilizing retention times and trapping procedures for gas chromatographic effluents into selective qualitative organic reagents (157), information as to the identity of certain peaks was obtained.

For butter culture distillations C and D and the heated milk distillation, the highly volatile fractions were separated from the aqueous distillate and were chromatographed by the direct concentrated vapor injection procedure described by Libbey et al. (86). Due to large amounts of carbon dioxide produced by the cultures and subsequently collected in the liquid nitrogen cold traps, certain manipulations of the cold traps to separate the major amount of carbon dioxide from the volatile flavor compounds were necessary. This was accomplished by connecting clean traps with the sample traps (immersed in liquid nitrogen) and reducing the pressure in the system to approximately one to three mm of mercury, closing the system to the vacuum pump and replacing the liquid nitrogen bath on the sample trap with an ethanol dry-ice slush bath while placing the liquid nitrogen bath on the clean trap. Manipulation of temperature and pressure effectively separated the carbon dioxide after several transfers.

The highly volatile fractions and the ethyl ether extracts of the distillates were chromatographed using a Model 20 Barber-Colman equipped with an argon, strontium-90 β-ionization detector. Both

polar and nonpolar columns were utilized for separations. The polar column was an 11 ft x 1/8 inch OD packed stainless steel column containing 5.68 g of 20 percent DEGS stationary phase on 100-120 mesh alkali-acid treated Celite 545. The nonpolar column was an 11 ft x 1/8 inch OD packed stainless steel column containing 5.89 g of 20 percent Apiezon M on 100-120 mesh alkali-acid treated Celite 545. The parameters of operation were: column temperature, 70° C; cell temperature, 200° C; injection port temperature, 165° C; column flow, 18-24 ml per min; cell scavenger flow, 120 ml per min; split flow, 20 ml per min; column gas (argon) pressure, 22 psi; and cell voltage, 1250 volts.

Gas Chromatography Combined with Fast-Scan Mass Spectrometry

The ethyl ether extracts of the various fractions of culture distillation D and the heated milk distillation were separated by temperature programmed gas chromatography. A Perkin-Elmer Model 800 gas chromatograph equipped with a hydrogen flame detector and containing a 300 ft. x 0.01 inch ID stainless steel capillary column coated with polypropylene glycol was used. Two different sets of operating conditions were employed in the analysis of the samples. For one set of conditions the instrument was temperature programmed from 30° C at 2.5° C per min for eight min, then at 25° C per min to 170° C and operated isothermally at 170° C until the

separation was complete. For the other set of conditions the column was maintained isothermally at 73° C for eight min, then temperature programmed at 25° C per min to 174° C and operated isothermally at 174° C until the separation was complete. Retention data were obtained for butter culture and heated milk volatiles and authentic compounds under these conditions. The same conditions were employed for gas chromatography analysis in combination with fast-scan mass spectrometry.

The mass spectrometric analysis were conducted with a Bendix Time-of-Flight Model 12 mass spectrometer as described by McFadden et al. (98), McFadden and Teranishi (99) and Teranishi et al. (148), except the positive ions occurring at m/e 43 (m/e is the mass to charge ratio) were monitored by gate two of the electron multiplier to obtain a concurrent strip chart recording of the gas chromatogram. The other electron multiplier gate was used to scan m/e 12 to 250 in two to six seconds and spectra were recorded by an oscillographic recorder. The spectra were observed concurrently on an oscilloscope which provided an important means of monitoring the effluent from the capillary column. The identification of flavor compounds was based on coincidence of gas chromatographic relative retention times with known compounds in conjunction with the analysis of the mass spectra and their comparison with standard mass spectral data.

Direct Vapor Injection Gas Chromatography

A procedure was developed, similar to one described by Jennings et al. (71), whereby suitable qualitative vapor analyses with the absence of contaminating peaks could be made. One hundred and twenty-five milliliter Erlenmeyer flasks fitted with (24/40) standard taper ground-glass joints were used. A one inch section of six mm ID glass tubing was fused to the male joint. The glass tubing was of such a diameter that a silicone-rubber septum normally used for a Barber-Colman Model 20 gas chromatograph injection port could be utilized as the closure. Glass hooks were welded on the flask and the top section to allow a strong rubber band to secure the ground-glass connection.

For analyses, 60 g samples were weighed into a clean flask and the unit was assembled before placing it in a 60° C water bath for 20 min with frequent shaking. One ml vapor samples were injected directly into an Aerograph Hy-Fi gas chromatograph equipped with a hydrogen flame detector and operating at maximum sensitivity.

For the analysis of butter cultures and butter samples for dimethyl sulfide, a 12 ft x 1/8 inch OD stainless steel column packed with five percent Ucon nonpolar stationary phase on 80-100 mesh alkali-acid treated Celite 545 was used. The column and injection port were maintained at 25° C.

A limited number of analyses for flavor compounds present in butter culture and butter headspace were made using a gas chromatographic technique developed by Hoff and Feit (65); the headspace vapors were brought into contact with selective qualitative organic classification reagents in the syringe used to inject the sample into the gas chromatograph. Certain compounds were tentatively identified in the headspace chromatograms using this technique along with relative retention time data.

Direct vapor injections were used for the evaluation of butter cultures exhibiting a green flavor defect. The techniques were the same as described above, but a different set of operating conditions were used. A nine ft x 1/8 inch OD stainless steel column packed with 20 percent Apiezon M stationary phase on 100-120 mesh alkali-acid treated Celite 545 was used. The column and injection port were maintained at 70° C.

Extra precautions were necessary to eliminate contaminating peaks in headspace analyses from sampling flasks, syringes and laboratory air. The glass sampling flasks and glass syringes (ground barrels) were rinsed in ethyl alcohol, then ethyl ether, and were placed in a vacuum oven maintained at ten mm of mercury and 60° C for at least 30 min immediately prior to use in analyses.

Churn Headspace Volatiles

These experiments were conducted primarily to isolate dimethyl sulfide as a flavor component of butter. Attempts were made to form the mercuric chloride derivative of dimethyl sulfide from the headspace of freshly churned sweet cream butter. A series of collection traps were connected to a (300 pound cream capacity) stainless steel butter churn immediately after draining the buttermilk. The churn was swept with 200 to 400 ml of nitrogen per min for 30 min into a train of trapping reagents; these were, in order, an empty safety trap, four percent mercuric cyanide, three percent mercuric chloride, half-saturated 2, 4-dinitrophenylhydrazine in 2. 5 N hydrochloric acid, 1. 0 N sodium hydroxide and an empty safety trap.

Since sufficient quantities of derivatives were not obtained for analyses by the reagent trapping procedures, a similar system was devised utilizing liquid nitrogen cold traps for concentration of the headspace volatiles. The series of cold traps were, in order, a two liter ethanol-dry-ice trap for excess water vapor removal connected to two liquid nitrogen traps arranged in a stopcock controlled "T" arrangement for collection in either or both of the traps. Finally a liquid nitrogen safety trap was placed on the end of the series to condense atmospheric oxygen and prevent extraneous air contaminants from entering the system during manipulation procedures. The

highly volatile fractions were concentrated by reduced-pressure, sublimation transfer techniques and analyzed by gas chromatography with a DEGS column at 70° C using the technique described by Libbey et al. (86).

Direct vapor sampling of cultured cream butter churn volatiles were made with subsequent analysis by an Aerograph Hy-Fi gas chromatograph operating at maximum sensitivity. Five milliliter headspace samples were separated with a 20 percent Apiezon M column at 70° C.

It should be mentioned that the attenuation factors for the different gas chromatographs used in these studies were not the same. An attenuation factor of 32 for the Aerograph Hy-Fi indicated that the instrument was operating at a less sensitive setting than at 16. The same relationship existed for the Perkin-Elmer instrument employed for the capillary column separations. However, in the case of the Barber-Colman gas chromatograph a gain setting of 30 was more sensitive than a setting at three.

2, 4-Dinitrophenylhydrazine Derivatives of Carbonyl Compounds

The properties of the 2, 4-dinitrophenylhydrazones (2, 4-DNP-hydrazones) of diacetyl, acetoin and alpha-acetolactic acid were investigated according to previously reported procedures (87). Further work concerning this investigation was carried out utilizing

infrared spectroscopy which enabled a more complete characterization of the DNP-derivatives under investigation.

The DNP-hydrazones obtained from a portion of the "acidic fraction" of culture distillation D were applied to a class separation column (136) to separate the monocarbonyl derivatives from the remainder of the mixture. The monocarbonyl derivatives were then applied to a hexane-nitromethane partition column (25) for resolution of the classes into chain-length groups. The ultraviolet absorption maxima were determined in chloroform and the tentative identity of the derivatives was determined by chromatographing the unknowns with authentic derivatives using the paper class separation method of Gaddis and Ellis (42).

Methods for Quantitative Determination of Important Flavor Compounds in Butter Culture and Butter

Determination of Diacetyl

In the earlier part of this investigation the method of Pien et al. (123) as described by Elliker (37) with a few modifications was used to determine the diacetyl content of butter culture and butter. The distillation procedure was altered by using a 50 g sample and by collecting 50 ml of distillate into a 50 ml volumetric flask. A ten ml aliquot of distillate was then reacted with 0.5 ml of concentrated hydrochloric acid and 0.1 ml of freshly prepared ortho-

diaminobenzidine hydrochloride reagent. Absorbance was determined at 345 mμ instead of 400 mμ. The samples were read against a reagent blank and the concentration of diacetyl was taken from a standard curve. The data used for the preparation of the standard curve are given in Table 1. Recovery of added diacetyl from acidified milk and butter was 90 percent and all quantitative data were corrected on this basis.

Recently, Pack et al. (107) reported the successful adaptation of the Owades and Jakovac method (106) for the quantitative determination of diacetyl in mixed-strain starters. In the latter stages of this investigation the method was used for quantitative diacetyl determinations in butter cultures with slight modification. The culture samples were weighed, and instead of diluting dimethylglyoxime for the standard, diacetyl (gas chromatographically pure) was used for dilutions in the preparation of the standard curve. The samples were read against a reagent blank at 530 mμ using a Beckman DU spectrophotometer. The data used for the preparation of the standard curve are given in Table 2. Recovery of diacetyl from acidified milk (pH 4. 6) was 90 percent and all quantitative data were corrected on this basis.

Table 1. Absorbance readings at 345 mμ for the concentrations of diacetyl used for the preparation of the standard curve.

μg Diacetyl per 50 g sample[a]	Corresponding p. p. m. concentration in 50 g sample	Absorbance at 345 mμ
25. 5	0. 51	0. 040
51. 0	1. 02	0. 071
102. 0	2. 04	0. 123
204. 0	4. 08	0. 262

[a] Absorbance was plotted against μg diacetyl per 50 g sample for standard curve; for use with the modified Pien et al. method (37).

Table 2. Absorbance readings at 530 mμ for the concentrations of diacetyl used for the preparation of the standard curve.

μg Diacetyl[a]	Corresponding p. p. m. concentration in 20 g sample	Absorbance at 530 mμ
10	0.5	0.060
20	1.0	0.122
40	2.0	0.235
60	3.0	0.384
80	4.0	0.495
100	5.0	0.590
160	8.0	0.981
200	10.0	1.200

[a] Absorbance was plotted against μg diacetyl for standard curve as sample size was varied; for use with the Pack et al. (107) method.

Determination of Acetaldehyde

The method of Sawicki et al. (130) for the selective quantitative determination of volatile aldehydes was adapted to the collection system described by Pack et al. (107). The method determines total volatile aldehydes, but since earlier experiments in the current investigation indicated that acetaldehyde comprised the major portion of the aldehyde fraction in butter culture, the method was modified on this basis.

Twenty grams (or 5 to 15 g of culture made to 20 g with distilled water) of butter culture were weighed into a 25 x 250 mm culture tube and a small amount of antifoam agent (spray-form) was added before unit assembly. The collection trap was similar to that described by Pack et al. (107), except that the 12 ml graduated centrifuge tube was omitted and the collection reagent was added directly to the 50 ml conical centrifuge tube. The collection reagent was prepared in each tube by the combination of 2.5 ml of 0.4 percent aqueous 3-methyl-2-benzothiazolone hydrazone hydrochloride, 2.5 ml of distilled water and 0.5 ml of dimethyl sulfoxide (DMSO). The collection system was assembled and butter culture samples along with a control were placed in a 65° C water bath and were purged with 100-125 ml of nitrogen per min for one hr. Upon completion of purging, a few drops of distilled water were used to rinse the purging-tips

into the collection tube and the samples were allowed to complete reacting by standing at room temperature for 25 min.

Twelve and one-half milliliters of 0. 2 percent ferric chloride in 0. 1 N hydrochloric acid were then added, mixed and allowed to stand for exactly 25 min, at which time 20 ml of acetone were added and mixed immediately to stop the oxidation reaction. The samples were then transferred to 50 ml volumetric flasks (rinsing with acetone) and brought to volume with acetone. The absorbance at 666 mμ was determined reading against a reagent blank with a Beckman Model DU spectrophotometer. A standard curve was prepared by adding acetaldehyde directly into the reagent. The data used for the standard curve are given in Table 3. The percent recovery of acetaldehyde from acidified milk (pH 4. 5) was found to be 80 percent and all quantitative data were corrected on this basis.

Table 3. Absorbance readings at 666 mμ for the concentrations of acetaldehyde used for preparation of the standard curve.

μg Acetaldehyde [a]	Corresponding p. p. m. concentration in 20 g sample	Absorbance at 666 mμ
1	0.05	0.027
5	0.25	0.158
10	0.50	0.308
15	0.75	0.473
20	1.00	0.628
30	1.50	0.933
50	2.50	1.520

[a] Absorbance was plotted against μg acetaldehyde for standard curve as culture sample size was varied.

Determination of Short Chain Acids

Free short chain organic acids in butter culture and butter were determined by the method of Wiseman and Irvin (162). Three grams of butter culture or butter serum were acidified with four drops of 50 percent sulfuric acid and mixed with sufficient dry silicic acid to give a free flowing powder. This material was added as a cap and the column was developed as described by Wiseman and Irvin (162). This procedure effectively separated in the order of elution, butyric acid and higher chain-length free fatty acids (as a single fraction), propionic, acetic, formic and lactic acids. The data for butyric and higher chain-length free fatty acids were not used in this investigation.

Butter serum was isolated by warming the butter to 40° C and centrifuging. Since the acids were measured from the serum phase of butter, it was necessary to determine their distribution in the milk fat-serum binary phase system to allow calculation of the total amount of each acid in the butter. The distributions of lactic, formic and acetic acids between 80 g of milk fat held at 40° to 50° C and 17 ml of six percent of aqueous sodium chloride solution were determined by adding known amounts of acids, shaking the mixture and titrating an aliquot of the aqueous phase with standard base. The percentages of acids partitioned into the aqueous phase were:

lactic, 100; formic, 91; and acetic, 86. The foregoing values were used to correct the amounts of acids found in butter serum, thereby allowing quantitative calculations of the total amount of each acid in the intact butter sample.

Determination of Volatile Esters

A modified hydroxamic acid method was adapted for the determination of volatile ester content of butter culture. One milliliter of one molar hydroxylamine hydrochloride and one ml of 1.8 N sodium hydroxide were pipetted into a 12 ml graduated conical centrifuge tube for use as the collection reagent. Twenty to forty grams of culture sample were weighed into a 25 x 250 mm culture tube and a small amount of antifoam agent (spray-form) was added to the sample before assembly and collection according to the procedure outlined by Pack et al. (107).

The culture samples along with a distilled water blank were then purged for 1.5 hr in a 60° C water bath with 100-125 ml of nitrogen per min. Upon completion of the collection and with the gas still flowing, the purging tips were rinsed with a few drops of distilled water into the hydroxylamine trap. The samples were allowed to stand at room temperature for 15 min and then were placed into the 60° C water bath for five min. Upon removal from the water bath, 0.1 ml of acetone and 1.5 ml of 2.5 N hydrochloric acid were

added and mixed thoroughly. When the tubes were cooled to room temperature, 0. 5 ml of ten percent ferric chloride in 0. 1 N hydrochloric acid was added and mixed immediately. The volume was adjusted to five ml with distilled water and again mixed.

Absorbances were read at 525 mμ against a reagent blank using a Beckman Model DU spectrophotometer and were referred to a standard curve. The data used for the preparation of the standard curve are given in Table 4. The percent recovery of added ethyl acetate from acidified homogenized milk (pH 4. 60) was found to be 88 percent and all quantitative data were corrected on this basis.

Table 4. Absorbance readings at 525 mμ for the concentrations of ethyl acetate used for the preparation of the standard curve.

μg Ethyl Acetate [a]	Corresponding p. p. m. Concentration in 20 g sample	Absorbance at 525 mμ
9. 8	0. 49	0. 014
19. 5	0. 98	0. 026
39. 0	1. 95	.0. 054
78. 0	3. 90	.0. 100
156. 0	7. 80	0. 220
312. 0	15. 60	0. 450

[a] Absorbance was plotted against μg ethyl acetate for standard curve as sample size was varied.

Determination of Optimum Levels of Dimethyl Sulfide

Butteroil was prepared by melting butter at 40° C, separation from butter serum and phospholipids and steam distillation under reduced pressure at 40° C to remove the major portion of the volatile flavor components. The resulting butteroil was bland in flavor.

Samples containing dimethyl sulfide were prepared by dilution from a stock solution. Stock solutions were prepared by placing sealed weighed vials of dimethyl sulfide in a cotton bag, immersing it in the oil and releasing the dimethyl sulfide by crushing the vial. A range of dilutions of dimethyl sulfide were prepared for butteroil (10-100 p. p. b.) and for butteroil containing 2. 5 p. p. m. of added diacetyl (25-100 p. p. b.), The samples were presented to an experienced nine-member panel in coded 3/4-ounce cups. The samples were prepared immediately before serving in butteroil tempered at 42° C. The panel members were asked to indicate the samples in which dimethyl sulfide could be detected and the sample which they preferred. From these data the average flavor threshold at the 50 percent positive response level and the approximate preferred concentration were established.

Determination of Optimum Diacetyl Content of Cultured Butters

One pound butter samples were coded, tempered at 5° C and evaluated by an eight to ten member experienced flavor panel. The

evaluations were carried out in a room free of extraneous odors and samples were displayed simultaneously so that panel members could make comparisons. A maximum of seven samples was displayed per evaluation period and three replicates of each sample were evaluated. The scoring ballot used was a seven-point hedonic scale type including six flavor characteristics believed to best discern differences in ripened cream butter flavor. The flavor characteristics included were aroma, diacetyl, flavor intensity, freshness, salt and over-all flavor. One ballot was used per judge per sample of butter and the data for the three replications were averaged to give an average score for each flavor characteristic. The data utilized for this investigation, however, include only that for the over-all flavor score as related to the chemically measured diacetyl content (37) to give an approximation of the optimum level of diacetyl in desirable highly flavored cultured butters.

Studies on Diacetyl and Acetaldehyde Production and Utilization in Lactic Cultures

Effect of Degree of Ripening on the Flavor and Aroma of Mixed-strain Butter Cultures

Commercial mixed-strain butter cultures, obtained from the same source as those used for the flavor isolation studies, were transferred every other day into 300 ml aliquots of the whole milk

medium. The length of incubation at 21° C and the percentage of inoculum were varied in order to control the degree of ripening. The titratable acidities of the cultures were used as indices of ripening. All samples were evaluated for flavor and aroma after cooling to 5° C and subsequent storage for 24 hr. In some of the trials, diacetyl was measured by the procedure described by Pack et al. (107) and acetaldehyde was determined by the modified Sawicki et al. (130) method.

Determination of the Ratio of Different Lactic Organisms in a Selected Commercial Mixed-strain Butter Culture

A commercial culture was analyzed after six transfers at 0. 65-0. 78 percent acidity and again after three successive transfers at 0. 85 - 0. 87 percent acidity for the numbers of various lactic organisms comprising the culture flora. The simplified method of general classification derived from the work of Sandine et al. (128) was used.

The cultures were plated after dilution in sterile 0. 9 percent physiological saline in and on lactic agar (38) using the conventional pour plate and surface spreader-inoculation techniques, respectively. Total plate counts were made from the pour plates. Approximately 100 colonies were picked from the surface inoculated plates into litmus milk and incubated for 48 hr at 30° C. The King's Test (81) for diacetyl and acetoin was then run on all cultures which had coagulated

the milk medium. The cultures which showed no acid production by coagulation were acidified to approximately pH 4.5 (0.1 ml of sterile 20 percent phosphoric acid per four ml of milk medium) and incubated an additional six hr at 30° C before performing the King's test. The criteria for classification were as follows:

Classification	King's Test	Acid Coagulation in 48 hr at 30° C
S. lactis or S. cremoris	negative	positive
S. lactis or cremoris var. aromaticus	slight positive	positive
S. diacetilactis	positive	positive
Leuconostoc sp.	positive after acidification	negative
Dead (or non-contributing organisms)	negative after acidification	negative

Determination of the Acetaldehyde-Diacetyl Ratio for Good Flavored Butter Culture

Five different mixed-strain lactic cultures were propagated from lyophilized powders. After two or more transfers for activity restoration, the resulting cultures were analyzed for diacetyl, acetaldehyde, pH, flavor and aroma. Diacetyl was determined as described by Pack et al. (107), and acetaldehyde was determined as previously described. All of the data were collected after holding the cultures for 24 hr at 5° C.

Evaluation of Single-Strain Cultures

Single-strain cultures of Streptococcus lactis C_2F, Streptococcus cremoris SC_1, Streptococcus diacetilactis 18-16, Leuconostoc citrovorum 91404 and Streptococcus lactis var. maltigenes M1, M3, M4 and M6 were obtained from the culture collection of the Department of Microbiology, Oregon State University. The cultures were propagated in the whole milk medium described earlier. One percent of inoculum was used and the cultures were incubated at 30° C for 14 hr, with an exception for the L. citrovorum cultures. Since these organisms typically produce little lactic acid in the prescribed incubation time, a sterile 20 percent phosphoric acid solution was used to lower the pH to 4.50 after 12 hr of incubation and then incubation was continued for an additional six hr.

The diacetyl and volatile aldehyde (expressed as acetaldehyde for all strains) content was measured by the Pack et al. (107) and modified Sawicki et al. (130) procedures, respectively. The pH was determined and each culture was evaluated for aroma and flavor.

Acetaldehyde Utilization by L. citrovorum 91404

A culture of L. citrovorum 91404 was incubated at 30° C for 18 hr and then divided into aliquots; one aliquot was not acidified (pH 6.5) and the second was acidified to pH 4.5 with sterile H_3PO_4.

Acetaldehyde was added to each aliquot (4. 6 p. p. m.) and incubation was then continued for 6 hr at 30° C. The acetaldehyde and diacetyl content were then determined as described.

The rate of acetaldehyde utilization by L. citrovorum 91404 was determined on acidified and non-acidified cultures held at 21° and 5° C at intervals up to nine hr. Diacetyl content of the cultures was determined simultaneously with acetaldehyde.

The effect of added acetaldehyde on the growth of L. citrovorum 91404 at 21° C was evaluated by adding from 5 to 25 p. p. m. of acetaldehyde to whole milk media at the time of inoculation with three percent of an 18 hr culture. Total lactic agar (38) counts were made at the initiation and after three and 18 hr of incubation at 21° C. Both acidified (pH 4. 5 at the time of inoculation) and non-acidified (pH 6. 5) cultures were examined.

Single-Strain Mixtures

Mixed-strain cultures containing various combinations of S. lactis C_2F, S. diacetilactis 18-16 and L. citrovorum 91404 were prepared by inoculating with appropriate single-strain cultures. The resulting cultures were analyzed for diacetyl and acetaldehyde and the pH, aroma and flavor were determined after 12 to 16 hr of incubation at 21° C.

The effect of storage at 5° C on the acetaldehyde content of an 18 hr culture of S. lactis C_2F was determined by measuring the acetaldehyde content at the time of cooling and again after 12 hr of storage. For comparison the acetaldehyde and diacetyl content of a commercial mixed-strain butter culture was measured immediately after cooling and after storage for 12 hr at 5° C.

Laboratory Scale Production of Synthetic Culture Flavored Products

Butter Culture

In the initial trial, 15 pounds of raw whole milk was heated for one hr in a boiling water bath. After cooling to 21° C, the pH was adjusted to 4.50 with 20 percent phosphoric acid and the following concentrations of flavor components were added:

3.0 p.p.m. of diacetyl
0.5 p.p.m. of acetaldehyde
1250.0 p.p.m. of acetic acid
40.0 p.p.b. of dimethyl sulfide

The sample was held at 5° C for 24 hr before evaluation. On the basis of the flavor evaluation for Trial one, a modified formulation was used in Trial two. In this trial, 15 pounds of raw whole milk was heated for one hr in a boiling water bath and cooled to 21° C. The milk was acidified to pH 4.50 with 20 percent phosphoric acid

and then a slow stream of carbon dioxide was purged through the milk for ten min. The modified flavor mixture was added at the following levels:

2.0 p. p. m. of diacetyl
0.5 p. p. m. of acetaldehyde
1250.0 p. p. m. of acetic acid
40.0 p. p. b. of dimethyl sulfide

The sample was evaluated after storage at 5° C for 24 hr. In a third trial, the effect of milk fat and heat treatment of milk were evaluated. The same levels of flavor compounds were added as in Trial two, except that the level of dimethyl sulfide was reduced from 40 p. p. b. to 25 p. p. b. Fifteen pound lots of homogenized whole milk, pasteurized skimmilk, skimmilk heated for one hr in a boiling water bath and raw whole milk heated for one hr in a boiling water bath were acidified to pH 4.50 with 20 percent phosphoric acid. The samples were purged slowly with carbon dioxide for ten min and then the flavor compounds were added. The samples were evaluated after 24 hr at 5° C.

Since the synthetically prepared butter cultures in the first three trials lacked the desirable viscous body of normal culture, further trials were conducted utilizing delta-gluconolactone as the acidogen. A procedure similar to that described by Deane and Hammond (31) was employed. In Trials four and five, raw whole milk was first

heated for one hr in a boiling water bath and then cooled to 5° C. The sample in Trial four was prepared by adjusting the temperature of the heated milk to 37° C and adding 10.98 g of delta-gluconolactone per kg of milk (calculated at the rate of 12.2 percent of acidogen times an assumed nine percent solids-not-fat content of the milk). The sample was held at 37° C for 1.5 hr, then cooled to 5° C and stored for 24 hr. After storage the following levels of flavor compounds were added:

2.0 p.p.m. of diacetyl
0.5 p.p.m. of acetaldehyde
1250.0 p.p.m. of acetic acid
25.0 p.p.b. of dimethyl sulfide

The final pH of the synthetic culture in Trial four was 4.00, and the flavor was not desirable. In an attempt to alter the apparent flavor balance, the pH of 500 g of the culture was adjusted to pH 4.50 with sodium bicarbonate.

