

AN ABSTRACT OF THE THESIS OF

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Title FACTORS INFLUENCING METHYL KETONE FORMATION  
IN MILK FAT

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Recent studies have shown that when milk fat is heated, a homologous series containing the n-alkyl members of methyl ketones with odd numbers of carbon in their chains are produced (48; 44; 38; and 3). The same series of compounds also is found in evaporated and dried whole milk and in these products the concentration increases during storage (70 and 46). It is believed by some investigators that the methyl ketones play an important role in flavor deterioration of milk fat and in the aforementioned concentrated products. At the present time, however, there is disagreement on the factors influencing methyl ketone production; some workers relate their formation to autoxidation (25), while there are others who report that heat and water are essential in the reaction (48; 38 and 3). Finally, a recent report indicates that anhydrous milk fat will give rise to methyl ketones when heated in the absence of oxygen (44).

The purpose of this investigation was to study the effect of various factors on the qualitative and quantitative composition of methyl ketones in heat treated milk fat. It is anticipated that the resulting information will contribute to a more thorough understanding of the reactions leading to ketone production in the fat; hence, to development of suitable processing measures for prevention of this type of deterioration in dairy products.

Milk fat was prepared from raw cream two days after milking. It was washed free from phospholipids, centrifuged at 30,000 x G for 20 minutes and degassed at two to five microns pressure for one hour. The fat was then heat treated in sealed vials at various temperatures and time periods under controlled conditions. The samples were quantitatively analyzed for methyl ketones by direct conversion of the ketones to 2,4-dinitrophenylhydrazine (DNP) derivatives in the intact fat sample. The derivatives were isolated from the fat, separated and identified by a combination of column and paper chromatographic methods and by their absorption spectra.

Methyl ketone formation in heated milk fat was shown to be non-oxidative. A plateau in ketone production was approached in the 120°C to 140°C range when the time of heat treatment was 30 minutes. Added water enhanced total methyl ketone production at 140°C but not at 200°C. Air did not hinder ketone production.

Maximum ketone production (1.733 mM/kg fat) was noted after three hours of heat treatment at two to five microns pressure, and 140°C. Milk fat centrifuged at 30,000 x G for 20 minutes and degassed at two to five microns pressure for one hour was found to contain 0.27% water. This quantity of water is sufficient for hydrolysis of  $\beta$ -keto-esters assuming them as the precursors of the methyl ketones. Conventional methods of preparing "anhydrous" milk fat were not adequate for removal of trace amounts of water. When milk fat was dried over calcium hydride (35) prior to heat treatment, total ketone formation was significantly reduced indicating that water is a limiting factor in ketone formation.

A homologous series of n-alkyl methyl ketones ( $C_3$ ,  $C_5$ ,  $C_7$ ,  $C_9$ ,  $C_{11}$ ,  $C_{13}$ ,  $C_{15}$ ) was isolated from heat treated samples. The ketones produced in large amounts were the  $C_3$ ,  $C_7$ , and  $C_{15}$ . When the heat treatment was for three hours or longer the  $C_4$  ketone was detected and composed approximately 11% of the total. The possible origin of the  $C_4$  ketone is discussed. The reaction of intact fat with DNP-hydrazine and the subsequent isolation and identification of methyl ketones were quantitatively evaluated.

FACTORS INFLUENCING METHYL KETONE FORMATION  
IN MILK FAT

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# FACTORS INFLUENCING METHYL KETONE FORMATION IN MILK FAT

## INTRODUCTION

The unique ability of milk fat to give rise to methyl ketones when heated has been recognized but the conditions influencing their formation have not been thoroughly investigated. Several investigators have reported that water and heat are necessary for ketone formation. One investigator reports that methyl ketones are formed in "anhydrous" milk fat heated in the absence of oxygen. Early investigators proposed that the methyl ketones formed via autoxidation of the milk fat. It is apparent, therefore, that confusion exists concerning the factors influencing methyl ketone formation.

This investigation was designed to study the effect of various factors on the qualitative and quantitative composition of methyl ketones formed in heat treated milk fat. It is believed that the information reported here will contribute to a more thorough understanding of the reactions leading to ketone formation in the fat and assist in the development of suitable processing measures for prevention of this type of deterioration in dairy products.