In Trial five, the temperature of one kg of heated milk was adjusted to 21° C and 9.76 g of delta-gluconolactone was added (calculated at the rate of 12.2 percent of acidogen times an assumed eight percent solids-not-fat content of the milk). The sample was then held at 21° C for 24 hr to allow complete hydrolysis of the acidogen (final pH 4.50). After cooling the sample to 5° C, the same levels of flavor components as those employed in Trial four were added and

the pH was adjusted to pH 4. 65 with sodium bicarbonate. The flavor balance of the synthetic culture was altered in two 100 ml aliquots. In one, the acetaldehyde content was raised to 1. 0 p. p. m. with the other components remaining constant; in the other, the dimethyl sulfide concentration was raised to 50 p. p. b. with the other components remaining constant. The samples were then evaluated.

Butter

Three lots of butter (one kg each) were obtained from a large churning of high quality sweet cream butter. The finished butter contained 1. 0 percent of salt. One lot was used as the control with no added flavor. The second lot was modified by adding the following levels of flavor compounds.

30. 0 p. p. m. of acetic acid
500. 0 p. p. m. of lactic acid
2. 5 p. p. m. of diacetyl

The third lot contained:

30. 0 p. p. m. of acetic acid
500. 0 p. p. m. of lactic acid
2. 5 p. p. m. of diacetyl
40. 0 p. p. b. of dimethyl sulfide

The butter samples were coded and held 12 hr at 5° C before evaluation by experienced judges.

RESULTS AND DISCUSSION

Identification of Volatile Flavor Components of Butter Culture and Heated Milk

Culture distillations A and B were carried out before refined methods of evaluation of isolated flavor material were available. However, information about the identity of certain compounds was obtained using retention times and gas chromatographic effluent trapping procedures. The compounds identified by these methods for culture distillation A are shown in Figure 4. Since a thermal conductivity detector was utilized in the gas chromatograph, it was possible to collect the peak effluents into qualitative organic reagents in a manner similar to that described by Walsh and Merritt (157). Ethyl ether, the extracting solvent, was identified by its characteristic odor. Diacetyl was characterized by its odor and the formation of its 2, 4-dinitrophenylhydrazone. Acetoin gave a 2, 4-dinitrophenylhydrazone identical to that for diacetyl. However, it also gave a positive nitrochromic acid ($K_2Cr_2O_7$ plus HNO_3) test (157) indicating the presence of a secondary or primary alcohol. No odor could be detected for acetoin in the effluent issuing from the gas chromatograph. Acetic acid was collected into a small amount of water and its presence was detected by its litmus paper reaction. Attempts to confirm the identity of the components in the flavor extract by

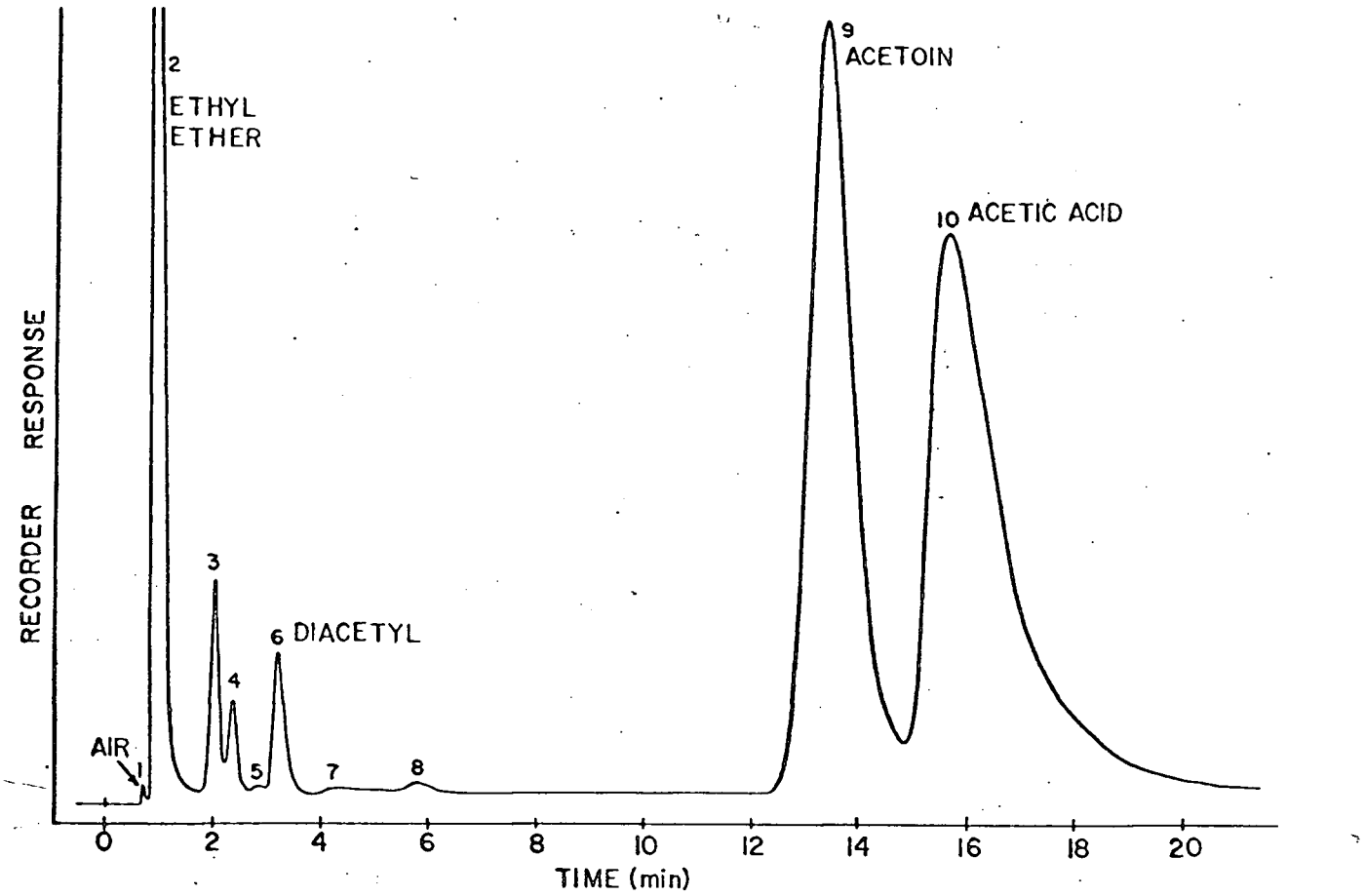


Figure 4. Gas chromatogram of butter culture distillate ether extract from distillation A using a DEGS column at 110° C and a gas chromatograph equipped with a thermal conductivity detector.

solvent collection and subsequent infrared analysis were unsuccessful. The small number of compounds observed in the gas chromatogram of culture distillation A was probably due to the low sensitivity of the instrument employed.

Culture distillations C and D were made using the selected commercial mixed-strain butter culture. These distillations exhibited qualitatively similar gas chromatographic patterns and the data presented herein are a result of a detailed analysis of culture distillation D.

Upon completion of the distillation the volatile flavor fraction was separated from the carbon dioxide produced by the culture organisms. This was accomplished by transfer procedures described earlier. A portion of the volatile flavor fraction was then analyzed by the vapor injection technique described by Libbey et al. (86). The gas chromatograms depicting the separation of the culture aroma concentrate at 70° C on DEGS and Apiezon M columns, respectively, are shown in Figures 5 and 6. The tentative identifications of the peaks shown in the chromatograms are listed in Tables 5 and 6, respectively. All gas chromatographic tentative identifications are based on the coincidence of the relative retention times of unknowns with those for authentic compounds. Since the culture used in distillation D contained 1.85 p.p.m. of diacetyl prior to distillation, this compound was chosen as the internal standard. The data

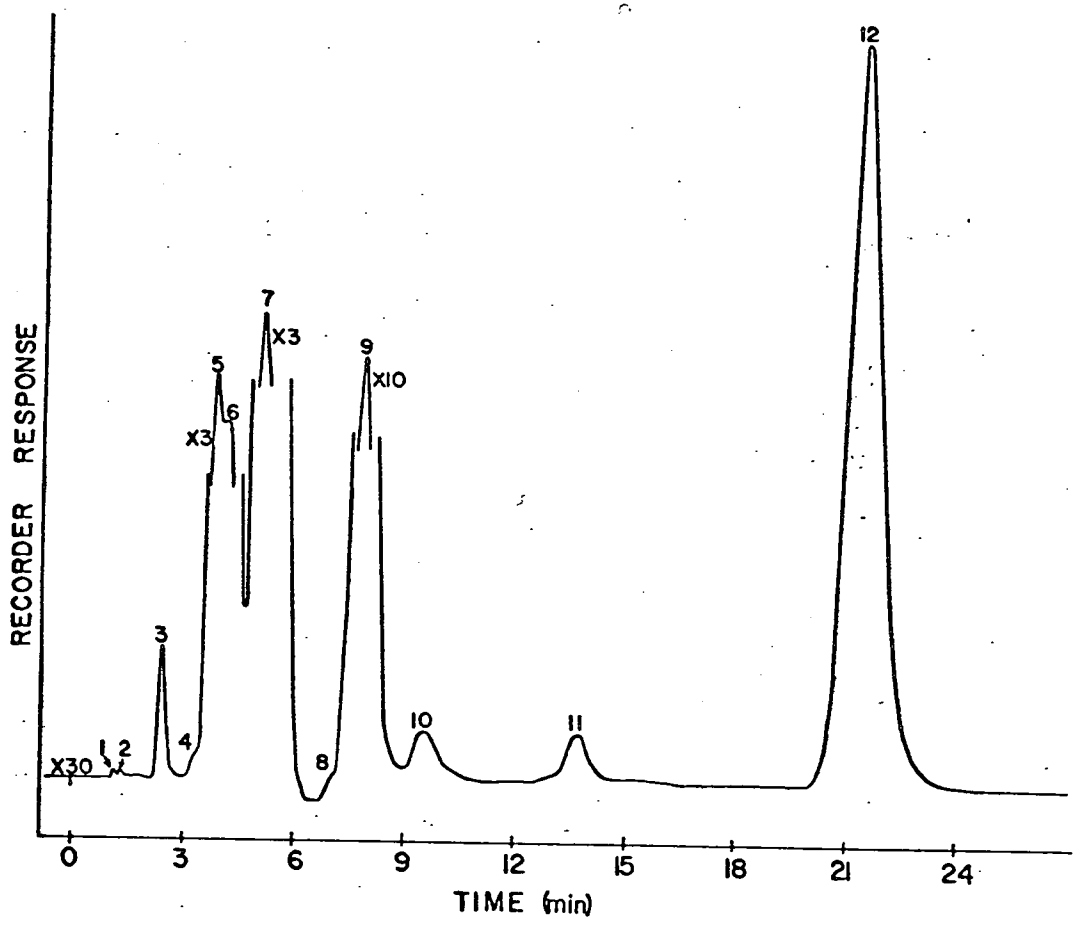


Figure 5. Gas chromatogram of butter culture volatiles analyzed by the vapor injection technique of Libbey et al. using a DEGS column at 70° C (see Table 5 for peak designations).

Table 5. Relative retention times of compounds tentatively identified from distillation D butter culture volatiles analyzed by vapor injection technique of Libbey et al. using a DEGS column; gas chromatogram shown in Figure 5.

Peak No.	Compound	t_R/t_R[a]	
		Butter Culture	Authentics
1	Hydrogen sulfide	0.122	0.103
2	Methyl mercaptan	0.138	0.158
3	Acetaldehyde	0.258	0.210
	Dimethyl sulfide		0.246
4	3-Methylbutanal	0.360	0.356
5	Ethyl formate	0.406	0.391
	Acetone		0.402
6	Ethyl acetate	0.478	0.489
7	Butanone	0.544	0.578
	Ethanol		0.610
8	Methyl butyrate	0.765	0.795
9	2-Pentanone	0.838	0.825
10	Diacetyl	1.000	1.000
11	2-Hexanone	1.454	1.380
12	2-Heptanone	2.272	2.270

[a] t_R/t_R = relative retention time based on diacetyl = 1.000. Packed column, 11 ft x 1/8 inch OD, 20 percent DEGS on 100-120 mesh alkali-acid treated Celite 545; column temperature: 70° C; Barber-Colman Model 20.

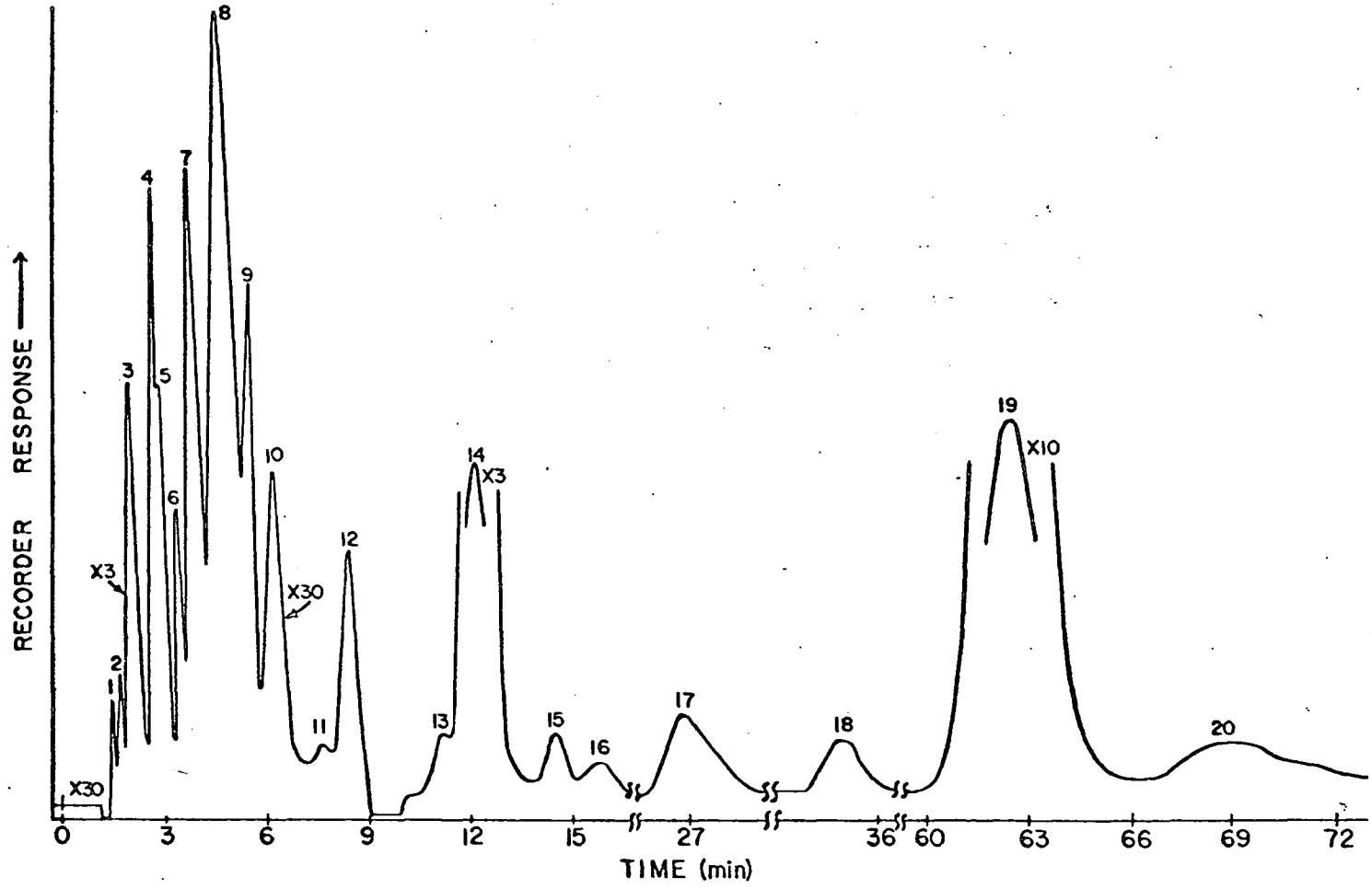


Figure 6. Gas chromatogram of butter culture volatiles analyzed by the vapor injection technique of Libbey et al. using an Apiezon M column at 70° C (see Table 6 for peak designations).

Table 6. Relative retention times of compounds tentatively identified from distillation D butter culture volatiles analyzed by vapor injection technique of Libbey et al. using an Apiezon M column; gas chromatogram shown in Figure 6.

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

a

t_R/t_R = relative retention time based on diacetyl = 1.000. Packed column, 11 ft x 1/8 inch OD, 20 percent Apiezon M on 100-120 mesh alkali-acid treated Celite 545; column temperature: 70° C; Barber-Colman Model 20.

reported for packed column gas chromatography are relative to diacetyl = 1.000. It can be seen by comparing Figures 5 and 6 that the Apiezon M column gave better separation of the volatile components than did the DEGS column. Only 12 peaks are apparent in Figure 5 for the DEGS column while 20 peaks are shown for the same material in Figure 6 for the Apiezon M column.

The ethyl ether extracts of the previously described aqueous fractions of culture distillation D were analyzed on both DEGS and Apiezon M columns at 70° C. Although the fractions were obtained from different cold traps in the distillation apparatus, somewhat similar gas chromatograms were obtained for the various fractions. The separation given by the Apiezon M column for the neutral fraction extract is shown in Figure 7. The gas chromatograms for the Apiezon M separations of the acidic fraction extract and the basic fraction extract are given in Figures 22 and 23, respectively, in the Appendix.

Figure 8 is a gas chromatogram showing the separation of the volatile compounds in the neutral extract fraction by temperature programmed capillary column gas chromatography. The separation and resolution of the flavor components is much greater by this technique than by conventional isothermal packed column gas chromatography. In comparing Figures 7 and 8, it can be seen that 20 components were obtained for the packed column separation of the

neutral fraction extract while the capillary column separation of the same material reveals 47 components. Figure 9 shows a similar separation of the acidic fraction extract and Figure 10 is a gas chromatogram showing the separation of the basic extract fraction of culture distillation D. The identifications of peaks shown in Figures 8, 9 and 10 are given in Tables 8, 9, and 10, respectively.

Identification of components for all capillary column gas chromatographic analyses are based on the coincidence of relative retention times and fast-scan mass spectral fragmentation patterns of unknowns with those of authentic compounds. Ethyl acetate was chosen as the standard and all capillary column separations are reported relative to ethyl acetate = 1.000. Supporting evidence for the identification of some of the compounds are given by packed column tentative identifications. In some instances, such as those for methane and methyl chloride in Table 8, 3-methylbutanal in Table 9 and 2-furfuryl acetate in Table 13, the mass spectral data were sufficient for tentative or positive identifications without corroborating gas chromatographic retention data. In these cases retention data for authentic compounds were not available. All identifications of compounds based on coincidence of gas chromatographic retention data only are considered as tentative identifications.

It can be noted in Tables 8, 9 and 10 that not all of the compounds are identified by mass spectral data. In some cases the

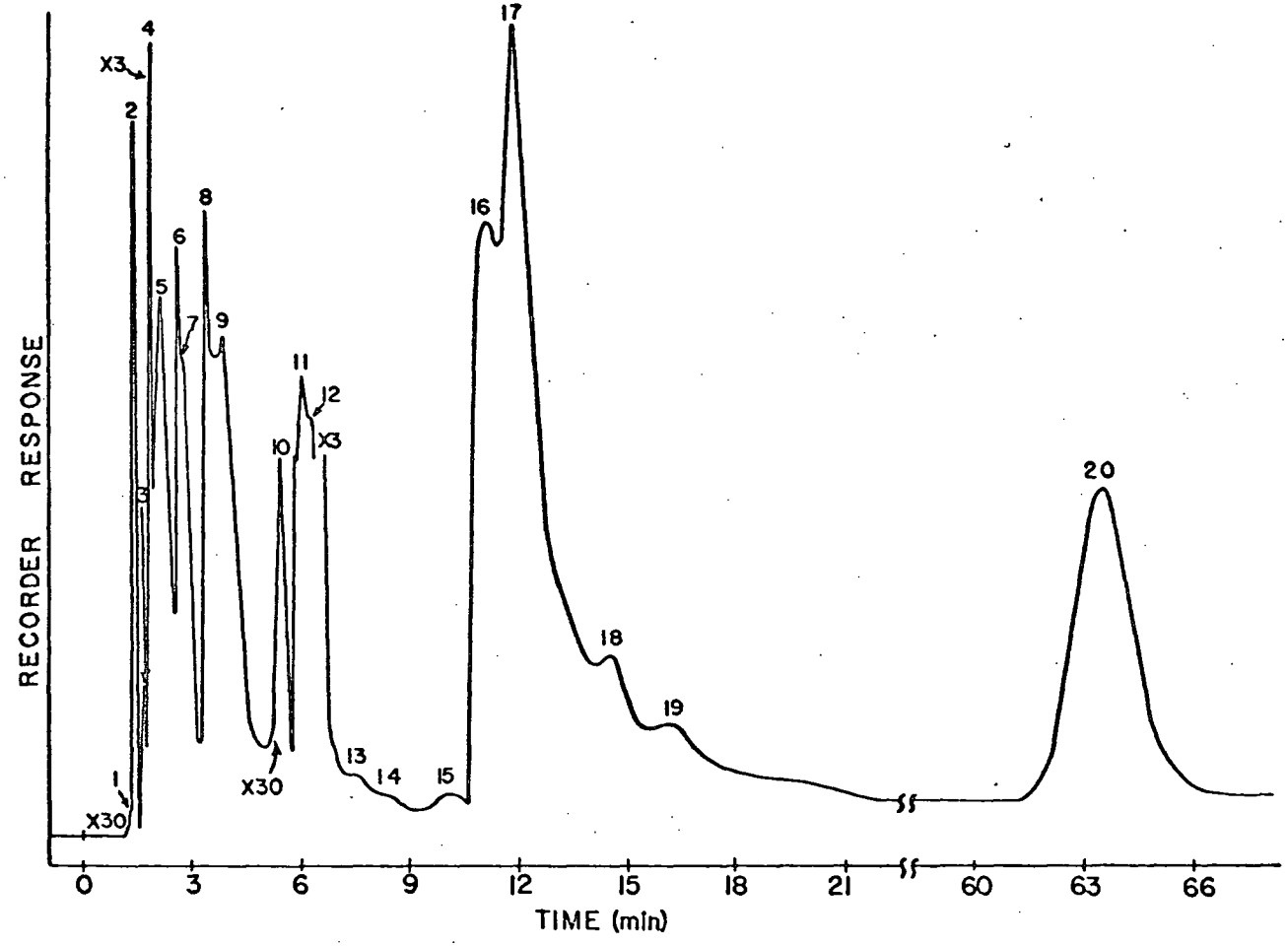


Figure 7. Gas chromatogram of butter culture distillate neutral fraction extract using an Apiezon M column at 70° C (see Table 7 for peak designations).

Table 7. Relative retention times of compounds tentatively identified from distillation D butter culture neutral extract using an Apiezon M column; gas chromatogram shown in Figure 7.

Peak No.	Compound	t_R/t_R[a] Butter Culture	t_R/t_R[a] Authentics
1	------	0.224	-----
2	Hydrogen sulfide	0.256	0.253
3	------	0.303	-----
4	Acetaldehyde	0.353	0.364
5	Methanol	0.423	0.416
6	Methyl mercaptan	0.473	0.466
7	Ethanol	0.541	0.502
8	Acetone	0.620	0.581
	Ethyl formate		0.634
9	Methyl acetate	0.713	0.686
	Ethyl ether		0.690
10	Diacetyl	1.000	1.000
11	n-Butanal	1.110	1.110
	Butanone		1.140
12	Ethyl acetate	1.140	1.140
13	2-Butanol	1.350	1.230
14	2-Mercaptoethanol	1.490	1.480
15	3-Methylbutanal	1.830	1.820
16	n-Butanol	2.020	2.120
17	2-Pentanone	2.170	2.240
18	n-Pentanal	2.620	2.510
19	Methyl butyrate	2.880	2.880
	Acetoin		2.980
20	2-Heptanone	11.320	11.700

[a] t_R/t_R = relative retention time based on diacetyl = 1.000. Packed column, 11 ft x 1/8 inch OD, 20 percent Apiezon M on 100-120 mesh alkali-acid treated Celite 545; column temperature: 70° C; Barber-Colman Model 20.

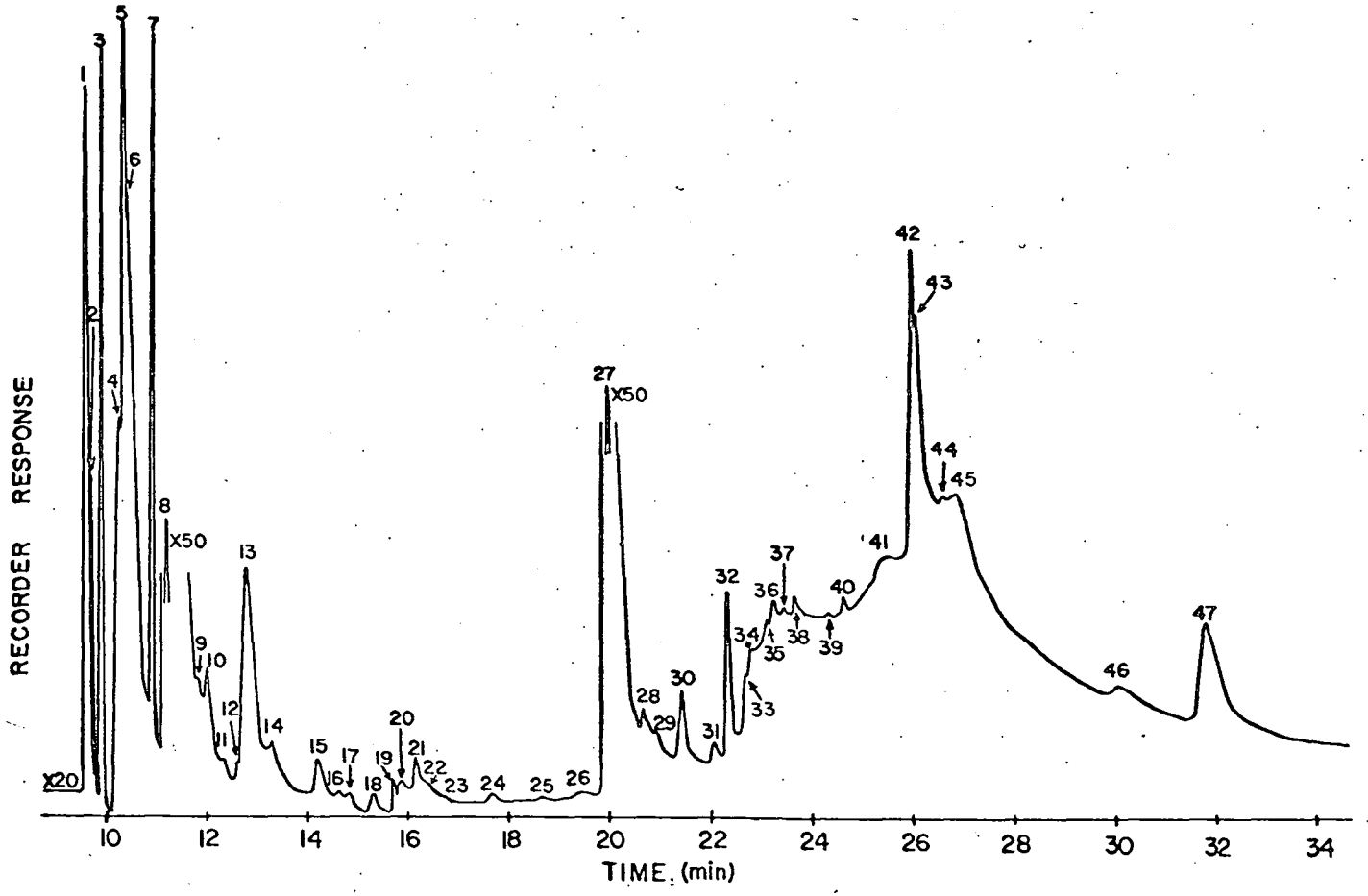


Figure 8. Gas chromatogram of butter culture distillate neutral fraction extract using a capillary column coated with polypropylene glycol; isothermal at 73° C for 8 min, then temperature programmed at 25° C/min to 174° C (see Table 8 for peak designations).

Table 8. Gas chromatographic and mass spectral identification of components of distillation D butter culture neutral extract fraction; gas chromatogram shown in Figure 8.[a]

Peak No.	Identity	t_R/t_R[b] Butter Culture	t_R/t_R Authentics	Confirmed by Packed Column GLC	Mass Spectral Identification	Reference for M. S.
1	Methane	0.856	-----	---	Positive	(1)
2	Methyl chloride	0.870	-----	---	Positive	(1)
3	Acetaldehyde	0.884	0.876	yes	Positive	(1)
	Ethyl ether		0.883	yes	Positive	(1)
4	Acetone	0.919	0.917	yes	Positive	(1)
5	Ethyl formate	0.933	0.924	yes	Positive	(1)
6	Methanol	0.975	0.938	yes	Positive	(4)
7	Ethanol	0.989	0.962	yes	Positive	(1)
8	Ethyl acetate	1.000	1.000	yes	Positive	(1)
9	Diacetyl	1.053	1.007	yes	------	----
10	---	1.067	-----	---	------	----
11	2-Butanol	1.102	1.097	yes	------	----
12	n-Pentanal	1.123	1.145	yes	------	----
13	2-Pentanone	1.144	1.152	yes	------	----
14	Methyl butyrate	1.186	1.200	yes	------	----
15	n-Butyl formate	1.263	1.214	---	-----	----
16	---	1.305	-----	---	------	----
17	---	1.326	-----	---	------	----
18	---	1.368	-----	---	------	----
19	n-Butanol	1.404	1.403	yes	------	----
20	Ethyl butyrate	1.425	1.414	yes	------	----
21	n-Butyl acetate	1.446	1.455	---	------	----
22	---	1.474	-----	---	------	----
23	---	1.495	-----	---	------	----
24	---	1.579	-----	---	------	----
25	Acetoin	1.670	1.676	yes	------	----
26	---	1.723	-----	---	------	----
27	2-Heptanone	1.789	1.834	yes	Positive	(1)
28	---	1.853	-----	---	------	----
29	---	1.864	-----	---	------	----
30	Methyl hexanoate	1.916	1.879	---	------	----
31	---	1.972	-----	---	------	----
32	2-Furfural	2.000	2.007	---	------	----
33	---	2.028	-----	---	------	----
34	Ethyl hexanoate	2.042	2.055	---	------	----
35	---	2.070	-----	---	------	----
36	---	2.084	-----	---	------	----
37	n-Octanal	2.098	2.097	---	------	----
38	---	2.119	-----	---	------	----
39	Methyl heptanoate	2.175	2.148	---	------	----

Table 8 Continued.

Peak No.	Identity	t_R/t_R Butter Culture[b]	t_R/t_R Authentics	Confirmed by Packed Column GLC	Mass Spectral Identification	Reference for M. S.
40	---	2.204	-----	---	------	----
41	2-Furfurol	2.295	2.345	---	------	----
42	2-Nonanone	2.330	2.359	---	Positive	(138)
43	Methyl octanoate	2.342	2.376	---	------	----
44	---	2.372	-----	---	------	----
45	Ethyl octanoate	2.400	2.510	---	------	----
46	Ethyl nonanoate	2.695	2.766	---	------	----
47	2-Undecanone	2.849	2.886	---	Positive	(138)

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

[b] t_R/t_R = relative retention time based on ethyl acetate = 1.000.

Stainless steel capillary, 300 ft x 0.01 inch ID coated with polypropylene glycol; operated at 73° C for eight min, then at 25° C/min to 174° C.

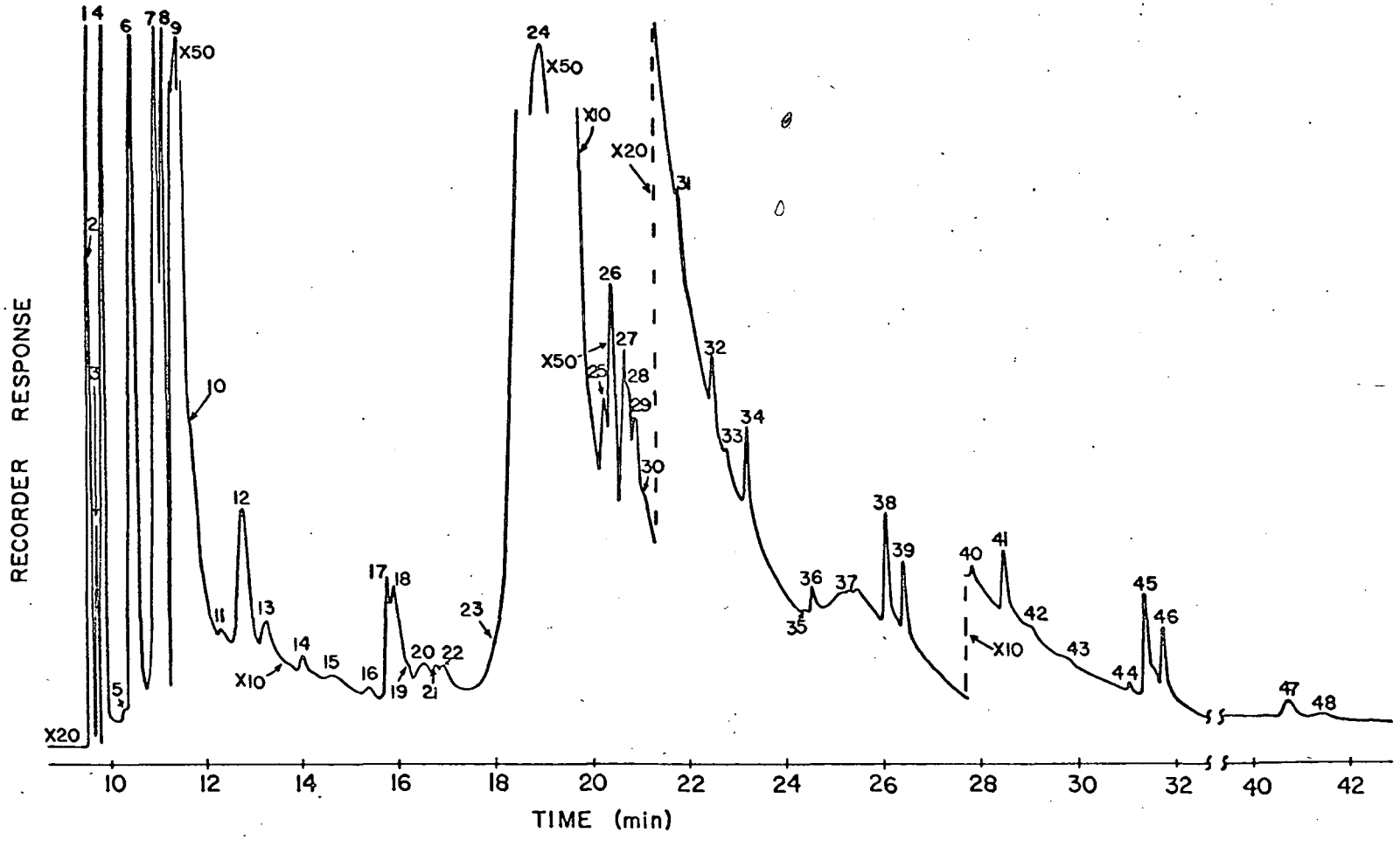


Figure 9. Gas chromatogram of butter culture distillate acidic fraction extract using a capillary column coated with polypropylene glycol; isothermal at 73° C for 8 min, then temperature programmed at 25° C/min to 174° C (see Table 9 for peak designations).

Table 9. Gas chromatographic and mass spectral identification of components of distillation D. butter culture acidic extract fraction; gas chromatogram shown in Figure 9. [a]

Peak No.	Identity	t_R/t_R [b] Butter Culture	t_R/t_R Authentics	Confirmed by Packed Column GLC	Mass Spectral Identification	Reference for M. S.
1	Methane	0.859	-----	---	Positive	(1)
2	Methyl chloride	0.863	-----	---	Positive	(1)
3	Acetaldehyde	0.873	0.876	yes	Positive	(1)
4	Ethyl ether	0.887	0.883	yes	Positive	(1)
5	Acetone	0.923	0.917	yes	Positive	(1)
6	Ethyl formate	0.937	0.924	yes	Positive	(1)
	Methyl acetate		0.928	yes	Positive	(4)
7	Ethanol	0.979	0.962	yes	Positive	(1)
8	Ethyl acetate	1.000	1.000	yes	Positive	(1)
9	Diacetyl	1.028	1.007	yes	Positive	(1)
10	2-Butanol	1.056	1.034	yes	Positive	(40)
	3-methylbutanal		-----	yes	Tentative	(45)
11	n-Pentanal	1.106	1.145	yes	------	----
12	2-Pentanone	1.148	1.151	yes	Positive	(138)
13	Methyl butyrate	1.190	1.200	yes	Positive	(1)
14	Butyl formate	1.254	1.214	---	------	----
15	---	1.310	-----	---	------	----
16	n-Butanol	1.380	1.403	yes	------	----
17	Ethyl butyrate	1.415	1.414	yes	Positive	(1)
18	---	1.430	-----	---	------	----
19	n-Butyl acetate	1.451	1.455	---	------	----
20	---	1.486	-----	---	------	----
21	---	1.507	-----	---	------	----
22	---	1.521	-----	---	------	----
23	---	1.620	-----	---	------	----
24	Acetoin	1.711	1.680	yes	Positive	(4)
25	---	1.824	------	---	------	----
26	2-Heptanone	1.838	1.834	yes	Positive	(1)
27	n-Pentanol	1.859	1.841	---	------	----
28	---	1.866	-----	---	------	----
29	Methyl hexanoate	1.884	1.879	---	Positive	(139)
30	---	1.894	-----	---	------	----
31	---	1.958	-----	---	------	----
32	2-Furfural	2.021	2.007	---	Positive	(4)
33	---	2.049	-----	---	------	----
34	Ethyl hexanoate	2.049	2.055	---	Positive	(139)
35	---	2.190	-----	---	------	---
36	Methyl heptanoate	2.204	2.148	---	------	---
37	2-Furfurol	2.275	2.345	---	Positive	(4)
38	2-Nonanone	2.338	2.359	---	Positive	(138)
39	Methyl octanoate	2.373	2.376	---	Positive	(4)
40	---	2.500	-----	---	------	----

Table 9. Continued.

Peak No.	Identity	t_R/t_R[b] Butter Culture	t_R/t_R Authentics	Confirmed by Packed Column GLC	Mass Spectral Identification	Reference for M. S.
41	Ethyl octanoate	2.563	2.510	---	Positive	(4)
42	Methyl nonanoate	2.613	2.607	---	------	----
43	----	2.676	-----	---	------	----
44	Ethyl nonanoate	2.789	2.766	---	------	----
45	----	2.820	-----	---	------	----
46	2-Undecanone	2.859	2.886	---	Positive	(138)
47	δ-Octalactone	3.655	3.400	---	------	----
48	2-Tridecanone	3.725	3.848	---	------	----

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

[b] t_R/t_R = relative retention time based on ethyl acetate = 1.000.

Stainless steel capillary, 300 ft x 0.01 inch ID coated with polypropylene glycol; operated at 73° C for eight min, then at 25° C/min to 174° C.

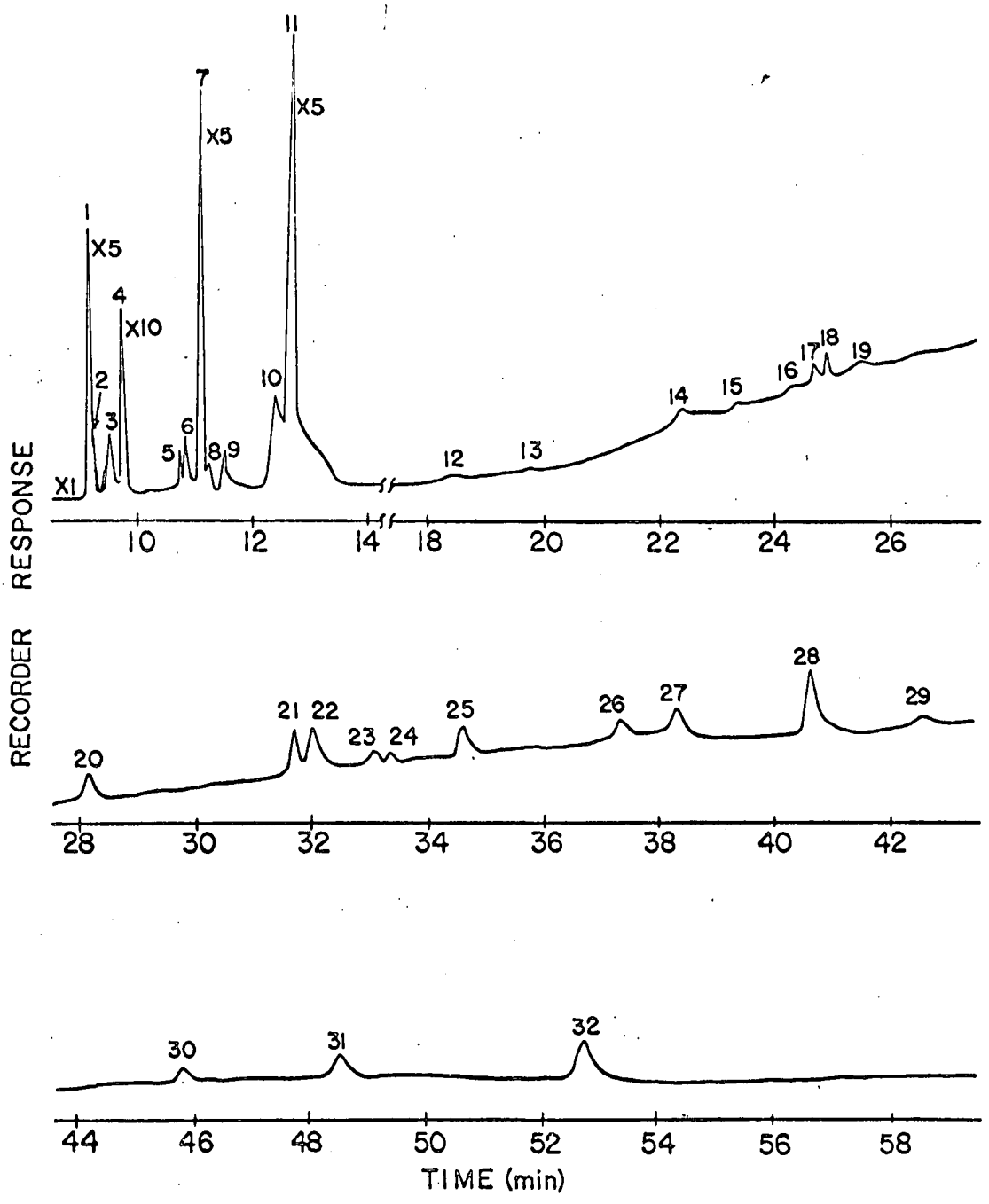


Figure 10. Gas chromatogram of butter culture distillate basic fraction extract using a capillary column coated with polypropylene glycol; temperature programmed from 30° C at 2.5° C/min for 8 min, then 25° C/min to 170° C (see Table 10 for peak designations).

Table 10. Gas chromatographic and mass spectral identification of components of distillation D butter culture basic extract fraction; gas chromatogram shown in Figure 10.[a]

Peak No.	Identity	t_R/t_R[b] Butter Culture	t_R/t_R Authentics	Confirmed by Packed Column GLC	Mass Spectral Identification	Reference for M. S.
1	----	0.725	-----	---	------	----
2	----	0.731	-----	---	------	----
3	Acetaldehyde	0.750	0.770	yes	Positive	(1)
4	Ethyl ether	0.769	0.789	yes	Positive	(1)
5	----	0.846	-----	---	------	----
6	Acetone	0.851	0.860	yes	Positive	(1)
7	Ethyl formate	0.883	0.880	yes	Positive	(1)
8	Methyl acetate	0.907	0.892	yes	------	----
9	Methanol	0.907	0.920	yes	Positive	(4)
10	Butanone	0.978	0.990	yes	Tentative	(1)
11	Ethyl acetate	1.000	1.000	yes	Positive	(1)
12	2-Butanol	1.457	1.300	---	Tentative	(40)
13	n-Butanol	1.543	1.550	yes	------	----
14	2-Heptanone	1.756	1.750	yes	Tentative	(1)
15	Ethyl hexanoate	1.840	1.850	---	Tentative	(139)
16	----	1.907	-----	---	------	----
17	2-Furfural	1.932	1.940	---	------	----
18	----	1.951	-----	---	------	----
19	Methyl heptanoate	1.994	2.048	---	------	----
20	2-Nonanone	2.210	2.130	---	Positive	(138)

Table 10 Continued.

Peak No.	Identity	$^{t}R/^{t}R^{b}$ Butter Culture	$^{t}R/^{t}R$ Authentics	Confirmed by Packed Column GLC	Mass Spectral Identification	Reference for M. S.
21	----	2.481	-----	---	------	----
22	2-Undecanone	2.536	2.580	---	Positive	(138)
23	----	2.596	-----	---	------	----
24	----	2.611	-----	---	------	----
25	Ethyl decanoate	2.707	2.780	---	------	----
26	----	2.926	-----	---	------	----
27	δ-Octalactone	3.000	3.040	---	Tentative	(97)
28	----	3.179	-----	---	------	----
29	2-Tridecanone	3.346	3.430	---	------	----
30	Methyl dodecanoate	3.586	3.460	---	------	----
31	Ethyl dodecanoate	3.821	3.800	---	------	----
32	δ-Decalactone	4.130	4.400	---	------	----

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

[b] $^{t}R/^{t}R$ = relative retention time calculated on the basis of ethyl acetate = 1.000.

Stainless steel capillary, 300 ft x 0.01 inch ID coated with polypropylene glycol; temperature programmed from 30° C at 2.5° C/min for eight min, then 25° C/min to 170° C.

quality of the mass spectra were adequate for identification of a given component in one fraction, but not in another. An example of this can be pointed out for acetoin in Figures 8 and 9. The gas chromatogram in Figure 8 was obtained from the neutral extract fraction of culture distillation D. Since the fraction was obtained from traps late in the trapping sequence, it contained only a small amount of acetoin (peak 25) because of its low volatility. On the other hand, the acidic extract fraction was obtained from the first cold trap in the series. In this case acetoin was present in large amounts (Figure 9, peak 24) and an adequate mass spectrum was obtained to allow identification. It should also be noted that in some cases satisfactory mass spectra were not obtained for any of the fractions and, hence, identification could not be made.

The possible formation of artifacts during isolation, concentration and identification procedures must always be considered in flavor chemistry studies. The presence of short chain fatty acids, especially acetic acid, and alcohols, such as ethanol, in the concentrated culture distillates led to speculation as to the actual existance of certain esters in the intact culture sample. For this reason, a portion of the aqueous distillate from the first cold trap in the distillation apparatus was made distinctly alkaline to litmus paper with sodium carbonate immediately after thawing. If the esters were not already formed, the existance of the acid components as sodium salts

during the preparative procedures should have prevented esterification. As can be seen in Figure 10 and Table 10, essentially the same esters were observed in the alkaline fraction extract as in the acidic fraction extract (Table 9). These data strongly support the theory that aliphatic esters normally exist in unaltered butter culture.

The control heated milk medium for culture distillation D was vacuum distilled under the same conditions as the culture. A portion of the volatile flavor material was separated by packed column gas chromatography using the vapor injection technique of Libbey et al. (86). The gas chromatograms for the Apiezon M and DEGS column separations at 70° C are shown in Figures 11 and 12, respectively. Again, the Apiezon M column showed a more efficient separation than did the DEGS column. The tentative identifications of the components are shown for the two columns in Tables 11 and 12. When the distillation apparatus was disassembled after the distillation, a very pungent ammonia-like odor was observed in some of the cold traps. Jenness and Patton (69, p. 354) state that ammonia is produced as a result of heat degradation of milk proteins, and that possibly urea may be a source of ammonia in heated milk. However, it should be noted that the organic acids produced by the lactic organisms lower the pH of butter culture to a level where pratically all amines would be present in the form of salts. Therefore, they would not be expected to contribute significantly to the flavor of

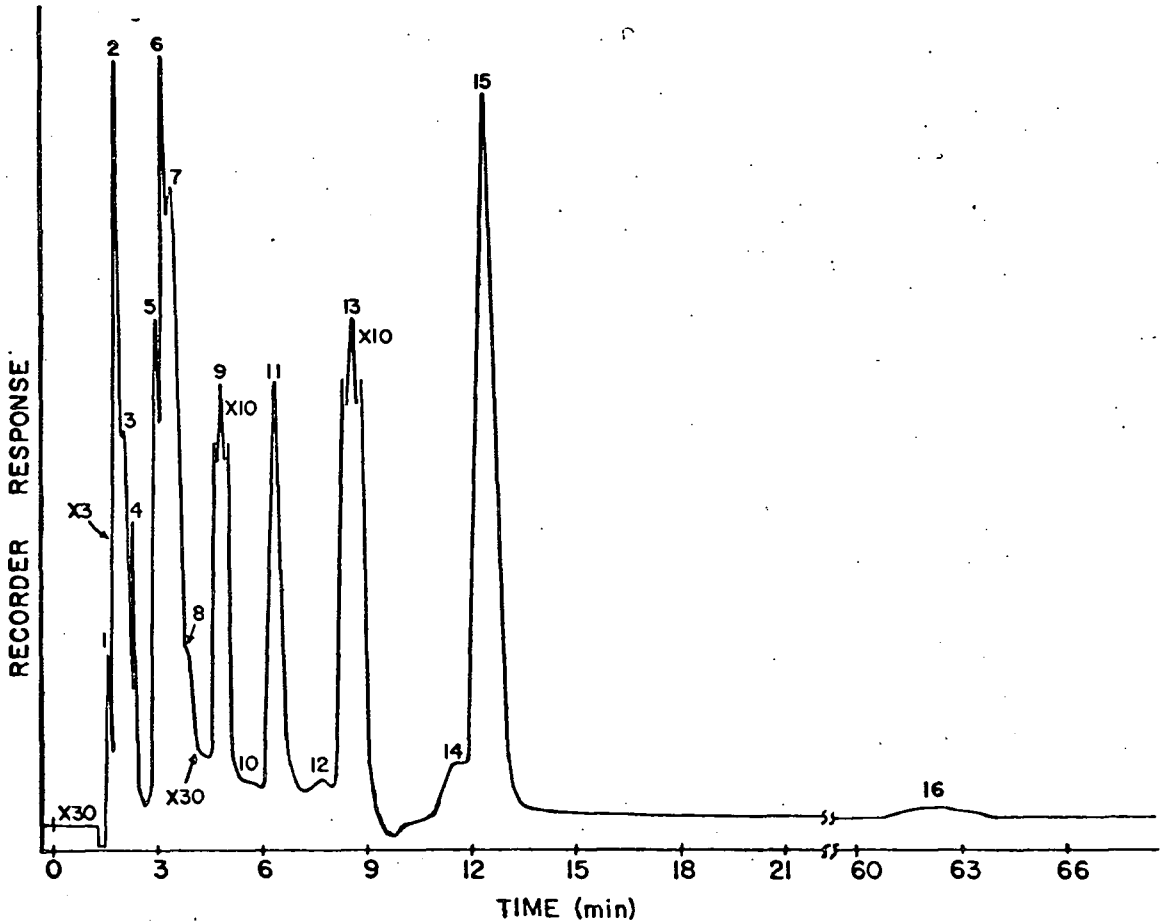


Figure 11. Gas chromatogram of heated milk volatiles analyzed by the vapor injection technique of Libbey et al. using an Apiezon M column at 70° C (see Table II for peak designations).

Table 11. Relative retention times of compounds tentatively identified in heated milk volatiles analyzed by vapor injection technique of Libbey et al. using an Apiezon M column; gas chromatogram shown in Figure 11.

Peak No.	Compound	t_R/t_R[a]	
		Heated Milk	Authentics
1	Hydrogen sulfide	0.280	0.253
2	------	0.350	-----
3	Acetaldehyde	0.356	0.364
4	Methanol	0.386	0.416
5	------	0.529	-----
6	Acetone	0.572	0.581
7	Ethyl formate	0.614	0.634
8	Methyl acetate	0.688	0.686
9	Dimethyl sulfide	0.879	0.846
10	n-Butanal	1.101	1.110
11	Ethyl acetate Butanone	1.155	1.140 1.140
12	------	1.388	-----
13	2-Mercaptoethanol	1.551	1.480
14	n-Butanol	2.118	2.212
15	2-Pentanone	2.235	2.240
16	2-Heptanone	11.562	11.700

[a] t_R/t_R = relative retention time based on diacetyl = 1.000. Packed column, 11 ft x 1/8 inch OD, 20 percent Apiezon M on 100-120 mesh alkali-acid treated Celite 545; column temperature: 70° C; Barber-Colman Model 20.