## REVIEW OF LITERATURE

### Phenomenon of Ketone Rancidity

The presence of ketones was recognized early as playing an important role in deteriorating fats (61, p. 371-412; 52 and 65). Stärkel was the first to report the formation of methyl ketones in the oxidative decomposition of milk fat. Five years later, in 1933, Täufel demonstrated (66) that ketones are produced by heating fatty acids. At the same time Schmalfuss et al.(52) reported that fats give rise to ketones via chemical decomposition and that the decomposition could be enhanced by light or heat. Later these workers (54) reported that soybean oil rapidly gave rise to ketones when exposed to light of wave lengths less than 410 millimicrons.

In 1938 Sporzynska (60) in a study on the spoilage of edible fats and oils concluded that those fats containing predominately saturated or monounstaured fatty acids i. e. lard, butter and olive oil, decomposed mainly to ketonic end products. Those fats containing predominately polyunsaturated acids decomposed mainly to aldehyde end products.

Stärkel (61, p. 412) concluded from his studies on the oxidative decomposition of milk fat that the methyl ketones were of

microbial origin. In 1939 Girshman (25) reported that methyl ketones were formed in milk fat under sterile conditions. This work indicated that methyl ketones were formed in milk fat via chemical decomposition of some precursor or precursors without the aid of microbial metabolism. Girshman suggested that ketone rancidity might be prevented by storing the fat at high temperatures to prevent enzymatic and microbial activity. Oxidation might be prevented by excluding oxygen from the sample or by addition of antioxidants to the system. In light of more recent information these suggestions would enhance rather than prevent the formation of ketones in milk fat.

### Evaluation of the Early Literature

The majority of the early work on the decomposition of fats shows only a very small part of the overall picture. These investigators were using the best analytical methods available to them at the time of their work; unfortunately, the methods failed to detect many important compounds that play a significant role in fat decomposition. In the early work the presence of ketones was demonstrated by the method of Täufel (64) or some modification thereof (16 and 53). This is based on the reaction of the carbonyl with salicylaldehyde in aqueous solution containing concentrated sulfuric acid. If a methyl

ketone is present, a red color develops and darkens with time in the organic layer. When the reaction mixture is warmed in a boiling water bath for 15 minutes the color will become darker. The literature lacks specific information on this color reaction for methyl ketones. Unfortunately the investigators using the reaction did not study the reaction mechanism nor did they characterize the product or products of the reaction. In light of recent literature on the decomposition of fats (21, p. 295; 1 and 20) it would appear that the color reaction probably was not specific for ketones and that aldehydes and other carbonyls were reported as ketones. This conclusion is supported by the work of Day and Lillard (10, p. 595) and Gaddis et al. (22) who found the majority of the monocarbonyl compounds from autoxidized fats were aldehydes.

#### Methyl Ketones in Milk Fat

The investigation of methyl ketone formation in milk fat was stimulated in 1958 by Wong et al. (70) who identified acetone, pentanone-2, and heptanone-2 in low temperature reduced pressure distillates from commercial evaporated milk. Their data indicated that heptanone-2 and pentanone-2 occurred in heated whole milk but not in raw milk. This led them to conclude that these compounds are heat generated. The largest quantity of ketones was isolated

from cream. Thus they concluded that the ketones arose from the lipid phase.

A year later Patton and Tharp (48) identified a homologous series of methyl ketones in the steam distillate and the unsaponifiable matter of milk fat. The series contained the n-alkyl members with odd numbers of carbons from 3 (acetone) through 15 (pentadecanone-2). They found that fresh unheated milk fat contained only acetone. Evidence was presented which suggested that fresh milk fat glycerides may contain ketonic carbonyl groups.

Parks and Patton (46) subsequently found the characteristic series of ketones in the low temperature vacuum distillates of reconstituted dry whole milks. When the dry whole milk was prepared from deodorized, i. e. steam stripped milk fat, methyl ketones were noticably absent. This data suggested that the ketones were heat generated and their precursors may be removed by steam stripping the fat. The ketones were reported to impart "stale" off-flavors to concentrated milks.

Nawar et al. (44) identified the same series of ketones found by Patton and Tharp (48), with the exception of pentadecanone-2, in heated milk fat. Trace quantities of even numbered methyl ketones butanone-2, hexanone-2, octanone-2 and n-alkanals were found. They reported the milk fat used was free of moisture.