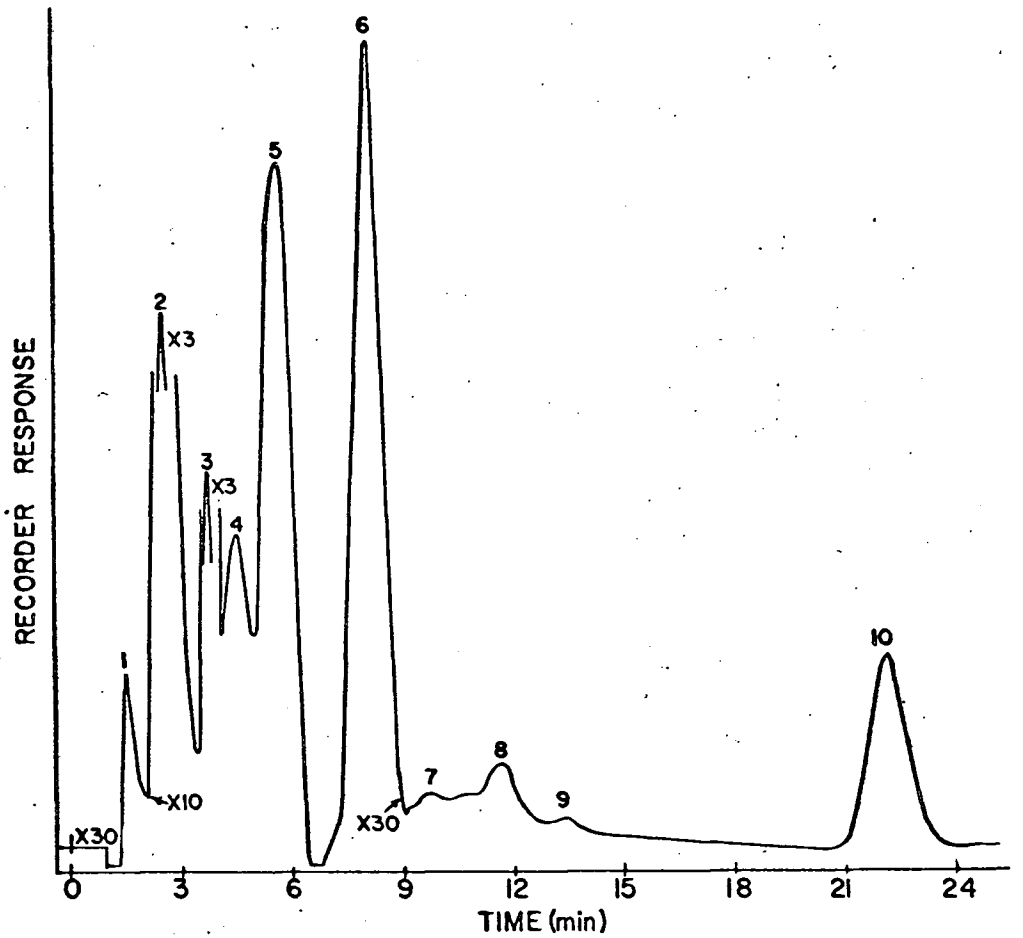


Figure 12. Gas chromatogram of heated milk volatiles analyzed by the vapor injection technique of Libbey et al. using a DEGS column at 70° C (see Table 12 for peak designations).

Table 12. Relative retention times of compounds tentatively identified in heated milk volatiles analyzed by vapor injection technique of Libbey et al. using a DEGS column; gas chromatogram shown in Figure 12.

Peak No.	Compound	t_R/t_R[a]	
		Heated Milk	Authentics
1	Methyl mercaptan	0.149	0.158
2	Acetaldehyde	0.246	0.210
	Dimethyl sulfide		0.246
3	Ethyl formate	0.384	0.391
	Acetone		0.402
4	n-Butanal	0.463	0.466
	Ethyl acetate		0.489
5	Butanone	0.579	0.578
6	2-Pentanone	0.832	0.825
7	Ethyl butyrate	1.012	0.920
8	-----	1.213	-----
9	2-Hexanone	1.390	1.380
10	2-Heptanone	2.280	2.270

[a] t_R/t_R = relative retention time based on diacetyl = 1.000. Packed column, 11 ft x 1/8 inch OD, 20 percent DEGS on 100-120 mesh alkali-acid treated Celite 545; column temperature: 70° C; Barber-Colman Model 20.

butter culture.

In comparing the odors of the distillates obtained from culture distillation D and the control heated milk, it was noted that the culture distillate exhibited a more intense methyl ketone aroma. The aroma of the culture distillate was very similar to that of 2-pentanone and 2-heptanone. In comparing the gas chromatographic patterns in Figures 5 and 6 for butter culture volatiles with those in Figures 11 and 12 for the heated milk volatiles, it can be seen that the peaks for 2-pentanone and 2-heptanone are much larger in the chromatograms for the culture volatiles. Both the culture and heated milk were prepared from raw whole milk and contained approximately 3.8 percent of milk fat. The origin of odd-numbered methyl ketones in milk fat has been found to be a result of decarboxylation of beta-ketoacids which have been hydrolyzed from milk glycerides (110). Gehrig and Knight (44) have demonstrated that the spores of Penicillium roqueforti convert octanoic acid to beta-ketooctanoic acid. Subsequent decarboxylation of the beta-ketoacid yielded 2-heptanone which is one of the important flavor components of blue cheese. Prolonged incubation of butter cultures does not give rise to a blue-cheese type aroma. This indicates that the methyl ketones are not a result of fatty acid oxidation by the culture organisms.

A reasonable explanation for the greater quantities of 2-pentanone and 2-heptanone observed in the culture distillate resides in the pH dependent rate of decarboxylation for beta-ketoacids. Both the culture and the heated milk received the same heat treatment of one hr at near 100° C. This treatment is sufficient to effect glyceride hydrolysis with the release of free beta-ketoacids (110). The free beta-ketoacids probably then are oriented near the surface of the individual fat globules due to the polar nature of the free carboxyl group and the non-polar character of the aliphatic chain, thus making the reactive portion of the molecules susceptible to the aqueous physical environment. Widmark (160) studied the rate of decarboxylation of acetoacetic acid and found the rate of decarboxylation was approximately 50 times greater in acid solution as in alkaline solution. According to Gould (50), the ketoacid decarboxylation reaction is favored over the ketoacid anion decarboxylation reaction because of a partial carboxyl proton transfer through intramolecular bonding to the keto group where it aids the decarboxylation reaction. West (159, p. 111) lists the pK_a of acetoacetic acid as 3.8 ($K_a = 1.6 \times 10^{-4}$), and it can reasonably be assumed that other short chain beta-ketoacids have similar pK_a values. The heated milk control had a pH of near 6.50 and thus more of the beta-ketoacids would exist in the anion form than in the butter culture where the pH was near 4.50. Therefore, methyl ketone production should be enhanced in butter

culture due to the more acid nature of the medium.

Davies (23) has reported that cultured cream required from 12 to 24 hr of refrigerated storage before a full cultured flavor developed. It was suggested that the storage period allowed production of diacetyl and thus accounted for the enhanced flavor. DeMann (33) found that alpha-acetolactic acid was liberated by aroma bacteria into the medium and could be subsequently oxidatively decarboxylated to form diacetyl. The formation of diacetyl by this mechanism has been generally considered as the main factor in the development of an enhanced flavor in butter cultures upon refrigerated storage. However, the evidence presented in this work indicates that other flavor components may also be formed during this period and thus contribute to the final flavor of the butter culture. In this respect, the work of Langler and Day (83) has shown that there are sufficient amounts of methyl ketone precursors available in whole milk to influence the flavor of beverage milk.

The absence of large amounts of acetic acid and carbon dioxide in the heated milk made it possible to combine all of the aqueous distillate from the cold traps into one fraction. While the culture flavor extracts exhibited an odor characteristic of commercial starter distillate, the heated milk extract did not exhibit a typical cooked milk aroma. An interesting observation on the odor of the heated milk extract was made when a strip of filter paper was saturated with the

concentrated flavor mixture. This was the appearance of an odor which was described as being very similar to that observed for "hot buttered popcorn". Attempts to isolate the compound (or compounds) responsible for the odor were unsuccessful. Figure 13 shows a gas chromatogram of the heated milk distillate separated by temperature programmed capillary column gas chromatography. The identifications of the peaks shown in Figure 13 are listed in Table 13. Again, positive identifications of compounds were based on coincidence of gas chromatographic retention times and mass spectral data.

To illustrate the type of data obtained by fast-scan mass spectrometry of capillary column effluents, mass spectral charts representing spectra for peaks 19 through 22 for the heated milk distillate (Figure 13) are presented in Figure 14. Spectra (a) and (b) were obtained from peak 19 and the tailing of peak 19. The ion peaks at m/e 43 ($OC_2H_3^+$) and 58 ($OC_3H_6^+$) are indicative of a methyl ketone. The ion fragment at m/e 114 is the parent ion for 2-heptanone. Comparison of the remainder of the two spectra, especially ion peaks 99, 85, 71 and 59, with the spectrum in the API tables (1) supports this interpretation. Coincidence of gas chromatography retention data for authentic 2-heptanone and chromatographic peak 19, Table 13, is sufficient evidence for confirming the identification. Spectrum (c), which represents chromatographic peak 22 in Figure 13, shows an

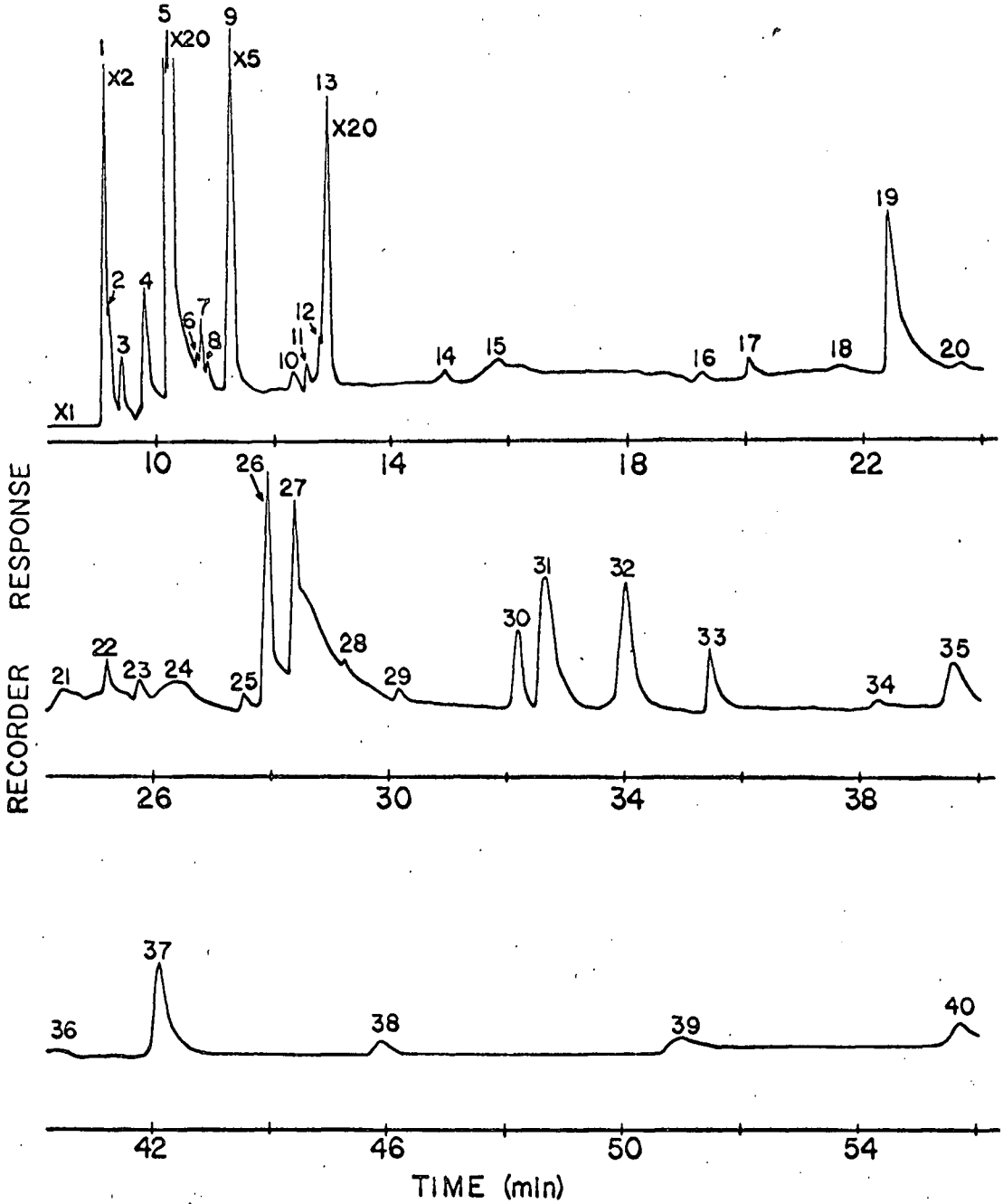


Figure 13. Gas chromatogram of heated milk distillate extract using a capillary column coated with polypropylene glycol; temperature programmed from 30° C at 2.5° C/min for 8 min, then at 25° C/min to 170° C (see Table 13 for peak designations).

Table 13. Gas chromatographic and mass spectral identification of components of heated milk distillate extract; gas chromatogram shown in Figure 13. [a]

Peak No.	Identity	t_R/t_R [b] Heated Milk	t_R/t_R Authentics	Confirmed by Packed Column GLC	Mass Spectral Identification	Reference for M. S.
1	---	0.709	-----	---	------	----
2	---	0.718	-----	---	------	----
3	---	0.727	-----	---	------	----
4	Acetaldehyde	0.758	0.770	yes	Positive	(1)
5	Ether	0.776	0.789	yes	Positive	(1)
6	Dimethyl sulfide	0.830	0.816	yes	------	----
7	Acetone	0.842	0.860	yes	------	----
8	---	0.852	-----	---	------	----
9	Ethyl formate	0.873	0.880	yes	Positive	(1)
	Methyl acetate		0.892	yes	Tentative	(4)
10	n-Butanal	0.945	0.958	yes	------	----
11	---	0.970	-----	---	------	----
12	Butanone	0.982	0.990	yes	Tentative	(1)
13	Ethyl acetate	1.000	1.000	yes	Positive	(1)
14	2-Pentanone	1.145	1.190	yes	------	----
15	Methyl butyrate	1.218	1.220	yes	------	----
16	Ethyl butyrate	1.431	1.430	---	------	----
17	2-Hexanone	1.552	1.480	---	Tentative	(1)
18	n-Butanol	1.648	1.550	yes	------	----
19	2-Heptanone	1.727	1.750	yes	Positive	(1)
20	---	1.828	-----	---	------	----

Table 13 Continued.

Peak No.	Identity	t_R/t_R[b] Heated Milk	t_R/t_R Authentics	Confirmed by Packed Column GLC	Mass Spectral Identification	Reference for M. S.
21	Ethyl hexanoate	1.891	1.850	---	------	----
22	2-Furfural	1.945	1.940	---	Positive	(4)
23	---	1.988	-----	---	------	----
24	Methyl heptanoate	2.031	2.048	yes	------	----
25	2-Furfuryl acetate	2.127	-----	---	Tentative	(4)
26	2-Nonanone	2.152	2.130	---	Positive	(138)
27	2-Furfurol	2.164	2.170	yes	Positive	(4)
28	Ethyl Octanoate	2.261	2.250	---	Positive	(4)
29	---	2.327	-----	---	------	----
30	---	2.485	-----	---	------	----
31	2-Undecanone	2.521	2.580	---	Positive	(138)
32	Methyl decanoate	2.624	2.580	---	Positive	(4)
33	Ethyl decanoate	2.739	2.780	---	------	----
34	---	2.958	-----	---	------	----
35	δ-Octalactone	3.055	3.040	---	------	----
36	---	3.115	-----	---	------	----
37	---	3.255	-----	---	------	----
38	2-Tridecanone	3.455	3.430	---	------	----
39	Ethyl dodecanoate	3.939	3.800	---	------	----
40	δ-Decalactone	4.303	4.400	---	------	----

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

[b] t_R/t_R = relative retention time calculated on the basis of ethyl acetate = 1.000. Stainless steel capillary, 300 ft x 0.01 inch ID coated with polypropylene glycol; temperature programmed from 30° C at 2.5° C/min for eight min, then at 25° C/min to 170° C.

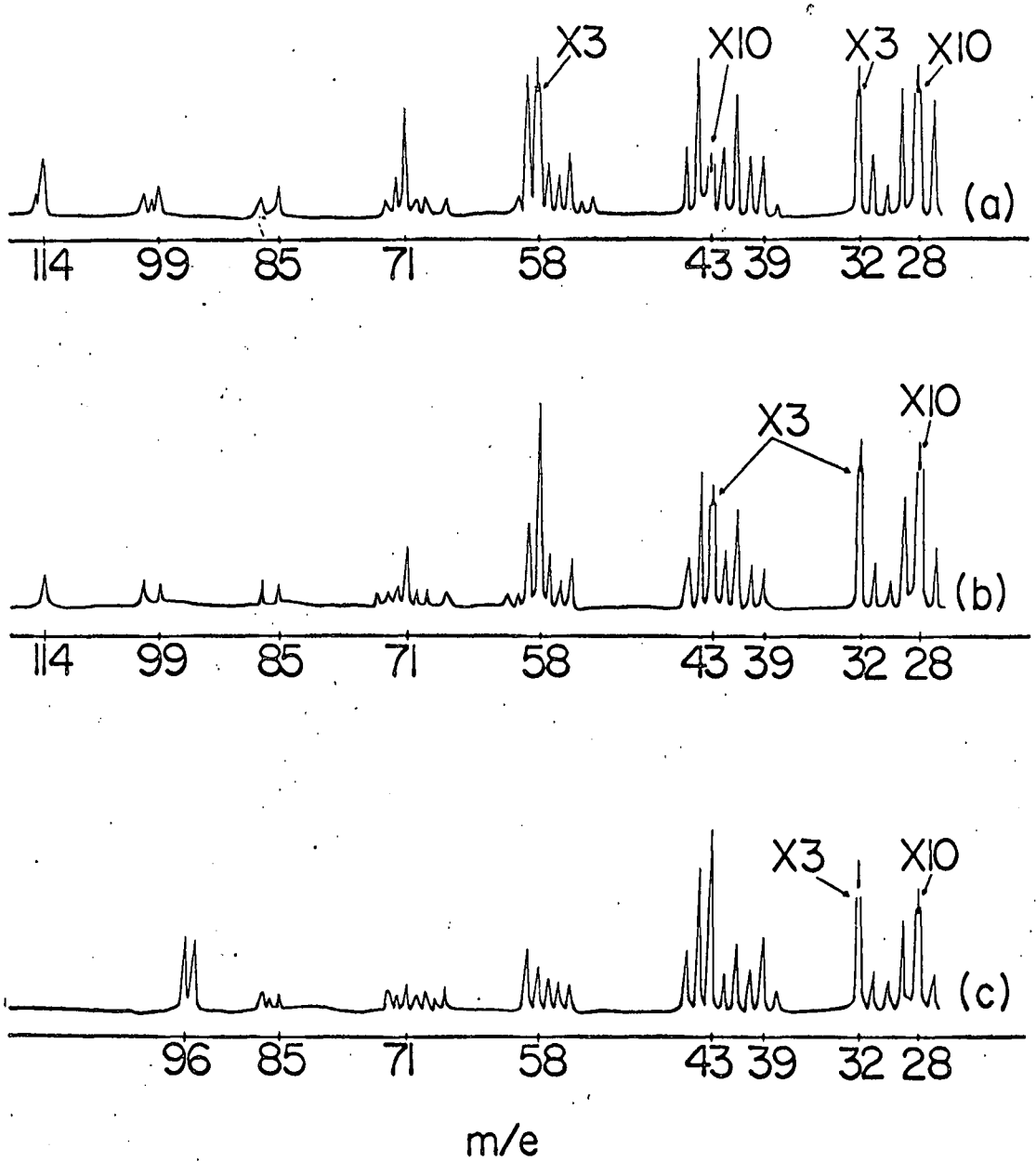


Figure 14. Mass spectral charts for chromatographic fractions shown in Figure 13 : (a) corresponds to peak 19; (b) = tailing of peak 19; (c) = peak 22.

ion peak at m/e 96. This is the parent ion and base peak for 2-furfural and represents the ion $C_5H_4O_2^+$. Comparison of the remainder of the spectrum, especially ion peaks 95, 67, 39 and 38, with the spectrum in the ASTM tables (4) supports this interpretation. Again, the gas chromatographic retention data for authentic 2-furfural and peak 22 in Table 13 are in agreement.

Another example of fast-scan mass spectral data obtained for closely spaced gas chromatographic peaks eluting from a capillary column is shown in Figure 15. Mass spectral patterns (a), (b) and (c) in Figure 15 correspond to gas chromatographic peaks 7, 8 and 9, respectively, in Figure 9. Spectrum (a) in Figure 15 shows a large ion peak at mass 31 which is indicative of a primary alcohol-type fragment (CH_3O^+). The base peak for ethanol is m/e 31 and its parent ion is m/e 46. Comparison of spectrum (a) with the spectrum for ethanol in the API tables (1) shows that the ion fragments at m/e 45 ($C_2H_5O^+$), 43 ($C_2H_3O^+$) and 42 ($C_2H_2O^+$) are present in both spectra and are in agreement. Table 9 shows the coincidence of gas chromatographic retention data for authentic ethanol and peak 7 in Figure 9, thus verifying the identification.

Spectrum (b) in Figure 15 shows the appearance of heavier ion fragments while certain characteristic peaks for ethanol are still apparent. There is also a large increase in the peak at m/e 43. This peak is the base peak for acetate esters and represents the

CH_3CO^+ fragment. The peak at m/e 88 is the parent peak of ethyl acetate. The ion fragment at mass 61 is a characteristic rearrangement peak for acetate esters (139). Comparison of the remainder of the spectrum with the API spectrum (1) reveals that the peaks at m/e 42, 70 and 73 are of significance in the interpretation. The gas chromatographic retention data also support this interpretation (Table 9). Spectrum (c) in Figure 15 shows the presence of a new ion fragment at m/e 86. It can also be seen that the peak at m/e 43 has greatly increased in magnitude. The parent ion for diacetyl is m/e 86 and the base peak is m/e 43 (1). The very large peak at m/e 43 can be justified by the effect of the residual ethyl acetate spectrum which is still apparent in spectrum (c) of Figure 15. Diacetyl was confirmed as the compound responsible for peak 9 in the gas chromatogram shown in Figure 9 by comparison of its retention time with that for the authentic compound (Table 9). The series of mass spectral charts in Figure 15 show the manner by which flavor compounds can be identified using gas chromatography combined with fast-scan mass spectrometry. It also demonstrates that complete separation of compounds is not absolutely necessary for identification of relatively simple organic compounds. This is best illustrated in spectra (c) of Figure 15, where ion fragments originating from ethanol, ethyl acetate and diacetyl are all observed.

Table 14 summarizes the compounds identified in butter culture

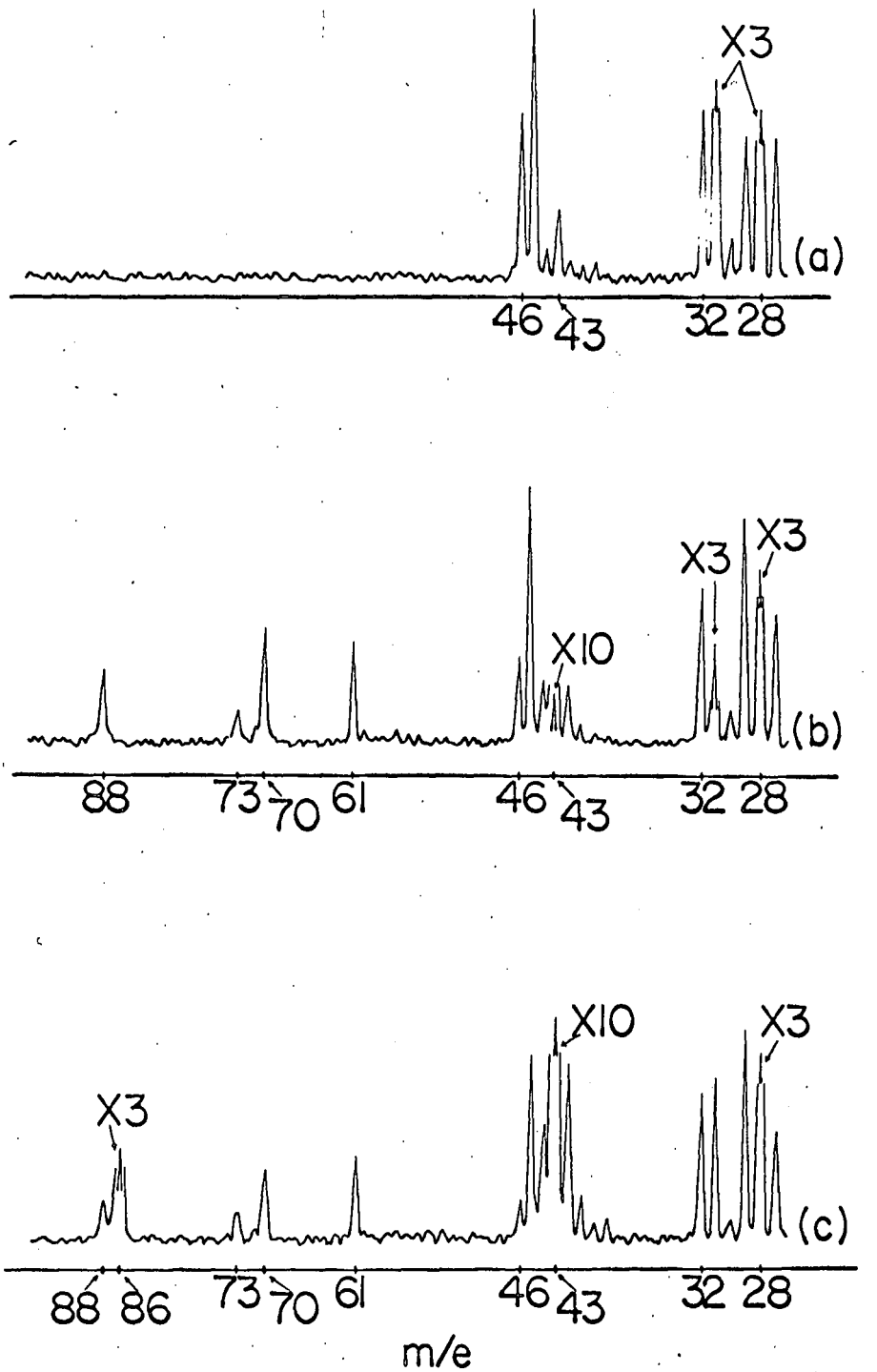


Figure 15. Mass spectral charts for chromatographic fractions shown in Figure 9 : (a) corresponds to chromatographic peak 7; (b) = peak 8; (c) = peak 9.

and heated milk distillates by gas chromatographic and mass spectral methods. The table includes both positive and tentative identifications. In comparing the data, it is obvious that many of the compounds present in the butter culture distillates are also present in the distillate obtained from the heated milk control. This suggests that at least part of normal butter culture flavor can be attributed to the compounds present in the original milk medium. Some compounds, such as ethanol, diacetyl, 2-butanol, acetoin and acetic acid, appear to be present only in the culture distillates. It also appears that all major culture produced flavor compounds have been isolated and identified. The qualitative data for heated milk and butter culture are very similar in many respects, however, quantitative differences may exist for many of the compounds found in the two materials. Although differences in relative concentrations of certain compounds in the distillates appeared to be significant, caution must be observed in interpreting such data. The distillation apparatus used for the isolation of flavor components was found to give 45 percent diacetyl removal when 20 percent by volume of culture was distilled. Thus, it becomes obvious that care must be taken in relating relative concentrations of recovered volatile compounds.

Further studies on the confirmation of identification of some of the compounds in butter cultures and cultured butter were carried

Table 14. Summary of volatile compounds identified in butter culture and control heated milk distillates. [a]

	GLC Packed Column				GLC Capillary		Mass Spectra	
	DEGS		Apiezon M					
Compound	Culture	Milk	Culture	Milk	Culture	Milk	Culture	Milk
Methane	-	-	-	-	-	-	+	-
Methyl Chloride	-	-	-	-	-	-	+	-
Acetaldehyde	+	+	+	+	+	+	+	+
Dimethyl Sulfide	+	+	+	+	-	+	-	-
Hydrogen Sulfide	+	-	+	+	-	-	-	-
Methyl mercaptan	+	+	+	-	-	-	-	-
Ethyl formate	+	+	+	+	+	+	+	+
Methyl acetate	-	-	+	+	+	+	+	±
Acetone	+	+	+	+	+	+	+	-
Methanol	-	-	+	+	+	-	+	-
Ethanol	+	-	+	-	+	-	+	-
Diacetyl	+	-	+	-	+	-	+	-
Ethyl acetate	+	+	+	+	+	+	+	+
Butanone	+	+	+	+	+	+	±	±
2-Butanol	-	-	+	-	+	-	+	-
n-Butanal	-	+	+	+	-	+	-	-
n-Butanol	-	-	+	+	-	+	-	-
Methyl butyrate	+	-	+	+	+	+	+	-
Ethyl butyrate	-	+	+	-	+	+	+	-
2-Pentanone	+	+	+	+	+	+	+	-
2-Hexanone	+	+	-	-	-	+	-	±
2-Heptanone	+	+	+	+	+	+	+	+
n-Pentanal	-	-	+	-	+	-	-	-
n-Pentanol	-	-	-	-	+	-	-	-
2-Mercaptoethanol	-	-	+	+	-	-	-	-
Acetoin	+	-	+	-	+	-	+	-
Acetic acid	+	-	-	-	-	-	-	-
n-Butyl formate	-	-	-	-	+	-	-	-
n-Butyl acetate	-	-	-	-	+	-	-	-
2-Methylbutanal	-	-	+	-	-	-	-	-
3-Methylbutanal	-	-	+	-	-	-	±	-
Methylpropanal	-	-	+	-	-	-	-	-
2-Furfural	-	-	-	-	+	+	+	+
2-Furfurol	-	-	-	-	+	+	+	+
2-Furfuryl acetate	-	-	-	-	-	-	-	±
Methyl hexanoate	-	-	-	-	+	-	+	-
Ethyl hexanoate	-	-	-	-	+	+	+	-
Methyl heptanoate	-	-	+	-	+	+	-	-
n-Octanal	-	-	-	-	+	-	-	-

Table 14 Continued.

Compound	GLC Packed Column				GLC Capillary		Mass Spectra	
	DEGS		Apiezon M					
	Culture	Milk	Culture	Milk	Culture	Milk	Culture	Milk
2-Nonanone	-	-	-	-	+	+	+	+
2-Undecanone	-	-	-	-	+	+	+	+
2-Tridecanone	-	-	-	-	+	+	-	-
Methyl benzoate	-	-	-	-	-	-	±	-
Methyl octanoate	-	-	-	-	+	-	+	-
Ethyl octanoate	-	-	-	-	+	+	+	+
Methyl nonanoate	-	-	-	-	+	-	-	-
Ethyl nonanoate	-	-	-	-	+	-	-	-
Methyl decanoate	-	-	-	-	-	+	-	+
Ethyl decanoate	-	-	-	-	+	+	-	-
Methyl dodecanoate	-	-	-	-	+	-	-	-
Ethyl dodecanoate	-	-	-	-	+	+	-	-
δ-Octalactone	-	-	-	-	+	+	±	-
δ-decalactone	-	-	-	-	-	+	-	-

[a]Includes both tentative and positive identifications.

out by other procedures. Figure 16 shows a gas chromatogram obtained from one ml of headspace by the technique described earlier. Peaks 1, 3 and 5 represent acetaldehyde, acetone and diacetyl, respectively. In addition to agreement of retention times with those for authentic compounds, some identifications were based on the syringe-reaction functional group analysis technique of Hoff and Feit (65). Contact of the volatiles in the syringe with hydroxylamine caused reduction or removal of the carbonyl compound peaks shown in the chromatogram. Treatment of the vapors with 1.25 N sodium hydroxide resulted in the disappearance of peaks 4 and 6, thus supporting their identifications as ethyl formate and ethyl acetate, respectively.

The identification of dimethyl sulfide posed a difficult problem, because of its low concentration and the difficulty of forming a suitable derivative. Tentative identification of dimethyl sulfide was made in the gas chromatographic analysis of culture distillates (Tables 5 and 6). Supporting evidence for dimethyl sulfide in butter culture is given in headspace gas chromatogram shown in Figure 16. The headspace samples were taken from containers previously described. Attempts to utilize two and five ml rubber stoppered serum vials and gas-tight syringes as described by Bassette et al. (8, 9) were unsuccessful. Contaminating peaks arising from the rubber caps and residual sample carry-over in the syringes were impossible

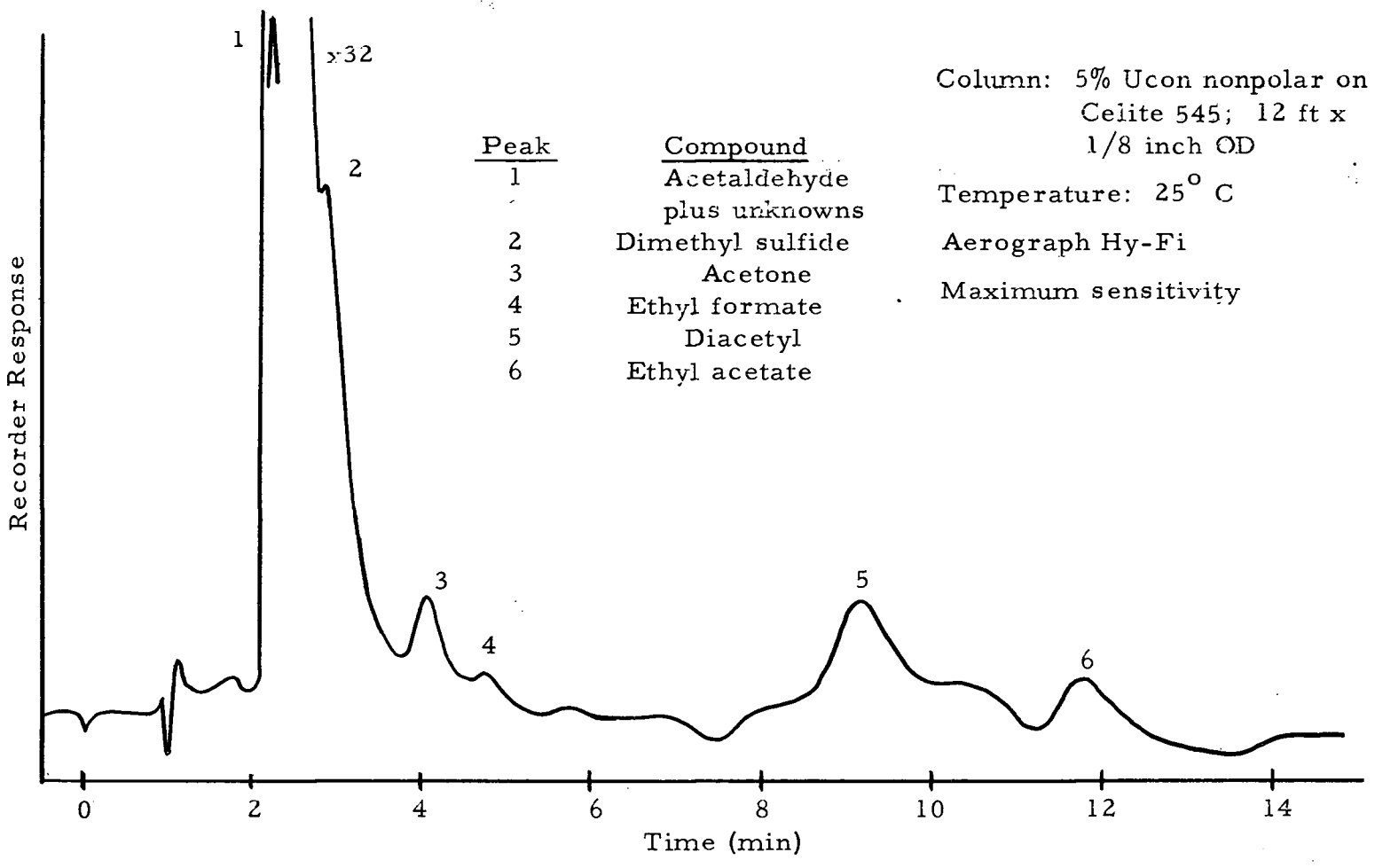


Figure 16. Gas chromatogram of one ml of headspace from high quality butter culture.

to eliminate from resulting chromatograms. Further evidence for dimethyl sulfide was obtained from headspace chromatograms of fresh cultured cream butter (Figure 17) using a packed 5% Ucon nonpolar column operated at 25° C in an Aerograph Hy-Fi operating at maximum sensitivity.

Since butter churns are usually quite well sealed during the churning process, it appeared that the churn headspace would be a good source of butter aroma compounds. After the buttermilk draining operation, nitrogen was swept through the churn into a series of previously described liquid nitrogen cold-traps. Figure 18 is a gas chromatogram showing the separation of a five ml gaseous sample obtained from a cold-trap after sweeping a churning of sweet cream butter with nitrogen for 30 min. A Barber-Colman Model 20 gas chromatograph equipped with a 20 percent Apiezon M column and operated at 70° C was employed. Figure 18 also includes a chromatogram for authentic dimethyl sulfide obtained under the same conditions for comparison. Cultured cream butter was also evaluated in a similar manner. Instead of syringe vapor injections, the collected aroma volatiles were evaluated by the concentrated vapor injection technique described by Libbey et al. (86). Figure 19 shows a gas chromatogram obtained using a DEGS column operated at 70° C. The tentative identifications of the peaks are given in Table 15 and the aroma volatiles indicated were previously observed in culture

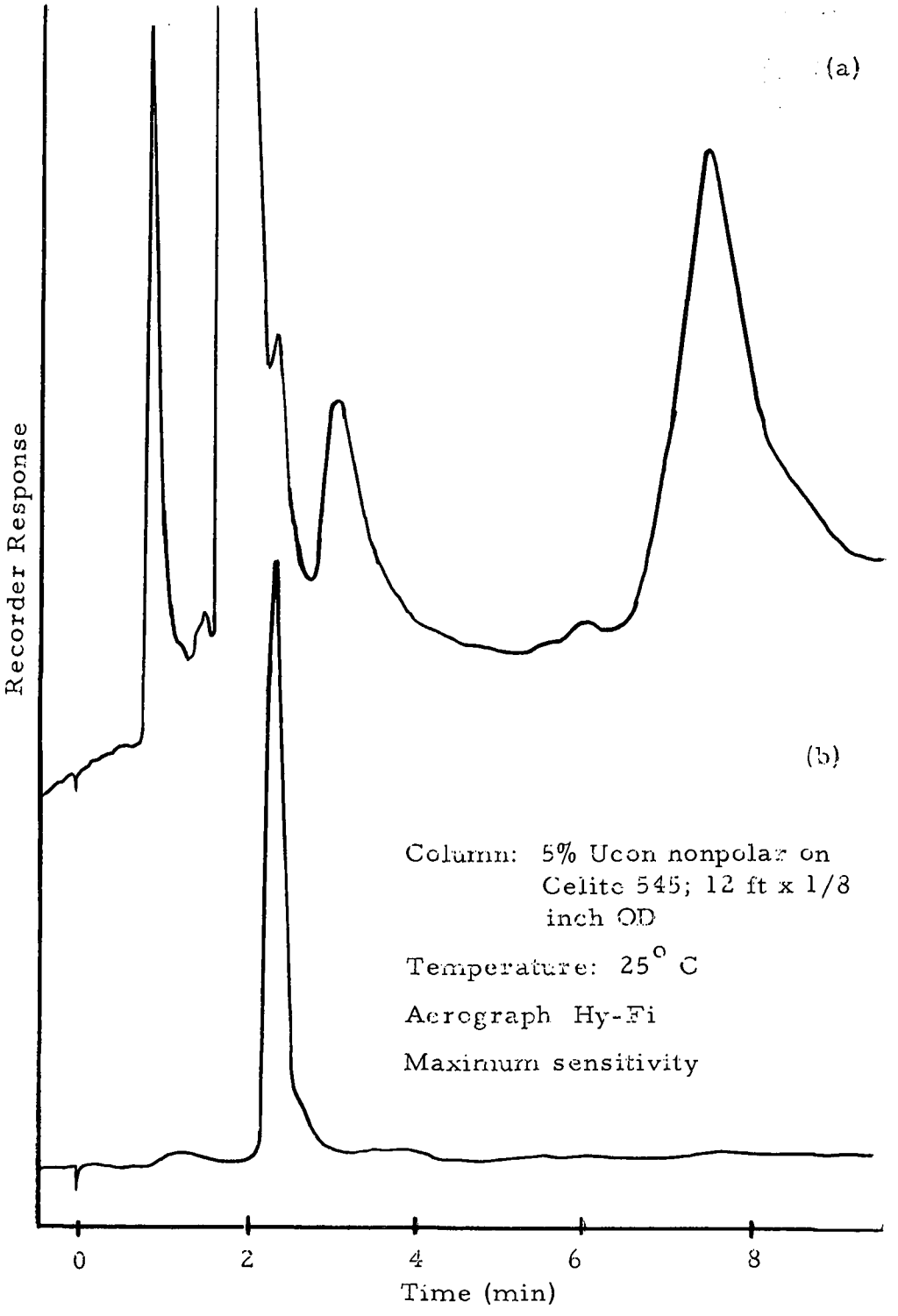


Figure 17. Gas chromatograms of (a) one ml headspace of fresh cultured cream butter, and (b) authentic dimethyl sulfide.

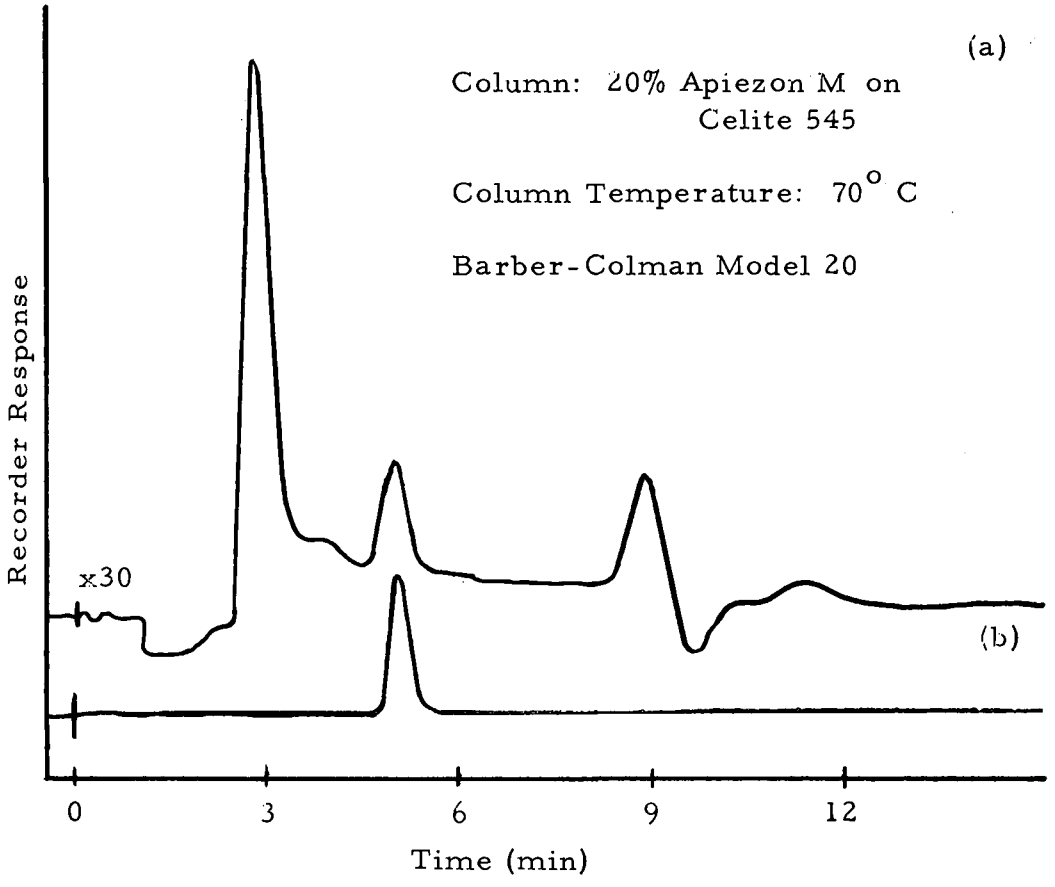


Figure 18. Gas chromatograms of (a) five ml of volatiles cold-trapped from a churn of sweet cream butter, and (b) authentic dimethyl sulfide.

distillates (Table 14).

A portion of the acidic fraction extract of culture distillation D was reacted with 2, 4-dinitrophenylhydrazine. The monocarbonyl derivatives were separated on a hexane-nitromethane liquid partition column (25). Column threshold volumes and the absorption maxima of the derivatives were determined. By comparison of authentic derivatives with the unknowns, tentative identifications for butanone, acetone and acetaldehyde were obtained (Table 16).

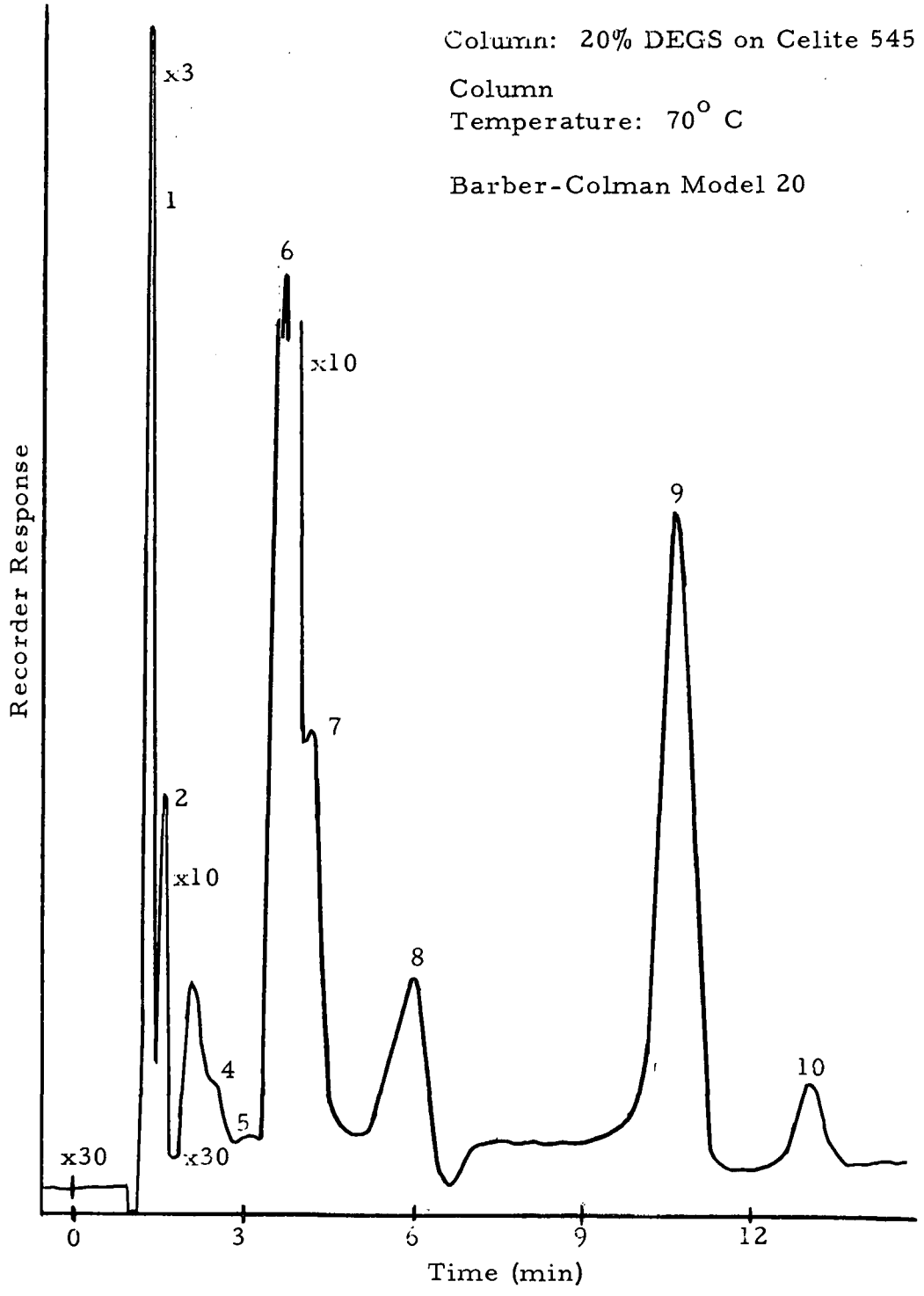


Figure 19. Gas chromatogram of cultured cream volatiles obtained from a churn of cultured cream butter. (See Table 15 for peak identifications).

Table 15. Relative retention times of compounds tentatively identified in cultured butter churn headspace analyzed by vapor injection technique of Libbey et al. using a DEGS column; gas chromatogram shown in Figure 19.

Peak No.	Compound	t_R/t_R[a]	
		Headspace	Authentics
1	Hydrogen sulfide	0.130	0.103
2	Methyl mercaptan	0.154	0.158
3	Acetaldehyde	0.209	0.210
4	Dimethyl sulfide	0.242	0.246
5	-----	0.308	-----
6	Ethyl formate	0.382	0.391
7	Acetone	0.411	0.404
8	Ethanol	0.604	0.610
9	Diacetyl	1.000	1.000
10	2-Hexanone	1.130	1.380

[a] t_R/t_R = relative retention time based on diacetyl = 1.000. Packed column, 11 ft x 1/8 inch OD, 20 percent DEGS on 100-120 mesh alkali-acid treated Celite 545; column temperature: 70° C; Barber-Colman Model 20.

The data for butanone lend support to mass spectral and gas chromatographic identifications of this compound.

In earlier work (culture distillation A), it was found that alpha-acetolactic acid and acetoin yielded three derivatives with 2, 4-dinitrophenylhydrazine (DNP): diacetyl bis-(DNP-hydrazone), diacetyl DNP-hydrazone and acetoin DNP-hydrazone (87). However, further studies showed that a fourth compound was produced in the reaction mixtures and it was subsequently identified as 2, 4-dinitroaniline (88), This compound apparently was produced in the reaction mixture by a reduction of excess 2, 4-dinitrophenylhydrazine with a corresponding oxidation of the alpha-hydroxyl group in the acetoin DNP-hydrazone to form a free carbonyl group (88). Properties of the four authentic derivatives are given in Table 17. Infrared spectra are also useful for distinguishing the derivatives provided special care is taken in purification and drying of materials. To circumvent interfering OH stretch due to water, the purified derivatives were dried at 60° C at pressure of 30 mm of Hg for 24 hr prior to analysis. The KBr used for making the pellets for analysis was ground for one min in a stainless steel capsule and then dried 24 hr at 360° C. All samples were handled rapidly immediately prior to analysis to prevent adsorption of excessive moisture.

The distinguishing characteristics for the infrared spectra for the derivatives are shown in Figure 20. Spectrum (a) for diacetyl

Table 16. Tentative identification of 2, 4-dinitrophylhydrazones of monocarbonyl compounds isolated from butter culture distillate.

Band	Probable 2, 4-DNP-hydrazone	Column Chromatographic Threshold Volume (ml) Unknown	Authentic[a]	λMax in $CHCl_3$ (mμ) Unknown	Known	Paper R_f Agreement[b]
1	C_7 and/or longer chain methyl ketones	Forerun	----	363	363	yes
2	Butanone	16.3	17.9	363	363	yes
3	Acetone	34.0	30.3	363	363	yes
4	Acetaldehyde	50.3	47.5	357	356	yes

[a] Method B, hexane-nitromethane partition column, Day et al. (25),
[b] Check for purity of carbonyl classes using petroleum ether paper chromatographic method of Gaddis and Ellis (42); suspected authentic compounds were spotted on respective paper strips and R_f values compared.

Table 17. Properties of some 2, 4-dinitrophenylhydrazine derivatives.

2, 4-DNP Hydrazone	Melting Point (°C)	λMax in $CHCl_3$	λMax Alcoholic KOH	Color in Alcoholic KOH
Bis-diacetyl	318d	430, 394	540	Purple
Diacetyl	175	355	500	Reddish pink
Acetoin	114	357	500	Red
2, 4-dinitroaniline[a]	178-9	327, 259	510, 332	Bright pink

[a] Soluble in water.

bis-(DNP hydrazone) shows an absence of significant OH or NH stretch and C=O stretch. Diacetyl DNP-hydrazone (Figure 20b) shows the absence of significant OH or NH stretch, but shows a relatively intense C=O stretch band near 1685 cm^{-1}. Acetoin DNP-hydrazone (Figure 20c) shows an intense band near 3380 cm^{-1}, but does not show a C=O stretch band. The band near 3380 cm^{-1} for acetoin DNP-hydrazone is a rather broad band, which is to be expected where hydrogen bonding through a hydroxyl group can be effected. The infrared spectrum for 2, 4-dinitroaniline (Figure 20d) shows two sharp absorption bands near 3333 cm^{-1} and 3460 cm^{-1}. The two bands are characteristic of the NH stretch resulting from the two NH bonds in the free amino group.