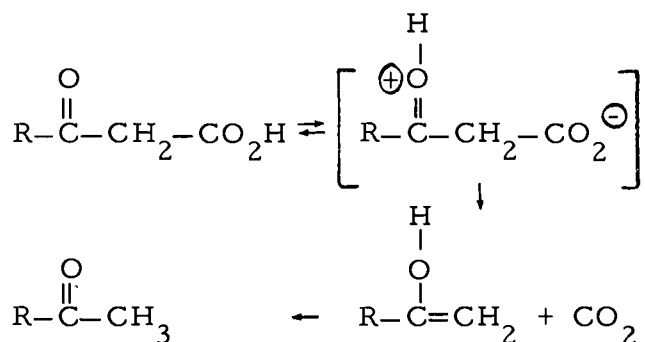
Recently Lawrence in New Zealand (38) and Boldingh and Taylor (3, p. 913) in the Netherlands have reported on the methyl ketones isolated from steam distillates of milk fat. Both papers report the same homologous series of ketones found by Patton and Tharp (48).

### Precursors of the Methyl Ketones

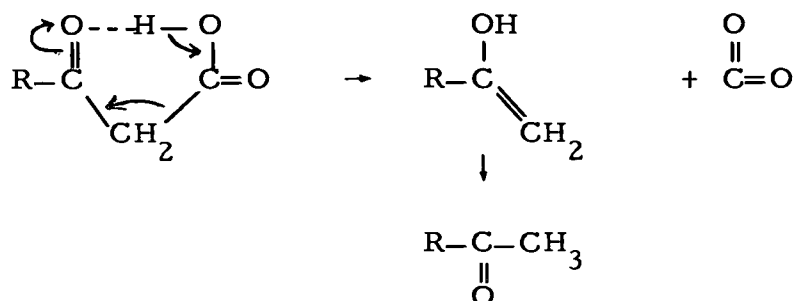
At the initiation of the work reported in this thesis the precursors of the methyl ketones in milk fat were unknown. Wong (70, p. 1704) had suggested that perhaps the ketones might arise from  $\beta$ -ketoacids present in the fat which upon heating decarboxylated to give methyl ketones. Hine reports that (26, p. 303-304)

If the R group of  $\text{RCO}_2^-$  contains a sufficiently basic functional group, it may accept a proton and greatly increase its electron-withdrawing power, thus facilitating the loss of carbon dioxide. This fact appears to be important in the decarboxylation of  $\beta$ -ketoacids.

The generally accepted reaction mechanism for the decarboxylation of  $\beta$ -ketoacids is as follows:



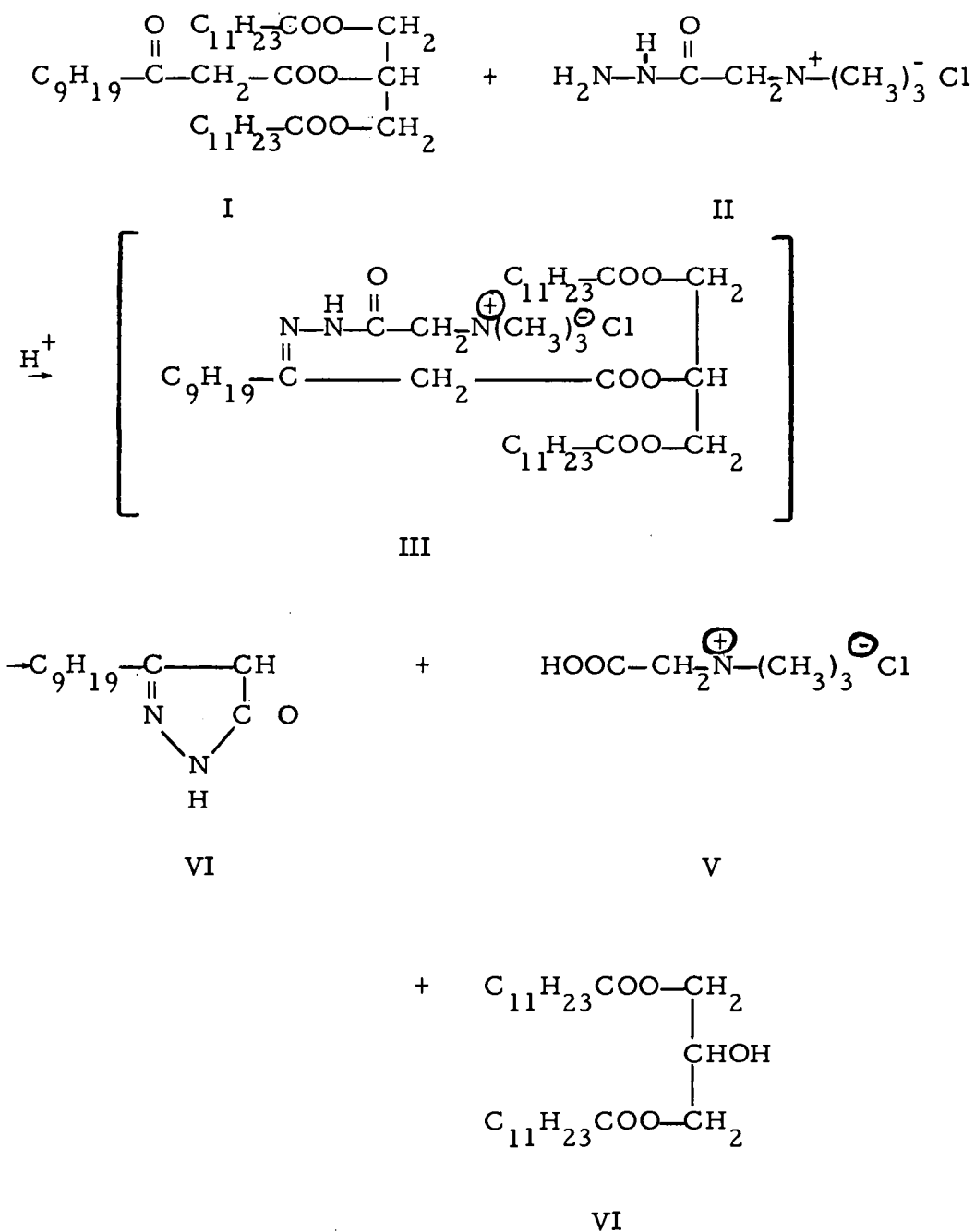
Gould (23, p. 346-347) points out that an intermediate of the following type:



in which there is a partial transfer of the carboxyl proton, in intramolecular hydrogen bonding, to the keto group facilitates the decarboxylation reaction.

In January 1963 van der Ven (68) and workers at the Unilever Research Laboratory in the Netherlands identified the precursors of the methyl ketones in milk fat. They used Girard-T reagent which forms hydrazones with carbonyl compounds. These hydrazones were separated from non-carbonyl compounds in the mixture (24, p. 1097; 17). They established that when the Girard-T reagent (II) reacted with a typical  $\beta$ -ketoacid glyceride (I) the hydrazone formed (III) decomposed to the pyrazolone (VI) and the diglyceride, dilaurin (IV), and they assumed that trimethylaminoacetic acid chloride (V) was split off from (III) during the course of the reaction.





These workers then reacted Girard-T reagent with milk fat and isolated a homologous series of six pyrazolones which corresponded to the six even numbered  $\beta$ -ketoacids with 6 up to and including 16

carbons. The  $\beta$ -ketoacids correspond to the precursors of the six odd numbered methyl ketones (5 through 15 carbons) that are formed when milk fat is subjected to steam-deodorization. The formation of the pyrazolones on reaction of milk fat with Girard-T reagent was considered by these workers to be proof that the precursors of methyl ketones exist as non-volatile  $\beta$ -ketoesters in milk fat. There is evidence to suggest that ketoacids are present in milk fat (48, p. 53; 33). The  $\beta$ -ketoesters, as such, have not yet been identified.

#### Origin of $\beta$ -ketoesters in Milk Fat

There are two sources that could account for  $\beta$ -ketoesters in milk fat. They might occur either as products of the oxidation of fatty acids or as intermediates in the biosynthesis of fatty acids by the mammary gland of the bovine.

Beta-oxidation was not considered very likely by Wong (11, p. 1705) who noted that autoxidation was a rather remote possibility in heated cream or evaporated milk because these media contain a wealth of reducing substances. If autoxidation played an important role in  $\beta$ -ketoester formation, then the carbonyls isolated from autoxidized milk fat via distillation should contain large quantities of methyl ketones. This indeed was not the case (10, p. 595; 40, p. 626; 22) with milk fat or other fats (22; 71, p. 195; 62) handled in a similar

manner. In consideration of the data presented in this thesis as well as that of Nawar (44), oxidation as a source of  $\beta$ -ketoesters or methyl ketones in milk fat would appear unlikely.