Most of the compounds either positively or tentatively identified in the culture and heated milk distillates have been previously observed in the volatile flavor fractions isolated from the different dairy products. The methyl and ethyl esters of the shorter chain fatty acids observed in the distillates are to be expected in view of the potential availability of both free acids and alcohols. The presence of methyl and ethyl alcohols in control heated milk would be necessary for the formation of esters, but the source of these compounds in heated milk has not been reported. Morgan et al. (101) have isolated 2-furfural from high-heat treated skimmilk. These workers have suggested that 2-furfural, which is a decomposition

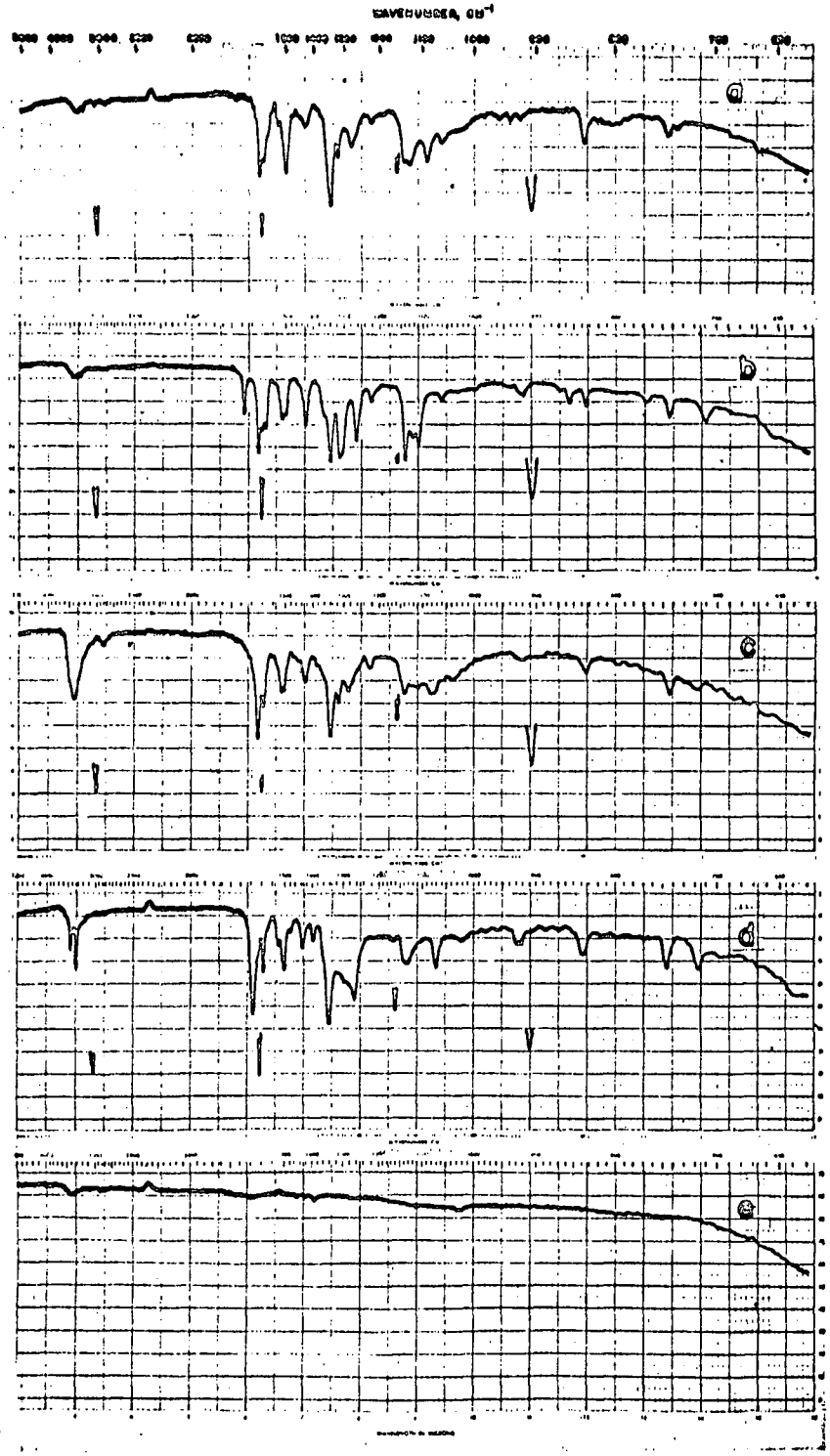


Figure 20. Infrared spectra of (a) diacetyl bis-(DNP-hydrazone), (b) diacetyl DNP-hydrazone, (c) acetoin DNP-hydrazone, (d) 2,4-dinitroaniline, (e) blank KBr pellet.

product of lactose, is an intermediate in the formation of 2-furfurol. They also isolated acetaldehyde from the heated skimmilk and Chou (18, p. 126) has reported the tentative identification of formaldehyde in buttermilk. It is possible that a reduction of these compounds in a manner similar to that described for 2-furfural in heated milk leads to the production of small quantities of the corresponding alcohols which would be sufficient for esterification. The formation of formic and acetic acids in heated skimmilk has been reported by Morr et al. (102).

The odd-numbered methyl ketones observed in both the control heated milk and culture distillates can be explained by heat induced glyceride hydrolysis which yields even-numbered beta-ketoacids and subsequent decarboxylation giving the corresponding methyl ketones (110). The origin of butanone in the distillates is obscure. Langler and Day (83) have observed the production of butanone during the heating of milk fat. However, its modes of formation through heating is not known. Scarpellino and Kosikowski (131) have proposed that butanone isolated from cheddar cheese originated from culture produced acetoin. Their proposed mechanism suggests that acetoin is first reduced to 2, 3-butanediol, then dehydrated to give butanone. Furthermore, butanone could then be reduced to 2-butanol, thus accounting for its presence. Delta-octalactone and delta-decalactone presumably are formed from the corresponding delta-hydroxyacids

in the same manner as that described by Boldingh and Taylor (14). Jenness and Patton (69, p. 354) state that hydrogen sulfide is produced by heat degradation of beta-lactoglobulin in milk, and thus accounts for its presence in the fresh culture and heated milk distillates.

Dimethyl sulfide was not identified in this study by mass spectral data, because it was probably lost during manipulation. However, its tentative identification using several gas chromatographic procedures and columns (Tables 14 and 15, and Figures 16, 17 and 18) give almost conclusive evidence for its presence in butter culture and butter. In addition, Patton et al. (119) have isolated and identified dimethyl sulfide from fresh whole milk and Day et al. (29) have verified its presence in sweet cream butter by the use of far ultraviolet spectroscopy. It is not known that dimethyl sulfide is produced by culture metabolism. Day et al. (29) have discussed possible precursors of dimethyl sulfide which are found in plant materials. These are dimethyl-beta-propiothetin and methylmethionine-sulfonium salts. In addition, methionine could serve as a possible precursor substance.

The production of acetaldehyde by butter cultures containing S. diacetilactis and cultures of S. lactis and S. cremoris has been reported by Badings and Galesloot (6, vol. B, p. 199-208). Its production by single-strains of similar organisms was also observed by

Harvey (59) and apparently is a result of the decarboxylation of pyruvate. In the current study acetaldehyde was by far the most abundant volatile aldehyde observed in cultures. The production of diacetyl, ethanol, acetoin and acetic acid by butter culture organisms is well documented (55). Compounds such as 3-methylbutanal, 2-methylbutanal and methylpropanal have been shown to be produced from amino acids by enzyme-catalyzed transamination and decarboxylation reactions (91). These compounds could also originate through Strecker degradation of amino acids during the heating of the milk medium.

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

Quantitative Determination of Important Flavor Compounds in Butter Culture and Cultured Butter

Comparison of the qualitative data on the composition of the heated milk and culture volatiles indicated that the characteristic

butter culture flavor was only partially due to the presence of certain culture produced flavor components. It appeared that the quantitative relationships of various compounds were equally important in establishing a desirable culture flavor. For these reasons, analyses of butter cultures and cultured cream butters were carried out to determine naturally existing levels of key flavor compounds.

It was necessary to modify some of the more commonly used culturing techniques to obtain cultures with high flavor producing characteristics. The addition of sodium citrate (0.2 percent) to the culture medium was employed for all cultures to insure adequate precursor for diacetyl synthesis. In addition, raw whole milk (approximately 3.8 percent milk fat), containing the added sodium citrate, was heated for one hr in a boiling water bath and immediately cooled to 5° C. The titratable acidity of ripened mother cultures intended for use in preparing high quality products was controlled between 0.68-0.75 percent by varying the incubation time and amount of inoculum. High quality butter cultures for evaluation were allowed to ripen to about 0.90 percent titratable acidity before cooling to 5° C and subsequent storage for 24 hr before evaluation. Cultured cream butters were prepared according to the manufacturing process previously outlined.

Table 18 gives the data obtained for the analysis of eight mixed-strain butter cultures for short chain organic acids, diacetyl and pH.

Table 18. The pH, short chain organic acid and diacetyl content of butter cultures used for manufacture of cultured cream butter.

Sample Number	Acetic Acid	Formic Acid	Lactic Acid	Diacetyl[b] ($\times 10^{-3}$)	pH
1	1.33[a]	0.20	8.80	3.68	4.70
2	1.48	0.29	9.03	1.20	4.60
3	1.16	0.16	8.50	0.87	4.50
4	1.27	0.18	8.27	1.58	4.60
5	1.12	0.17	7.14	2.51	4.90
6	1.09	0.13	7.68	1.89	4.50
7	1.11	0.11	7.28	2.78	4.90
8	1.23	0.14	7.21	1.58	4.45
Average	1.22	0.17	7.99	2.01	4.64

[a] Expressed as g per kg culture for diacetyl, and acetic, formic and lactic acids.

[b] Method of Elliker (37).

It can be seen that the diacetyl content varied considerably (0. 87 to 3. 68 p. p. m.). The short chain acids were separated and measured by a modification of the column procedure of Wiseman and Irvin (30). The acetic acid concentration was found to vary between 1. 09 to 1. 48 g per kg with an average of 1. 22 g per kg. The lactic acid content of the cultures varied considerably, but there does not appear to be a close correlation between pH and measured lactic acid. The amount of formic acid found in the cultures showed a range of 0. 11 to 0. 29 g per kg. It is of interest to note that Morr et al. (102) found that skimmilk containing 0. 5 percent added disodium phosphate and heated for one hr at 100° C contained an average of 0. 10 g formic acid per kg. The average concentration of formic acid found in butter cultures in this study was 0. 17 g per kg. Although no trials were conducted to determine the amount of formic acid in the heated milk medium containing 0. 2 percent added sodium citrate, it may be speculated that at least part of the formic acid present in butter cultures results from the heat treatment. Formic acid has been reported to be an end product of lactose and glucose metabolism in non-milk media by S. cremoris, S. lactis and L. dextranicum (41, 124).

Table 19 shows the corrected data for the analysis of butters for short chain acids. The acids in the serum of the butter samples were determined by the modified method of Wiseman and Ervin (30). Corrections were based on experimentally determined percentages

Table 19. Short chain organic acid and diacetyl content of cultured butters and pH of corresponding butter serum; butters were manufactured using cultures listed in Table 18.

Sample Number	Acetic Acid[a]	Formic Acid	Lactic Acid	Diacetyl[b] ($\times 10^{-3}$)	pH of Butter Serum
1	0.08	0.004	0.75	3.22	5.40
2	0.07	0.006	0.53	2.71	5.10
3	0.02	0.003	0.42	2.84	5.50
4	0.02	0.004	0.61	3.42	4.90
5	0.03	0.002	0.50	1.82	5.15
6	0.01	0.002	0.70	2.13	5.20
7	0.01	0.001	0.37	2.04	5.31
8	0.01	0.002	0.49	2.34	5.15
Average	0.03	0.003	0.54	2.56	5.21

[a] Expressed as grams per kilogram butter for diacetyl, and acetic, formic and lactic acids.

[b] Method of Elliker (37).

of acids partitioned between a previously described milk fat-water system. The percentage of acids partitioned into the aqueous phase were: lactic, 100; formic, 91; and acetic, 86. It can be seen by comparing Table 18 and Table 19 that the concentrations of short chain acids are much less in the butters than in the corresponding cultures. The average concentration of acetic acid was found to be 0.03 g per kg of butter, but there was a large range of concentrations represented (0.01 to 0.08 g per kg). Formic acid was present on an average to an extent of only 0.003 g per kg. Lactic acid also showed considerable variation (0.37 to 0.75 g per kg), and the average concentration was found to be 0.54 g per kg of cultured cream butter. Hammer and Sherwood (57) and Knudsen (82) have reported that small quantities of propionic acid were formed in good butter cultures. However, in the current investigation, no evidence for propionic acid was obtained. It can also be seen that the diacetyl concentration in a culture does not necessarily reflect the amount of diacetyl that will be present in the resulting cultured butter. For example, note Sample 3 in Tables 18 and 19 where it can be seen that the diacetyl content of the resulting butter was much higher (2.84 p. p. m.) than in the culture (0.87 p. p. m.) from which it was prepared. The reverse was true for Sample 5 where the culture contained 2.51 p. p. m. of diacetyl and the resulting butter contained 1.82 p. p. m. of diacetyl. Apparently, factors encountered in the manufacturing process and the

characteristics of the culture organisms are responsible for the variations. The amount of diacetyl precursor, alpha-acetolactic acid, and diacetyl reductase produced by the culture organisms are probably involved. All of the cultures included in Table 18 were considered of high quality. In this respect it can be seen that the pH of the cultures varied between 4.45 and 4.90. The serum from the corresponding butters showed pH values ranging from 4.90 to 5.50 (Table 19).

Sixteen cultured cream butters were evaluated by an eight- to ten-member experienced flavor panel in an effort to estimate the optimum level of diacetyl in desirable cultured butters. The scoring ballot used was a seven-point hedonic scale type where 7.0 was very desirable and 1.0 was very undesirable. A maximum of seven samples was displayed per evaluation period and three replications for each sample were carried out. The judges were allowed to compare samples. This judging procedure did not give real flavor scores on samples, yet it magnified differences which is essential when attempting to chemically define a flavor. The chemically determined diacetyl concentrations and the average overall flavor scores for the butters are given in Table 20. Although a large number of samples were not available for evaluation, it can be seen that butters containing from 1.82 to 3.42 p.p.m. of diacetyl were usually scored above 5.0. It is significant to note that samples containing less than

Table 20. Relationship of diacetyl content to overall flavor score of cultured cream butter.

Sample Number	Diacetyl [c] (p. p. m.)	Average Overall Flavor Score [a]
1[b]	0.05	4.06
2	0.16	2.71
3	0.50	4.36
4	0.72	4.72
5	1.82	5.50
6	2.04	5.25
7	2.13	5.63
8	2.14	5.72
9	2.32	5.00
10	2.35	5.07
11	2.62	5.45
12	2.71	5.31
13	2.75	5.15
14	2.84	5.14
15	3.22	4.69
16	3.42	5.72

[a] From a seven-point hedonic scale where 7.0 = very desirable and 1.0 = very undesirable.

[b] Experimental sweet cream butter.

[c] Method of Elliker (37).

1.0 p. p. m. of diacetyl scored lower in the rating system. A graphic plot of the concentration of diacetyl versus the overall flavor score indicated that there was a trend towards a maximum in the overall flavor score for butters containing 2.0 to 2.5 p. p. m. of diacetyl. The lack of sufficient numbers of representative samples precluded statistical analysis of the data. In view of previous reports by Babel and Hammer (5), Davies (23) and Riel and Gibson (127), the desirability of butters containing more than 2.0 p. p. m. of diacetyl would appear questionable. However, the butters prepared and evaluated in the present investigation were highly acceptable to the flavor judges. It was also noted informally that excellent butter cultures often contained at least 2.0 p. p. m. of diacetyl. The proper balance of other flavor compounds is undoubtedly very important in determining a desirable level of diacetyl for butter cultures and cultured cream butters.

Since standard sensitive chemical or instrumental methods for the quantitation of dimethyl sulfide were not available, taste panel evaluations were used to determine desirable levels of dimethyl sulfide in butter oil. These determinations were designed to give an approximation of levels of dimethyl sulfide which could be applied to butter and butter culture flavor formulations.

The flavor threshold was determined in a butter oil that had been stripped of volatiles by vacuum steam distillation at 40° C. Stock

solutions were prepared as previously described. A range of dilutions prepared from the stock solution was presented to a nine-member panel in 3/4-ounce cups. The panel members were asked to indicate the sample in which dimethyl sulfide could be detected and the sample they preferred. The data obtained from the trials are presented in Table 21. The average flavor threshold, at the 50 percent level, was 24 p. p. b. and the panel preferred a concentration of 40 p. p. b. in the oil. Patton et al. (119) have determined the flavor threshold for dimethyl sulfide in water using similar methods and found it to be 12 p. p. b. Day et al. (29) have reported that experiments conducted in Australia revealed that Federal Butter Graders found 10 to 20 p. p. b. of dimethyl sulfide the optimum addition to salted sweet cream butter. It was suggested that the 10 to 20 p. p. b. of added dimethyl sulfide were probably superimposed on some naturally occuring dimethyl sulfide and thus, the actual absolute preferred concentration would be elevated. A limited number of dimethyl sulfide flavor threshold trials were conducted in the current investigation using the same bland butter oil containing 2. 5 p. p. m. of added diacetyl. In these trials it was found that average flavor threshold, at the 50 percent level, was approximately 50 p. p. b. of dimethyl sulfide. From these results it is apparent that added diacetyl flavor blends with the dimethyl sulfide and as a result the flavor threshold for dimethyl sulfide is approximately doubled.

Table 21. Determination of dimethyl sulfide flavor threshold at the 50 percent level and preferred level in bland butteroil.

Concentration (p. p. b.)	Observations[a]	Percent Positive Response	Percent Preference[b]
0	45	13.3	14.25
10	36	25.0	20.00
25	45	53.3	0.29
40	63	63.5	31.40
60	27	77.7	0.86
75	35	82.8	14.30
100	18	94.4	0.57

[a] Different combinations of dilutions were presented to the panel during trials.

[b] Total of 35 judgements by panel members; higher percent preferences at lower and higher dilutions were influenced by individuals who extremely liked or disliked dimethyl sulfide.

Volatile esters, such as ethyl acetate and ethyl butyrate, have long been considered to contribute to the flavor of butter cultures and cultured cream butter (5, 55). In view of the presence of the large number of esters noted in the culture distillates of this study (Table 14), this point was investigated further. A spectrophotometric method was developed in an attempt to quantitatively determine the volatile ester content of butter culture. The method was based on the reaction of esters with alkaline hydroxylamine to form hydroxamic acid derivaties which yield red-colored complexes under acid conditions in the presence of ferric chloride. The collection system utilized was the same as that described by Pack et al. (107) for diacetyl and the reaction conditions were derived from the works of Bassette and Keeney (7), Goddu et al. (47) and Mattick and Robinson (96). The volatile ester content of four high quality butter cultures are given in Table 22. As can be seen in the table, less than 1.0 p.p.m. of esters were found. The recovery of added ethyl acetate was found to be 88 percent from acidified milk (pH 4.60). Goddu et al. (47) have reported that the molar absorptivity of the ferric-hydroxamate complexes of short chain aliphatic esters is near 1,000. The calculated molar absorptivity for ethyl acetate in the current study was near 800. Thus, the determination is not highly sensitive. In addition, many of the longer chain esters found in the culture distillates (Table 14) would not be expected to volatilize and be

collected in the system employed. However, it is of significance to note that the flavor threshold of ethyl acetate in water was found in this investigation to vary between 5.0 and 10.0 p.p.m. at the 50 percent level. Therefore, it appears safe to assume that short chain esters, such as ethyl formate and ethyl acetate, do not play a major role in culture flavor. However, this observation does not preclude the possibility that some esters may contribute to the overall flavor balance of high quality butter cultures and cultured cream butter.

Acetaldehyde has been reported to cause the green flavor defect of butter cultures (6, vol. B, p. 199-208), and it was also observed in this investigation to comprise the major portion of the aldehyde fraction of butter culture distillates. Harvey (59) has described a 2, 4-dinitrophenylhydrazone spectrophotometric procedure for determining acetaldehyde in lactic cultures. However, the analysis was very time consuming and difficult to perform. In view of this, it appeared that the 3-methyl-2-benzothiazolone hydrazone method (130) for acetaldehyde measurement could be employed in conjunction with the recovery system described by Pack et al. (107) for rapid diacetyl determination in lactic cultures. The modified procedure has been described earlier in the Experimental chapter.

In the development of the acetaldehyde procedure, it was found that the ferric chloride oxidation time was very critical in obtaining reproducible data. In the original procedure of Sawicki et al. (130),

Table 22. Volatile ester content of butter cultures as determined by the modified hydroxamate procedure.

Sample	Culture Sample Size (g)	Absorbance at 525 mμ	Volatile Esters[a] (p. p. m.)
1	40	0.010	0.22
2	40	0.030	0.62
3	20	0.008	0.34
4	20	0.006	0.23

[a] Expressed as ethyl acetate.

the oxidation time was five min after addition of ferric chloride whereas in the present study absorbance values on duplicate samples were not reproducible until after a 25 min oxidation time (Table 23). Furthermore, termination of the reaction after 25 min by the immediate addition of acetone is important. The absorption spectrum for the acetaldehyde-dye complex exhibits a distinct maximum at 666 mμ and a shoulder at 635 mμ. Sawicki et al. (130) reported a molar absorptivity of 51, 000 for the acetaldehyde-dye complex, whereas a value of 69, 000 was obtained by the procedure described herein. The higher sensitivity probably is due to the longer oxidation time employed in the modified procedure.

The acetaldehyde-dye complex obeys Beer's Law over the concentration range employed. Since it is not possible to dilute successfully the final dye complex to obtain absorbance readings (Table 24, Trials 13 and 14), dilution of cultures in which acetaldehyde concentrations exceed 2. 5 p. p. m. are necessary. With experience, one can ascertain where dilutions are required simply by tasting or smelling the culture.

The average percent recovery of added acetaldehyde from eight samples of heated milk medium (acidified to pH 4. 5) was 80 percent. Heating the milk medium for one hr in a boiling water bath produced small amounts of acetaldehyde (0. 06 to 0. 15 p. p. m., Table 25). The recovery from distilled water was only slightly greater than

Table 23. Effect of ferric chloride oxidation time on sensitivity and reproducibility of determinations for 0. 02 mg acetaldehyde.

Oxidation Time (min)	Absorbance at 666 mμ [a]
5	0. 405
	0. 310
10	0. 560
	0. 605
15	0. 624
	0. 575
25	0. 630 [b]
	0. 630

[a] All samples were read against appropriate reagent blank.

[b] Obtained using final modified procedure.

Table 24. The effect of various conditions and reagents on the modified 3-methyl-2-benzothiazolone hydrazone aldehyde determination.

Trial No.	Sample	Variable	Absorbance[a] at 666 mμ
1[b]	20 g distilled water	nitrogen and water	0.000
2	20 g distilled water	antifoam agent	0.000
3	20 g of 20 percent aqueous phosphoric acid	phosphoric acid	0.000
4	20 g of 20 percent aqueous lactic acid	commercial lactic acid	too dense to read
5	20 g pH 4.5 milk plus 0.01 mg acetaldehyde	one hour nitrogen purging	0.246
6	20 g pH 4.5 milk plus 0.01 mg acetaldehyde	1.5 hour nitrogen purging	0.248
7[c]	0.01 mg acetaldehyde direct to reagent	dioxane replacing dimethyl sulfoxide	too dense to read
8	0.01 mg acetaldehyde direct to reagent	unmodified reagent	0.303
9	0.01 mg acetaldehyde direct to reagent	modified reagent containing dimethyl sulfoxide	0.308
10	0.24 mg diacetyl direct to reagent	high diacetyl content	0.000
11	200 mg ethanol direct to reagent	high ethanol content	0.063
12	200 mg methanol direct to reagent	high methanol content	0.054
13	0.02 mg acetaldehyde direct to reagent	----------	0.625
14	0.02 mg acetaldehyde direct to reagent	final dye solution (Trial 13) diluted 1:1 with acetone	0.015

[a] Average of two duplicates read against appropriate reagent blank.

[b] Trials 1-6 utilized nitrogen-purging at 100-125 ml per min.

[c] Trials 7-14 were direct additions to the collecting reagent.

that from the acidified milk medium.

The influence of a number of variables on the validity of the test are summarized in Table 24. Nitrogen used as the purging gas, antifoam agent and phosphoric acid did not contribute to the color reaction. Commercial lactic acid contained large quantities of aldehyde as shown in Trial four and thus could not be used for adjusting the pH of control milk samples. Phosphoric acid should be used for this purpose. As indicated in Trials five and six there appeared to be no advantage in purging the sample longer than one hr.

The presence of diacetyl in samples, which is transferred to the collection tube by the purging treatment, results in the formation of a water-insoluble hydrazone. The precipitate formed caused difficulties in the quantitative transfer of the reaction mixture from the collection tube to the volumetric flask. Initial efforts to overcome the problem involved addition of 0. 5 ml of dioxane to the collection reagent. The dioxane was not satisfactory, however, as evidenced by the gross contamination with acetaldehyde or other reactants (Table 24, Trial seven). The addition of 0. 5 ml of dimethyl sulfoxide to the collection reagent prevented formation of the diacetyl derivative precipitate and had no adverse effect on the absorbance values (see Trials eight and nine). Under these conditions, a large excess of diacetyl did not interfere with the acetaldehyde determination (Trial ten). Additional studies showed that sufficient reagent

Table 25. Analyses of fresh raw milk and heated milk for acetaldehyde and diacetyl.

	Raw Milk		Heated Milk [a]	
Trial	Acetaldehyde (p. p. m.)	Diacetyl (p. p. m.)	Acetaldehyde (p. p. m.)	Diacetyl (p. p. m.)
1	0. 002	0. 000	0. 150	0. 000
2	0. 004	0. 000	0. 115	0. 000
3	0. 002	0. 000	0. 065	0. 000
4	0. 012	0. 000	0. 080	0. 000
5	0. 002	0. 000	0. 060	0. 000

[a] Raw whole milk heated for 1. 0 hr in a boiling water bath.

was available to react with the equivalent of ten p. p. m. of acetaldehyde in the presence of ten p. p. m. of diacetyl. Hence, the excess reagent should be adequate for most applications.

It has been reported that dimethyl sulfoxide will mediate the oxidation of certain alcohols to aldehydes (149). Since earlier work had demonstrated the presence of both methanol and ethanol in culture distillates, 200 mg of each alcohol was added directly to separate tubes of collection reagent. Trials 11 and 12 in Table 24 show that the high concentrations of alcohols gave only small absorbance values and aldehyde contaminants in the commercial solvents were probably responsible.

Table 26 shows the results of analyses of cultures for diacetyl and acetaldehyde. The flavor, aroma and pH of each culture also are listed. All single-strain lactic streptococci cultures contained concentrations of acetaldehyde that were well above the taste threshold of 0. 4 p. p. m. reported by Harvey (59). The L. citrovorum 91404 culture contained less acetaldehyde than did the control milk. S. lactis C_2F and S. cremoris SC_1 produced negligible amounts of diacetyl, but the S. lactis var. maltigenes strains showed production of up to 0. 5 p. p. m. of diacetyl. This is consistent with the observations of Gordon et al. (48) that many strains of S. lactis var. maltigenes gave positive Voges-Proskauer reactions.

As indicated in Table 26, all S. lactis var. maltigenes cultures

Table 26. Diacetyl and acetaldehyde content of lactic cultures in whole milk medium.

Culture		pH	Diacetyl[c] (p. p. m.)	Acetaldehyde[a] (p. p. m.)	Flavor and Aroma
S. lactis C_2F		4.40	0.10	2.84	green
S. lactis var. maltigenes	M1	4.20	0.40	3.24	malty
	M3	4.40	0.30	3.04	malty
	M4	4.30	0.45	2.60	malty
	M6	4.40	0.55	3.00	malty
S. cremoris SC_1		4.60	0.15	2.34	green
S. diacetilactis 18-16		4.90	2.80	2.50	diacetyl, green
L. citrovorum 91404[b]		4.50	3.24	0.08	diacetyl, not full
Control heated milk[b]		4.50	0.00	0.15	strong cooked

[a] Volatile aldehydes for all strains expressed as acetaldehyde.

[b] Incubated at 30° C for 12 hr, then acidified with sterile 20 percent phosphoric acid to pH 4.5 and incubated an additional 6 hr at 30° C.

[c] Method of Pack et al. (107).

gave a malty flavor. Zuraw and Morgan (168) have reported that malty strains of S. lactis liberate acetaldehye, and later work by Jackson and Morgan (68) implicated 3-methylbutanal as the malty flavor component. Subsequent work by MacLeod and Morgan (92) revealed that these organisms are capable of producing 3-methylbutanal, 2-methylbutanal, methylpropanal, 3-methylthiopropanal and phenylacetaldehyde. Hence, the data for the malty cultures in Table 26 undoubtedly include a partial measurement of some of the aforementioned aldehydes. Sawicki et al. (130) reported that the molar absorptivity of the methylpropanal dye complex determined at its wavelength maximum of 664 mμ was only about 1/3 of that found for acetaldehyde. Other branched-chain aldehydes, such as 3-methylbutanal appear to behave similarly. Thus, their contribution to the absorbance of the dye complex would not be equivalent to acetaldehyde.

The method is reasonably fast in that a number of samples can be analyzed at the same time. Adaption of the method for use with the same collection system employed for diacetyl measurements (107) allows simultaneous measurements of culture aliquots for both flavor compounds. Results obtained were accurate and reproducible. For duplicate analyses on 26 different cultures, the average deviation of each duplicate from its mean was $\pm$ 0.18 μg of acetaldehyde per sample with a deviation range of $\pm$ 0.00 to $\pm$ 0.50 μg per sample.

The method is extremely sensitive for acetaldehyde with the dye complex giving a molar absorptivity of 69, 000 as compared to a molar absorptivity of approximately 20, 000 (72) for the corresponding 2, 4-dinitrophenylhydrazone.

Diacetyl and Acetaldehyde Production and Utilization in Lactic Cultures

Under certain ill-defined conditions, butter cultures develop a flavor defect that is described as green or yogurt-like. Badings and Galesloot (6, vol. B, p. 199-208) have shown that culture produced acetaldehyde is responsible for the defect. These same workers have observed that L. citrovorum in some manner transforms acetaldehyde produced by lactic streptococci in mixed-strain butter cultures. During this investigation of the flavor chemistry of butter cultures, it was necessary to utilize cultures of optimum flavor as a source of flavor isolates. By far the most serious problem encountered was the development of a green flavor. Since a procedure for rapid quantitative determination of acetaldehyde was developed, it was possible to study the nature of the defect and methods for overcoming it.

Figure 21 shows the comparison of two gas chromatograms obtained for one ml of headspace from a green flavored culture and an excellent flavored butter culture. Peak three in the chromatograms

represents acetaldehyde. It can be seen in the figure that acetaldehyde is present in greater quantity in the green flavored butter culture. However, of greater significance is the fact that acetaldehyde is still present in the excellent flavored butter culture. Badings and Galesloot (6, vol. B, p. 199-208) have reported gas chromatograms similar to Figure 21a for green flavored butter cultures. However, their data for butter cultures lacking a green flavor showed the absence of any significant amounts of acetaldehyde. This was attributed to the removal of acetaldehyde by high numbers of L. citrovorum in the cultures. In this respect, it was noted in the present investigation that pure cultures of L. citrovorum prepared in a manner similar to that described by Mather and Babel (95) often exhibited a harsh diacetyl-type flavor.

Table 27 relates the effect of the ratio of diacetyl to acetaldehyde on the types of flavor developed by the cultures. Further data also are presented in Tables 38 and 39 in the Appendix. Absolute quantities of the two compounds determine the intensity, but the relative amounts which affect the flavor balance are of greater importance. Cultures containing diacetyl to acetaldehyde ratios of 13:1 to 5.5:1 had flavors described as harsh or lacking the full, balanced flavor. Cultures exhibiting diacetyl to acetaldehyde ratios of 4.4:1 to 3.2:1 had desirable flavors. A green flavor was apparent when the ratio dropped below 3.2:1. The preceding data suggest that a small

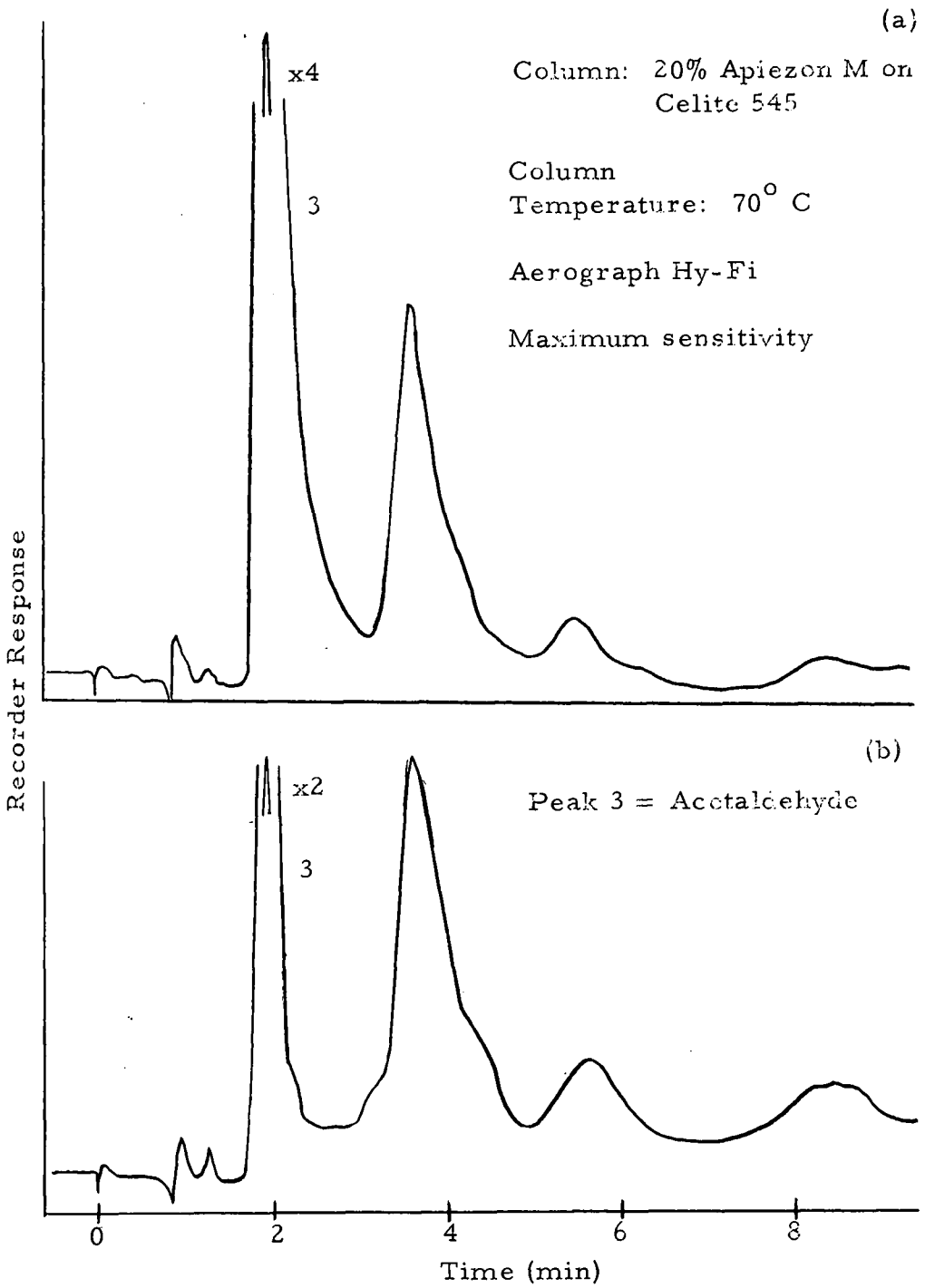


Figure 21. Gas chromatograms of (a) one ml of headspace of green flavored butter culture, and (b) one ml of headspace of an excellent flavored butter culture.

amount of acetaldehyde is necessary in the desired full flavored culture and excessive amounts give rise to the green flavor defect.

Results of initial studies on acetaldehyde utilization by L. citrovorum 91404 are shown in Table 28. All samples were incubated at 30° C for 18 hr before acetaldehyde was added and all samples were subjected to the additional six hr incubation before diacetyl and acetaldehyde were determined. The stability of acetaldehyde in acidified heated milk (Treatment two) eliminated the possibility that acetaldehyde removal was due to a reaction with the milk proteins (100). Almost complete acetaldehyde removal by the Leuconostoc organisms was apparent in all treatments with the non-acidified sample (Treatment five) showing the greatest reduction.

The rate of acetaldehyde utilization and diacetyl production by L. citrovorum 91404 at 21° C in both acidified and non-acidified cultures is shown in Table 29. Complete acetaldehyde utilization was observed after three hr in the non-acidified culture while the acidified culture showed a reduction of 54 percent in a corresponding time. The acidified culture removed all of the acetaldehyde after nine hr at 21° C. The King's Test (81) for diacetyl and acetoin was performed on the non-acidified culture after three hr of incubation and the results were negative. This indicated that the acetaldehyde was not shunted to acetoin.

Table 27. Ratio of diacetyl to acetaldehyde in mixed-strain butter cultures and its relation to flavor and aroma.

Culture	pH	Diacetyl [a, b] (A) (p. p. m.)	Acetaldehyde (B) (p. p. m.)	Ratio A:B	Flavor and Aroma
1	4. 70	7. 80	0. 60	13. 0:1	harsh
2	4. 65	4. 44	0. 44	10. 0:1	harsh
3	4. 60	1. 01	0. 13	7. 8:1	not full
4	4. 60	3. 33	0. 45	7. 4:1	harsh
5	4. 60	1. 60	0. 25	6. 4:1	not full
6	4. 80	3. 66	0. 66	5. 5:1	harsh
7	4. 60	1. 65	0. 35	4. 7:1	good
8	4. 55	2. 30	0. 52	4. 4:1	good
9	4. 50	2. 44	0. 59	4. 1:1	good
10	4. 60	1. 50	0. 42	3. 6:1	good
11	4. 65	1. 20	0. 38	3. 2:1	good
12	4. 80	2. 40	2. 20	1. 1:1	green
13	4. 70	0. 96	1. 20	0. 8:1	green
14	4. 50	2. 32	3. 88	0. 6:1	green
15	4. 80	0. 80	1. 65	0. 5:1	green
16	4. 70	1. 00	2. 30	0. 4:1	green

[a] Average of duplicate analyses.

[b] Method of Pack et al. (107).

Table 28. Acetaldehyde utilization by an 18 hour L. citrovorum 91404 culture after holding 6 hr at 30° C.

Sample Treatment	Acetaldehyde[a] (p. p. m.)	Diacetyl[a, c] (p. p. m.)
1. Control heated milk medium	0. 15	0. 00
2. Heated milk plus acetaldehyde and adjusted to pH 4. 5[b]	4. 50	0. 00
3. Heated milk plus culture and adjusted to pH 4. 5[b]	0. 17	7. 75
4. Heated milk plus culture plus acetaldehyde and adjusted to pH 4. 5[b]	0. 17	7. 80
5. Heated milk plus culture plus acetaldehyde	0. 05	0. 00

[a] Average of duplicate analyses.

[b] Sterile 20% phosphoric acid used.

[c] Method of Pack et al. (107).

Table 29. Acetaldehyde utilization by L. citrovorum 91404[a] at 21° C.

Time after Acetaldehyde Addition (hr)	Diacetyl[d] (p. p. m.)	Acetaldehyde (p. p. m.)	Percent Utilization of Initial Acetaldehyde
Trial 1 (acidified milk, pH 4.50)[b]			
0.1	0.17	6.60	----
0.5	0.75	6.60	0.0
1.0	1.60	5.50	16.7
3.0	4.00	3.00	54.5
9.0	8.25	0.10	98.5
Trial 2 (non-acidified milk, pH 6.50)			
0.1	0.00	6.65	----
0.5	----	5.00	24.8
1.0	----	3.80	43.0
2.0	----	0.83	87.5
3.0	0.00[c]	0.13	98.0
Heated Control Milk	0.00	0.08	----

[a] Total count of 47.5×10^6 organisms per ml.

[b] Acidified with sterile 20 percent phosphoric acid at the time of acetaldehyde addition.

[c] Also negative to King's test.

[d] Method of Pack et al. (107).

Table 30 shows the utilization of acetaldehyde at 5° C. Both acidified and non-acidified cultures showed approximately the same reduction in acetaldehyde content after 12 hr. Further reduction was not noted in the acidified culture after an additional 12 hr storage period while in the non-acidified culture after an additional 12 hr storage period almost complete removal of acetaldehyde was observed.

In order to determine the effect of added acetaldehyde upon the growth of L. citrovorum 91404, total lactic agar plate counts were made at the initiation of the trial and after incubation periods of three and 18 hr at 21° C. In Table 31, it can be seen that non-acidified cultures containing acetaldehyde showed a trend towards increased cell members after three hr, and after 18 hr the growth stimulation was very marked with almost three times as many organisms present in cultures containing acetaldehyde as in control cultures. Levels of acetaldehyde higher than 5.0 p. p. m. did not appear to increase growth. The growth stimulation effects of acetaldehyde are not as apparent in acidified cultures. This may be explained by the fact that at the low pH of the milk medium the organisms were able to utilize citrate. The corresponding rapid production of diacetyl after acidification is amply demonstrated in Table 30.

Table 30. Acetaldehyde utilization by an 18 hr L. citrovorum 91404 culture at 5° C.

Sample	Storage Time (hr)	pH	Diacetyl [d] (p. p. m.)	Acetaldehyde (p. p. m.)	Flavor and Aroma
L. citrovorum 91404[a] plus acetaldehyde and acid[b]	0	4.60	0.17	6.60	very green
	12	4.50	1.40	4.13	green
	24	4.50	2.10	3.90	green
L. citrovorum 91404 plus acetaldehyde	0	6.50	0.00	6.60	very green
	12	6.50	----	3.92	green
	24	6.50	0.00[c]	0.15	cooked milk
Heated milk control	--	6.50	0.00	0.08	cooked milk

[a] An 18 hr at 30° C culture; total count: 47.5×10^6 organisms per ml.

[b] Acetaldehyde and 20% H_3PO_4 added at time of initiation of experiment.

[c] Negative King's test.

[d] Method of Pack et al. (107).

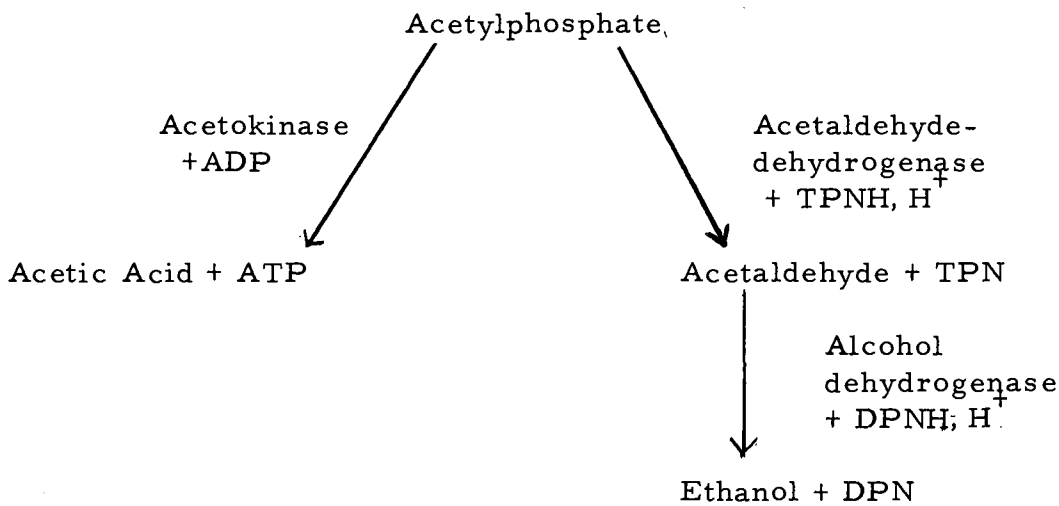
Table 31. The effect of various levels of added acetaldehyde on the growth of L. citrovorum 91404 in whole milk medium.

Incubation Time (hr) [a]	Added Acetaldehyde			
	0 p. p. m.	5 p. p. m.	15 p. p. m.	25 p. p. m.
	(Plate Counts per ml x 10^4)			
Trial 1 (pH 6. 6)				
0	11. 8	9. 1	8. 3	9. 2
3	20. 0	24. 0	23. 0	30. 0
18	360. 0	1160. 0	----[b]	1170. 0
Trial 2 (pH 6. 6)				
0	20. 1	11. 8	14. 3	20. 8
3	42. 0	35. 0[b]	40. 0	33. 0
18	670. 0	----[b]	1790. 0	1830. 0
Trial 3 (pH 4. 5)				
0	22. 9	----	23. 5	----
3	57. 0	----	122. 0	----
18	1420. 0	----	1660. 0	----

[a] Incubation at 21° C after addition of 3% inoculum of an 18 hr culture and appropriate amounts of acetaldehyde and phosphoric acid.

[b] Accurate counts not available.

It may be possible to explain the observed growth stimulation of L. citrovorum by acetaldehyde in the following manner. The terminal compounds of heterofermentative carbohydrate metabolism in addition to D (-)-lactic acid are (73):



Galesloot (43) has pointed out that the reduction of acetylphosphate to ethanol in normal fermentation is a waste of considerable energy, but the Leuconostoc organisms are compelled to do this in order to consume the hydrogen generated by the conversion of hexose phosphate to 6-phosphogluconic acid and the subsequent conversion of the latter compound to carbon dioxide and ribulose-5-phosphate. Leuconostoc organisms tend to avoid the reduction of acetylphosphate to ethanol and prefer to convert the former to acetic acid with the production of one ATP. The addition of acetaldehyde to a culture of Leuconostoc would supply a hydrogen acceptor for the regeneration

of reduced pyridine nucleotides without loss of energy. Although it would be possible for the organisms to form acetylphosphate and acetic acid from free acetaldehyde, it would not appear favorable because of a corresponding formation of more reduced pyridine nucleotide.

Harvey (59) has reported that in a milk medium, strains of S. lactis produced from 0.4 to 4.5 mg of acetaldehyde per liter; S. cremoris strains gave from 0.5 to 9.0 mg/l and a strain of S. diacetilactis produced from 11 to 13 mg/l. The findings in the current investigation indicate that acetaldehyde production and utilization in mixed-strain cultures constitutes a type of associative relationship between culture organisms. Leuconostoc organisms derive a beneficial growth stimulation from acetaldehyde produced by lactic streptococci. This effect would probably be more pronounced in earlier stages of growth, especially when the pH of the medium is above 5.0, and this may prevent a complete dominance by the lactic streptococci. In this respect Glenn and Prouty (46) found that approximately five percent of the total microbial population of a mixed-strain culture was L. citrovorum through the first ten hr of incubation at 22° C in a milk medium. After 16 hr of incubation the numbers of L. citrovorum organisms decreased to about one percent of the population and there was a corresponding marked decrease in the pH of the medium to 4.85. After extended incubation to 25 hr

L. citrovorum again accounted for about four percent of the total population.

The cause of the green flavor defect in mixed-strain butter cultures has been attributed primarily to an overgrowth of S. diacetilactis (43). Vedamuthu et al. (155) observed that the addition of 0.2 percent citrate to skimmilk medium caused a dominance of a strain of S. diacetilactis over strains of either S. lactis or S. cremoris in two-strain cultures. Further work (61) using a culture containing two strains of S. diacetilactis, a strain of S. cremoris and a strain of S. lactis gave similar results. The dominance of S. diacetilactis was attributed to its ability to utilize citrate as an additional carbon source and possibly involved antibiotic production and greater acid tolerance. DeMann and Galesloot (34) have shown that fluctuations in numbers of Leuconostoc organisms may be due, in part, to seasonal variations in the manganese content of milk. These workers were able to prevent variation in numbers of Leuconostoc organisms by the addition of 0.05 p.p.m. of Mn^{++}.

According to Galesloot (43), a small percentage of inoculum, an increased incubation temperature and transfers at low acidity favor a shift in the aroma bacteria population of butter cultures towards S. diacetilactis while the opposite method of propagation favors increases in the number of Leuconostoc organisms. However, it was found in the present study that at least some commer-

commercial mixed-strain butter cultures containing S. diacetilactis require mother culture transfers at low acidities in order to prevent the development of a green flavor and to achieve a desirable culture flavor in the final product. An example of the effects of over-incubation on the flavor of cultures prepared by successive transfers is given in Table 32. The culture represented in the table contained strains of S. lactis, S. cremoris, S. diacetilactis and L. citrovorum and produced a fine-flavored cultured cream butter when handled properly. However, if the acidity of mother culture was allowed to exceed 0. 78 percent in the whole milk medium, the culture gave a green flavor on subsequent transfers. This is illustrated in Table 32 where the first three transfers were controlled so that the acidity did not exceed 0. 76 percent. Under these conditions a clean, mildly green and diacetyl type flavor was observed. The acidity was allowed to reach 0. 96 percent after the fourth transfer and the resulting culture exhibited a desirable flavor. However, in succeeding transfers from the culture after it had reached 0. 96 percent acidity, the flavor was consistently green regardless of the acidity attained and the desirable culture flavor could not be restored.

Since the green flavor has been attributed to an overgrowth of S. diacetilactis, it appeared desirable to compare the numbers of various types of culture organisms present in a green flavored culture with those for a good flavored culture. However, the attempts

Table 32. The effect of ripening to different titratable acidities on the flavor of a commercial mixed-strain butter culture.

Successive Transfers[a]	Percent Inoculum	Incubation at 21°C (hr)	Percent Acidity	Flavor and Aroma
lyophilized culture	---	10.0	0.66	clean, sl. green & diacetyl
1	2.0	9.5	0.76	clean, sl. green & diacetyl
2	0.6	12.0	0.76	clean, sl. green & diacetyl
3	0.6	11.0	0.72	clean, sl. green & diacetyl
4	1.0	14.0	0.96	very good, balanced
5	0.6	12.0	0.81	some diacetyl, green
6	0.6	12.0	0.89	very green, low diacetyl
7	0.6	10.0	0.73	very green
8	2.0	12.0	0.92	green, not much other flavor

[a] All transfers made into 300 ml whole milk medium.

to produce a green flavor with the selected commercial culture for this purpose were unsuccessful. Apparently the milk used for the medium favored the growth of Leuconostoc organisms, or the composition of the strains used in commercial lyophilized culture has been altered by the manufacturer.

Instead of the originally planned experiment, the percent distribution of lactic organisms in the mixed-strain culture were compared for the effect of different degrees of ripening. The data obtained are presented in Table 33. The culture for Series one in Table 33 was not fully ripened and gave a titratable acidity of 0.68 percent (see Table 39 in the Appendix). The culture represented in Series two (Table 33) was a culture derived from the culture examined in Series one; however, the subcultures had been allowed to ripen to developed acidities of 0.85-0.87 percent (see Table 39 in the Appendix). It can be seen in Table 33 that no great differences were apparent in the flora of the two cultures. The greatest differences were noted for the percentages of S. diacetilactis and Leuconostoc sp. It should be pointed out that care must be taken in interpreting the small changes that were observed. Only one trial was made for each series, thus errors could easily be encountered in carrying out the enumerations.

Further experiments were carried out to determine the role of the different culture bacteria in the production of the green flavor

Table 33. Percent distribution of lactic organisms in a commercial mixed-strain butter culture.

Classification	Acid Coagulation of Milk in 48 hr	King's Test	Number of Colonies	Percent of Total Colonies
Series 1 [a]				
S. lactis, *S. cremoris*	+	-	84	90.32
S. lactis or *cremoris* var. *aromaticus*	+	±	3	3.23
S. diacetilactis	+	+	4	4.30
Leuconostoc sp. [b]	—	+	2	2.15
		Total count = 1480×10^6 per ml		
Series 2 [c]				
S. lactis, *S. cremoris*	+	-	94	88.70
S. lactis or *cremoris* var. *aromaticus*	+	±	6	5.66
S. diacetilactis	+	+	2	1.89
Leuconostoc sp. [b]	-	+	4	3.77
		Total count = 2600×10^6 per ml		

[a] Culture previously transferred six times with developed acidities of 0.65-0.75 percent.

[b] Positive King's test after acidification of 48 hr culture with sterile phosphoric acid to pH 4.50 and subsequent six hr incubation at 30°C.

[c] Stock culture from Series 1 was subcultured daily for three days with developed acidities of 0.85-0.87 percent and then evaluated (see Table 39).

defect. From these data, it appeared that mixed-strain cultures with high acetaldehyde production from other lactic streptococci besides S. diacetilactis in conjunction with a low Leuconostoc population also give rise to the flavor. This is demonstrated in Table 34 which shows results obtained by mixing selected single-strains of lactic organisms. Sample one, which contained only S. lactis and L. citrovorum, gave a very green flavor after 12 hr of incubation, but after an additional four hr of incubation the flavor was markedly improved. During this period the Leuconostoc activity was sufficient to lower the acetaldehyde content from 3.40 to 0.38 p.p.m. and to raise the diacetyl content from 0.80 to 1.50 p.p.m. Culture two in which the stock Leuconostoc culture was diluted x 10^4 prior to inoculation gave a very green flavor and analyses showed that 2.62 p.p.m. of acetaldehyde and 0.55 p.p.m. of diacetyl were present. Cultures three and four containing both S. lactis and S. diacetilactis, again demonstrate the importance of adequate Leuconostoc activity in mixed-strain lactic cultures. S. lactis produces large amounts of acetaldehyde and insignificant amounts of diacetyl while S. diacetilactis produces relatively high levels of both flavor compounds. In comparing the flavor of single-strain cultures of S. lactis and S. diacetilactis with that of mixed-strain butter cultures, it was noted that the typical green flavor defect of mixed-strain butter cultures was more like the flavor of S. diacetilactis than that of S. lactis.

Table 34. Acetaldehyde and diacetyl production by selected single-strain mixtures of lactic organisms.

Culture Sample	Mixture	Incubation time at 21° C (hr)	pH	Diacetyl[c] (p. p. m.)	Acetaldehyde (p. p. m.)	Flavor and Aroma
1	2% S. lactis C_2F[a] 1% L. citrovorum 91404[b]	12	4.70	0.80	3.40	very green
		16	4.65	1.50	0.38	good
2	2% S. lactis C_2F 1% L. citrovorum 91404 diluted 10^4	16	4.70	0.55	2.62	very green
3	1% S. lactis C_2F 1% S. diacetilactis 18-16 1% L. citrovorum 91404	16	4.80	2.16	0.66	good
4	1% S. lactis C_2F 1% S. diacetilactis 18-16 1% L. citrovorum 91404 diluted 10^4	16	4.80	1.50	6.72	very green

[a] Total count: 1.5×10^9 organisms per ml.
[b] Total count: 47.5×10^6 organisms per ml.
[c] Method of Pack et al. (107).

Apparently diacetyl in conjunction with high level of acetaldehyde contributes to the typical green flavor in mixed-strain butter cultures.

The effect of storage at 5° C on the diacetyl content of ripened lactic cultures is shown in Table 35. The acetaldehyde content remained essentially constant over storage periods up to 120 hr in both the S. lactis C_2F culture and the mixed-strain cultures. These data indicate that under storage conditions where carbohydrate metabolism is reduced, little, if any, acetaldehyde accumulation takes place. There was a large increase in diacetyl content in mixed-strain cultures during storage which was probably due to the breakdown of the diacetyl precursor, alpha-acetolactic acid (33). Reduction of diacetyl to acetoin by diacetyl reductase (137, p. 78) was avoided in the mixed-strain cultures by controlling the incubation period to 16 hr or less to prevent overripening.

Laboratory Scale Production of Synthetic Culture Flavored Products

Laboratory scale lots of synthetic butter culture and synthetic culture flavored butters were prepared to evaluate synthetic culture flavor formulas. The initial flavor formulas were derived from the quantitative analyses of high quality naturally cultured products for key culture flavor components.

For the initial trial, raw whole milk was heated for one hr in a boiling water bath, then cooled to 21° C and the pH was adjusted

Table 35. Effect of storage at 5° C on the diacetyl and acetaldehyde content of ripened lactic cultures.

Culture Sample	Storage Time (hr)	pH	Percent Acidity	Diacetyl[b] (p. p. m.)	Acetaldehyde (p. p. m.)	Flavor and Aroma
S. lactis C_2F	0	4. 40	0. 98	0. 10	4. 40	green
	12	4. 40	0. 98	0. 10	4. 40	green
A[a]	0	4. 60	0. 93	1. 50	0. 42	good
	120	4. 60	0. 93	3. 33	0. 45	harsh
B[a]	0	4. 70	0. 86	3, 81	0. 45	harsh
	24	4. 70	0. 86	7. 80	0. 60	harsh diacetyl

[a] Commercial mixed-strain butter cultures.

[b] Method of Pack et al. (107).

to 4. 50 with 20 percent phosphoric acid. The following concentrations of flavor compounds were added:

3. 0 p. p. m. of diacetyl
0. 5 p. p. m. of acetaldehyde
1250. 0 p. p. m. of acetic acid
40. 0 p. p. b. of dimethyl sulfide

After storage at 5° C for 24 hr, the sample was evaluated. The synthetic culture exhibited a harsh diacetyl flavor, a sharp mineral acid flavor and lacked the "tangy" effect that normal butter cultures often exhibit due to the presence of carbon dioxide. The sample also had a very poor body. In the second trial, similar preparative methods were used, except that 2. 0 p. p. m. of diacetyl was added instead of 3. 0 p. p. m., and a slow steam of carbon dioxide was purged through the heated milk prior to the addition of the flavor compounds. The aroma was improved, but was then criticized for high dimethyl sulfide content. The carbon dioxide was noted to definitely improve the overall flavor, and the body was again very poor.

A third trial was carried out to evaluate the effect of the presence of milk fat and the effect of the heat treatment of the milk on the synthetic butter culture flavor formulation. The same levels of flavor compounds were added as in the second trial, except that the level of dimethyl sulfide was reduced from 40 p. p. b. to 25 p. p. b.

The samples which were evaluated included homogenized whole milk, pasteurized skimmilk, skimmilk heated for one hr in a boiling water bath and raw whole milk heated for one hr in a boiling water bath. All samples were acidified to pH 4. 50 with 20 percent phosphoric acid and were purged with a slow steam of carbon dioxide prior to the addition of flavor compounds. After holding for 24 hr at 5° C, the samples were evaluated.

The synthetic culture prepared with pasteurized skimmilk had the poorest flavor of the four samples evaluated. The homogenized whole milk synthetic culture was judged to be slightly better than the pasteurized skimmilk sample. Both of these samples were severely criticized for an atypical, unbalanced butter culture aroma and flavor. The synthetic butter culture prepared from heated skimmilk was found to have a fairly desirable culture aroma and flavor. However, it was criticized for lack of flavor balance. On the other hand, the synthetic culture prepared from raw whole milk heated for one hr in a boiling water bath was found to possess a very desirable butter culture aroma. When compared to a high quality natural butter culture, the synthetic culture was found to exhibit a very similar, if not identical, aroma. However, as in the previous trials, the body was very thin and undesirable.

Since Deane and Hammond (31) had reported the successful use of delta-gluconolactone for producing a firm coagulum for cheese-

making, two more trials were initiated. Delta-gluconolactone, when used as the acidogen, slowly hydrolyzes to gluconic acid, and thereby lowers the pH of the milk slowly to produce a firm coagulum. Raw whole milk heated for one hr in a boiling water bath was used as the starting material. For Trial four, 10.98 g of delta-gluconolactone was added to one kg of heated milk and held at 37° C for 1.5 hr. The sample was then cooled to 5° C and stored for 24 hr. After storage the following levels of flavor compounds were added:

2.0 p.p.m. of diacetyl
0.5 p.p.m. of acetaldehyde
1250.0 p.p.m. of acetic acid
25.0 p.p.b. of dimethyl sulfide

When the synthetic culture was first evaluated, the flavor and aroma were not typical and acetic acid appeared to predominate in the aroma. The pH was then measured and was found to be 4.00. This value was considerably lower than that normally encountered in butter cultures (pH 4.50-4.90). Adjustment of the pH to 4.50 with sodium bicarbonate, caused the synthetic culture to possess a very typical, natural culture aroma and flavor. For Trial five, 9.76 g of the acidogen was added to one kg of the heated whole milk. The final pH obtained was 4.50. Sodium bicarbonate was used to adjust the pH to 4.65, and thus carbon dioxide was introduced into the synthetic culture. Flavor compounds were then added at the

same levels as Trial four. The resulting synthetic culture possessed a highly desirable typical butter culture aroma and flavor. In both trials utilizing delta-gluconolactone, a very desirable body was obtained. However, an astringent flavor persisted after tasting the samples and was attributed to the acidogen. Deane and Thomas (32) have also observed this defect. These workers have found that the astringent flavor can be avoided by using citric acid to lower the pH to just above the coagulation point, and then adding a small amount of delta-gluconolactone which hydrolyzes slowly to complete the formation of a firm coagulum.

To test the balance of the flavor components used in Trial five, the levels of some of the flavor components were altered in aliquots of the original desirably flavored synthetic culture. In one aliquot the acetaldehyde content was raised to 1.0 p. p. m. while the other components were held constant. The flavor of the sample then became typically green. When the dimethyl sulfide content was raised to 50 p. p. b., the resulting synthetic culture was criticized as being "feedy".

The studies on the formulation of a synthetic culture flavor revealed that the addition of 2.0 p. p. m. of diacetyl, 0.5 p. p. m. of acetaldehyde, 1250.0 p. p. m. of acetic acid, 25.0 p. p. b. of dimethyl sulfide and a small amount of sodium bicarbonate to raw whole milk heated in a boiling water bath for one hr gave a very typical natural

butter culture aroma and flavor. It was found that the heat treatment of milk, especially whole milk, is critical for the background flavor of synthetic cultures. This indicates that constituents derived from milk fat contribute to a desirable culture flavor. In addition, maintaining the pH of the synthetic butter culture between 4. 50 and 4. 65 was very important in obtaining a desirable balance of the flavor components investigated. When the pH was lower than pH 4. 50, acetic acid appeared to predominate in an unbalanced aroma. The addition of sodium bicarbonate introduced carbon dioxide into the synthetic culture and was observed to markedly improve the overall flavor. In this respect, Andersen (2) has reported that the addition 0. 05 to 0. 15 percent of sodium bicarbonate to lactic cultures improved their flavor.

For the present study, it can be said that the flavor of the butter cultures prepared under the described conditions has been chemically reproduced to a close degree in synthetic butter cultures. The data indicate that an interaction of the proper levels of added synthetic flavor chemicals with the flavor compounds present in heated milk gives rise to the typical aroma "bouquet" of high quality natural butter culture.

Synthetic butter culture flavor formulations were added to sweet cream butter to evaluate their possible applications in standardizing and improving the flavor of butter. Butter containing 30. 0 p. p. m.

of acetic acid, 500 p. p. m. of lactic acid and 2. 5 p. p. m. of diacetyl was found to possess a characteristic cultured flavor, but was criticized for being "harsh" in flavor. However, the addition of 40. 0 p. p. b. of dimethyl sulfide to the formulation produced a butter with a smooth desirable flavor. Although further trials were not conducted on the use of synthetic flavor formulations in butter, it was apparent that a desirable natural culture flavor could be synthetically produced for butter. In a recent report by Ernstrom (39), it was stated that the lack of a suitable source of culture flavor was one of the main problems in the development of commercial methods for the production of sour cream and cottage cheese without the use of natural starters. It would seem very probable that in the future the basic flavor formulas derived from this work will be modified and used for standardizing and improving the flavor of these products.

SUMMARY AND CONCLUSIONS

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

Compounds that were positively identified in butter culture include ethanol, acetone, ethyl formate, methyl acetate, acetaldehyde, diacetyl, ethyl acetate, dimethyl sulfide, butanone, 2-butanol, methyl butyrate, ethyl butyrate, methane, methyl chloride, carbon dioxide and methanol; also included were 2-pentanone, 2-heptanone, acetoin, formic acid, acetic acid, lactic acid, 2-furfural, 2-furfurol, methyl hexanoate, ethyl hexanoate, 2-nonanone, 2-undecanone,

methyl octanoate and ethyl octanoate. Compounds that were tentatively identified in butter culture include hydrogen sulfide, methyl mercaptan, n-butanal, n-butanol, 2-hexanone, n-pentanal, n-pentanol, 2-mercaptoethanol. n-butyl formate, n-butyl acetate, 2-methylbutanal, 3-methylbutanal, methylpropanal, methyl heptanoate, n-octanal, 2-tridecanone, methyl benzoate, methyl nonanoate, ethyl nonanoate, ethyl decanoate, methyl dodecanoate, ethyl dodecanoate, delta-octalactone and delta-decalactone.

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

culture fractions. This was attributed to their presence in low concentrations, chemical instability or inefficient recovery.

A modified 3-methyl-2-benzothiazolone hydrazone spectrophotometric procedure was adapted for the determination of acetaldehyde produced in lactic starter cultures. The procedure was applied in conjunction with diacetyl measurements in studying single- and mixed-strain lactic cultures. The diacetyl to acetaldehyde ratio was found to be approximately 4:1 in desirable flavored mixed-strain butter cultures. When the ratio of the two compounds was lower than 3:1 a green flavor was observed. Acetaldehyde utilization at 21° C by Leuconostoc citrovorum 91404 was very rapid in both acidified (pH 4. 5) and non-acidified (pH 6. 5) milk cultures. The addition of five p. p. m. of acetaldehyde to non-acidified milk media prior to inoculation greatly enhanced growth of L. citrovorum 91404 during incubation at 21° C. Combinations of single-strain organisms demonstrated that the green flavor defect can result from excess numbers of Streptococcus lactis or Streptococcus diacetilactis in relation to the L. citrovorum population.

Diacetyl, dimethyl sulfide, acetaldehyde, acetic acid and carbon dioxide were found to be "key" compounds in natural butter culture flavor. Optimum levels of these compounds in butter culture were ascertained by chemical and flavor panel evaluations. On the basis of these determinations, a synthetic butter culture prepared with

heated whole milk and delta-gluconolactone (final pH 4. 65) was flavored with 2. 0 p. p. m. of diacetyl, 0. 5 p. p. m. of acetaldehyde, 1250 p. p. m. of acetic acid, 25. 0 p. p. b. of dimethyl sulfide and a small amount of sodium bicarbonate for production of carbon dioxide. The resulting synthetic butter culture exhibited the typical aroma, flavor and body characteristics found in natural high quality butter cultures, except that the delta-gluconolactone was found to contribute an astringent flavor.

The results of this investigation that are believed to be of significance in the flavor chemistry of butter culture were as follows:

1. As a result of gas chromatographic, mass spectrometric and other chemical analyses, 30 flavor compounds were positively identified in butter culture. Tentative identifications were also obtained for 24 additional flavor compounds in butter culture. Similar analysis of control heated milk yielded data for the positive identification of nine flavor components. In addition, 23 other flavor compounds were tentatively identified.

2. The qualitative composition of flavor distillates obtained from butter culture and control heated milk were found to be very similar.

3. Diacetyl, dimethyl sulfide, acetaldehyde, acetic acid and carbon dioxide were found to be "key" compounds in natural butter culture flavor.

4. Treatment of a fresh butter culture distillate with sodium carbonate did not prevent the isolation of esters, thus indicating that esters are normal components of culture.

5. A spectrophotometric ferric-hydroxamate procedure was developed for measurement of volatile esters in lactic cultures. Analyses of high quality butter cultures demonstrated that the levels of volatile esters in butter culture were not sufficient to allow them to assume a major role in butter culture flavor.

6. A modified 3-methyl-2-benzothiazalone spectrophotometric procedure was adapted for the determination of acetaldehyde in lactic cultures. The diacetyl to acetaldehyde ratio was found to be approximately 4:1 in desirably flavored mixed-strain butter cultures. When the ratio of the two compounds was lower than 3:1 a green flavor was observed.

7. Acetaldehyde utilization at 21° C by Leuconostoc citrovorum 91404 was very rapid in both acidified (pH 4.5) and non-acidified (pH 6.5) milk cultures. The addition of five p.p.m. of acetaldehyde to non-acidified milk media prior to inoculation greatly enhanced growth of L. citrovorum 91404 during incubation at 21° C.

8. Synthetic butter culture was prepared by adding synthetic flavor chemicals to heated whole milk which had been acidified with delta-gluconolactone (final pH 4.65). The added flavor mixture which was found to very closely simulate natural butter culture contained

2.0 p. p. m. of diacetyl, 0.5 p. p. m. of acetaldehyde, 1250 p. p. m. of acetic acid, 25.0 p. p. b. of dimethyl sulfide, and a small amount of carbon dioxide.

9. It was found important to control the pH of the synthetic butter culture between 4.50 and 4.65 in order to maintain a desirable flavor balance. Heating whole milk for one hr in a boiling water bath was critical for producing necessary background flavor in the synthetic butter cultures. Delta-gluconolactone produces an astringent flavor when used as the acidogen in synthetic cultures.

10. A limited number of trials indicated that a synthetic culture flavor could be successfully adapted for the production of culture flavored butter.

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APPENDIX

Table 36. Relative retention times of compounds tentatively identified from distillation D butter culture acidic extract using an Apiezon M column; gas chromatogram shown in Figure 22.

Peak No.		t_R/t_R[a]	
		Butter Culture	Authentics
1	Hydrogen sulfide	0. 252	0. 253
2	Acetaldehyde	0. 363	0. 364
3	Ethanol	0. 517	0. 541
4	Ethyl formate	0. 641	0. 634
5	Methyl acetate	0. 726	0. 686
	Ethyl ether		0. 690
6	Dimethyl sulfide	0. 813	0. 846
7	Diacetyl	1. 000	1. 000
8	n-Butanal	1. 130	1. 110
	Ethyl acetate		1. 140
	Butanone		1. 140
9	n-Butanol	1. 996	2. 120
	2-Pentanone		2. 240
10	Methyl butyrate	2. 740	2. 880
	Acetoin		2. 980

[a] t_R/t_R = relative retention time based on diacetyl = 1. 000; Packed column, 11 ft x 1/8 inch OD, 20 percent Apiezon M on 100-120 mesh alkali-acid treated Celite 545; column temperature: 70° C; Barber-Colman Model 20.

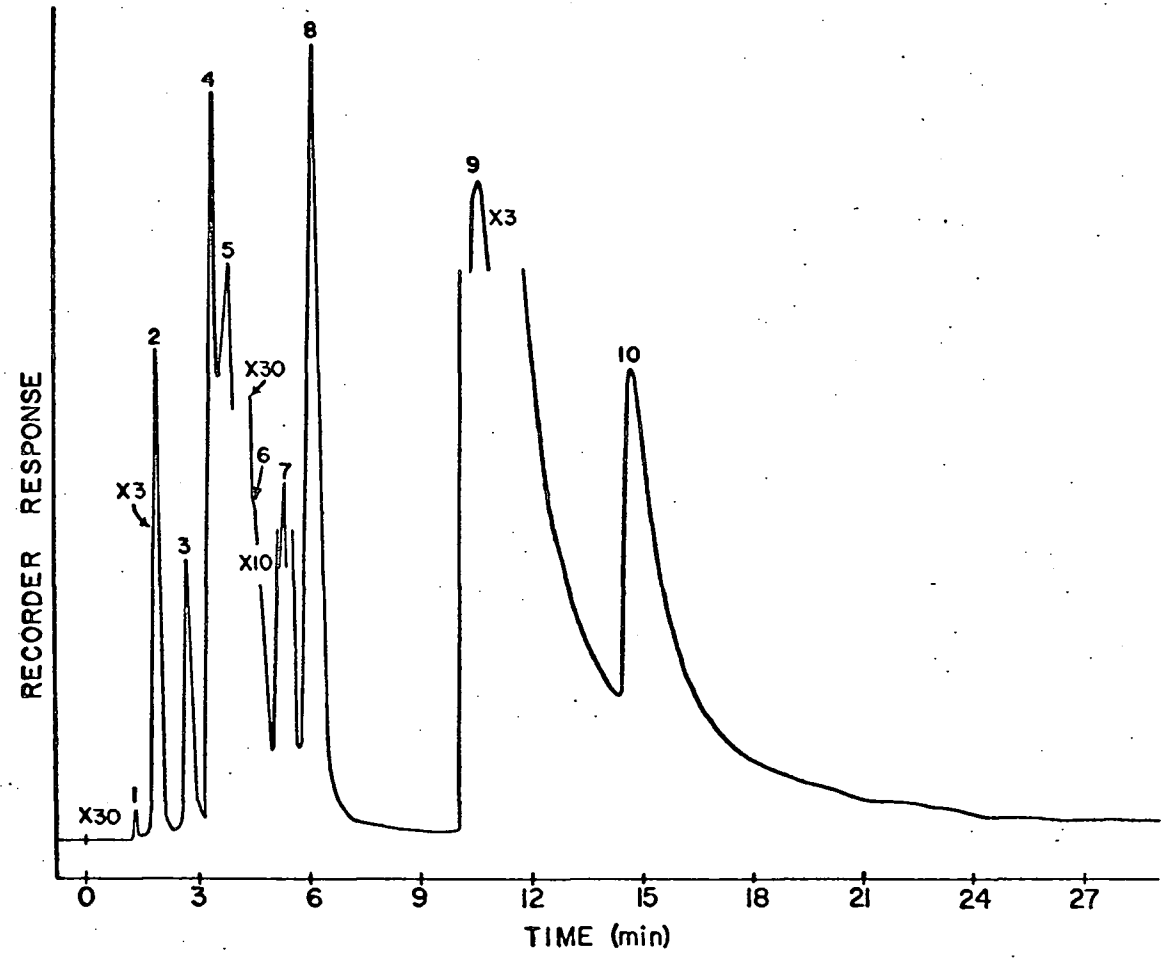

Figure 22. Gas chromatogram of butter culture distillate acidic fraction extract using an Apiezon M column at 70° C (see Table 36 for peak designations).

Table 37. Relative retention times of compounds tentatively identified from distillation D butter culture basic extract using an Apiezon M column; gas chromatogram shown in Figure 23.

Peak No.	Compound	t_R/t_R[a]	
		Butter Culture	Authentics
1	-------	0.224	-----
2	Hydrogen sulfide	0.256	0.253
3	-------	0.316	-----
4	Acetaldehyde	0.346	0.364
5	-------	0.384	-----
6	Methanol	0.406	0.416
7	Methyl mercaptan	0.487	0.466
8	Ethanol	0.513	0.541
	Acetone		0.581
9	Ethyl formate	0.628	0.634
10	Methyl acetate	0.722	0.686
	Ethyl ether		0.690
11	Diacetyl	1.000	1.000
12	n-Butanal	1.110	1.110
	Ethyl acetate		1.140
13	Butanone	1.150	1.140
14	2-Mercaptoethanol	1.410	1.480
15	3-Methylbutanal	1.830	1.820
16	n-Butanol	2.020	2.120
	2-Pentanone		2.240
17	n-Pentanal	2.590	2.510
18	Methyl butyrate	2.820	2.880
	Acetoin		2.980
19	2-Heptanone	11.210	11.700

[a] t_R/t_R = relative retention time based on diacetyl = 1.000. Packed column, 11 ft x 1/8 inch OD, 20 percent Apiezon M on 100-120 mesh alkali-acid treated Celite 545; column temperature: 70° C; Barber-Colman Model 20.

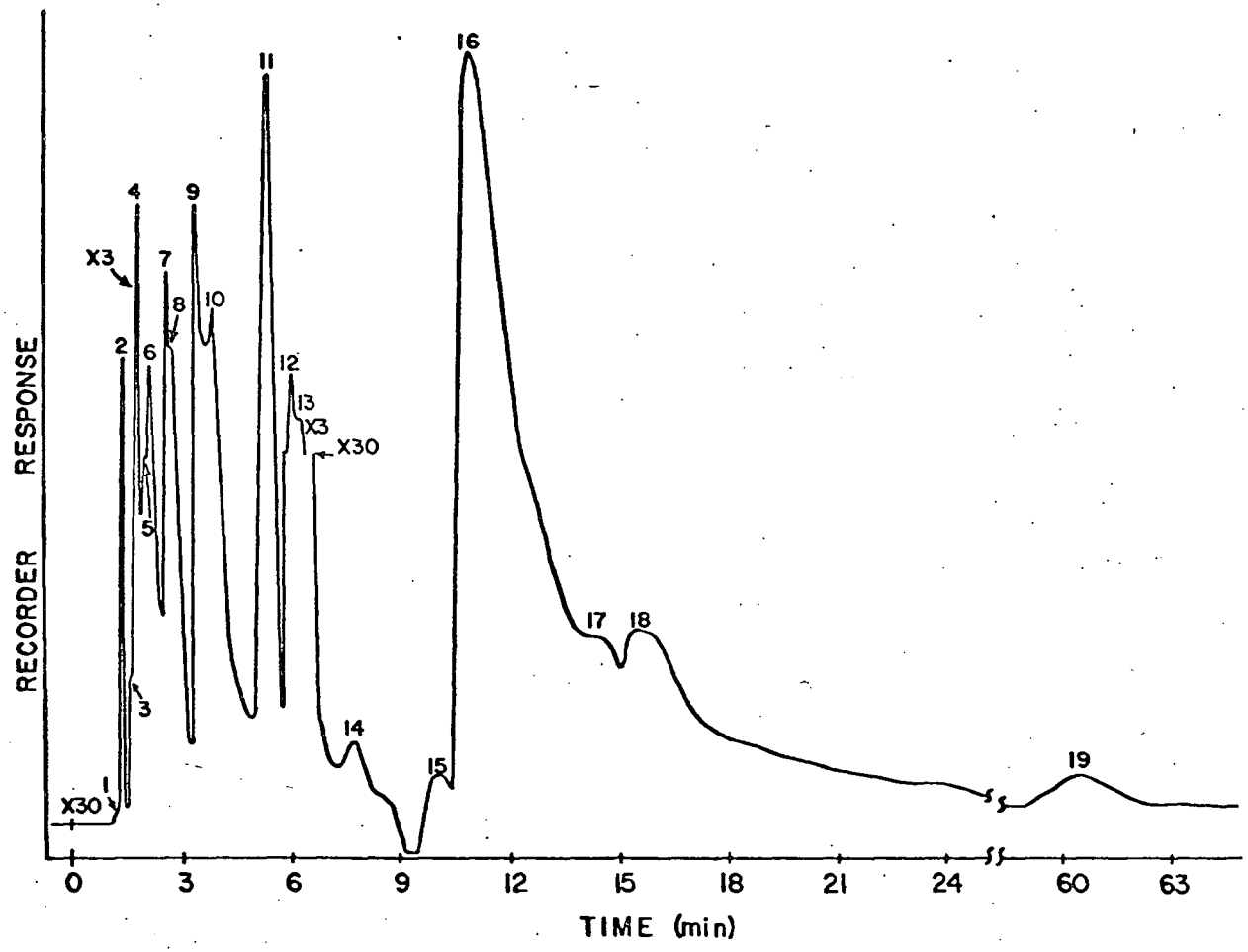

Figure 23. Gas chromatogram of butter culture distillate basic fraction extract using an Apiezon M column at 70° C (see Table 37 for peak designations).

Table 38. Analyses of a commercial mixed-strain butter culture for diacetyl and acetaldehyde after successive transfers in whole milk medium.

Transfer [a, c] (Sample)	Percent Inoculum	Incubation at 21° C (hr)	Percent Acidity	pH	Diacetyl [b, d] (p. p. m.) A	Acetaldehyde [b] (p. p. m.) B	Ratio A:B	Flavor and Aroma
Series 1								
1	3	11	0.74	4.90	5.85	0.55	10.6:1	harsh
2	1	11	0.77	4.90	7.31	2.78	2.6:1	coarse
3	3	14	0.80	4.70	6.05	0.58	10.4:1	harsh
4	3	12	0.83	4.70	7.80	0.60	13.0:1	harsh
Series 2								
1	3	11	0.74	4.90	5.85	0.55	10.6:1	harsh
2	3	14	0.92	4.60	3.24	0.44	7.4:1	sl. harsh
3	1	12	0.77	4.70	3.80	0.43	8.8:1	sl. harsh
Series 3								
1	3	18	0.96	4.50	1.60	0.46	3.5:1	good
2	1	18	0.83	4.65	5.45	0.41	13.3:1	harsh
3	3	14	0.83	4.65	2.50	0.54	4.6:1	good
Series 4								
1	3	18	0.96	4.50	1.60	0.46	3.5:1	good
2	3	14	0.89	4.60	6.60	0.40	16.5:1	harsh
3A	3	14	0.84	4.65	4.44	0.44	10.0:1	harsh
3B	1	12	0.76	4.75	2.30	0.38	6.1:1	sl. harsh

[a] All samples held 24 hr at 5° C before analyses.

[b] Average of duplicate analyses.

[c] Initital stock culture prepared from lyophilized powder.

[d] Method of Pack et al. (107).

Table 39. Analyses of a commercial mixed-strain butter culture for diacetyl and acetaldehyde after successive transfers in whole milk medium.

Transfer (sample)	Percent Inoculum	Incubation at 21° C (hr)	Percent Acidity	pH	Diacetyl [c, e] (p. p. m.) A	Acetaldehyde [c] (p. p. m.) B	Ratio A:B	Flavor and Aroma
Initial [a, b, d]	1	12	0.68	4.80	2.40	2.20	1.1:1	green
Series 1								
1	3	14	0.87	4.50	4.50	0.26	17.3:1	harsh
2	3	9	0.77	4.70	0.90	0.20	4.5:1	not full
3	3	14	0.84	4.50	1.65	0.15	11.0:1	not full
Series 2								
1	3	9	0.75	4.70	2.44	0.32	7.6:1	sl. harsh
2	3	9	0.74	4.70	1.00	1.12	0.9:1	green
3	3	9	0.75	4.70	0.96	1.20	0.8:1	green
Series 3								
1	3	12	0.87	4.50	5.20	0.26	20.0:1	harsh
2	3	9	0.77	4.70	0.90	0.20	4.5:1	lacking
3	3	14	0.88	4.50	1.95	0.20	9.7:1	not full
Series 4								
1	3	9	0.75	4.70	2.44	0.32	7.6:1	sl. harsh
2	3	9	0.74	4.70	1.00	1.12	0.9:1	green
3	3	14	0.86	4.50	2.14	0.15	14.2:1	harsh
Series 5								
1	3	12	0.87	4.50	5.20	0.26	20.0:1	harsh
2	3	14	0.85	4.50	4.00	0.23	17.4:1	harsh
3 [b]	3	14	0.87	4.50	3.40	0.20	17.4:1	harsh
Heated milk	-	14	0.21	6.55	0.00	0.15	-----	strong cooked

[a] All samples held 24 hr at 5° C before analyses.

[b] Plated on lactic agar for selecting colonies (see Table 33).

[c] Average of dulicate analyses.

[d] Stock culture used for initial transfers; previously transferred six times with developed acidities of 0.65-0.75 percent.

[e] Method of Pack et al. (107).