The most logical explanation for the presence of  $\beta$ -ketoesters in milk fat appears to be via incomplete biosynthesis of fatty acids in the mammary gland. It would be worthwhile therefore, to discuss the composition of milk fat and its biosynthesis.

### Milk Fat Composition

Milk fat is very complex and its composition is not entirely known or understood (30, p. 1-25). Jenness and Patton reported (31, p. 34) the presence of the following constituents: triglycerides - 98-99%, phospholipids - 0.2-1.0%, sterols - 0.25-0.40%, free fatty acids - traces, waxes - traces, squalene - traces, and fat soluble vitamins - traces. The major concern here is with the triglycerides and their fatty acids. Fatty acids with an even number of carbon atoms predominate in milk fat. The quantities of the shorter chain fatty acids (four to eight carbons) are much greater in the milk fat of ruminants than non-ruminants.

In 1960 Jack (29) reported that milk fat contains a variety of individual fatty acids ranging in chain length from 4 to 26 carbons and in differing degrees of unsaturation from monoenes to pentaenes.

Minor saturated fatty acids (less than 1.0%) with odd-number of carbons, branched chains, and normal even numbered high molecular weight fatty acids were also reported. Although Patton and Tharp (48, p. 53) gave evidence suggestive of keto functions in milk fat, the amount must be so small that they were not detected by Jack or other workers.

Keeney et al. (33) found that per gram of milk fat, there is 5 to 15 micromoles of predominantly saturated ketoesters in the glycerides. The major ketoester fraction contained ketostearate. Smaller amounts of the saturated 10 to 16 carbon ketoacids as well as unsaturated 18 carbon ketoacids were found. Beta-ketoacids were not reported.

### Milk Fat Synthesis

It is well known that the average milk cow secretes about twice as much fat in milk as is ingested in the feed. The cow must therefore synthesize one half or more of its daily production of milk fat from non-lipid sources.

In a review article by Luick (41, p. 1345) data were presented that indicate ingested lipids undergo extensive changes during the first step of digestion in the rumen. Rumen lipids and blood lipids compared fairly closely in molar percentage of fatty acids. The plasma lipids were normally 80% cholesterol esters, 7.5% triglycerides,

6.5% sterols, 3.8% phospholipids, 2.5% unidentified lipids and only traces of free fatty acids.

Milk fat has been found to contain about 98% triglycerides, 1.0% phospholipids and less than 1.0% cholesterol esters (40, p. 1346). This represents quite a difference from the plasma lipids. A difference exists in fatty acid composition between the plasma triglycerides and milk fat triglycerides that is accounted for by the presence of the 4 to 14 carbon fatty acids in milk fat. Luick points out that of the components of blood lipids, only the plasma triglycerides are absorbed by the mammary gland in great enough quantities to contribute significantly to milk fat synthesis (41, p. 1347).

Milk fat synthesis in the mammary gland is not entirely understood at this time. The long chain fatty acids are thought to be derived mainly from the plasma lipids (50 and 42) as mentioned previously. It appears now that enough evidence has accumulated to state that the shorter chain fatty acids (4 to 16 carbons), which are the main difference between the plasma triglycerides and milk fat triglycerides, are synthesized from acetate in the mammary gland (4; 37; 12 and 49). Beta-hydroxybutyrate from blood has been shown to be utilized for synthesis when there was a shortage of acetate in the blood (37). However, acetate was preferentially taken up by the perfused lactating bovine udder when an adequate pool occurred. Recent

work (39) using milk fat from a cow injected intravenously with radioactive acetate has shown that the methyl ketones isolated by steam distillation of the fat, excepting acetone, have been found to be radioactive. This points to acetate as an intermediate in the biosynthesis of the ketone precursors.

The biosynthesis of fatty acids from acetate is now believed to be far more complex than a simple reversal of  $\beta$ -oxidation (69, p. 376-391). Elongation of fatty acids is thought to pass through a step in which there is a  $\beta$ -ketoacid-acyl-enzyme complex each time the fatty acid chain is lengthened by two carbons (69, p. 390). Popják et al. (49) and Dils and Popják (12) reported that the overall process of synthesis of short chain fatty acids by goat mammary gland and rat mammary gland was analogous to, but not equivalent to the reversal of  $\beta$ -oxidation. Presumably at the  $\beta$ -ketoacid-acyl-enzyme step the  $\beta$ -ketoacid might mistakenly be esterified onto the glycerol moiety thus giving rise to the  $\beta$ -ketoglycerides in milk fat. These  $\beta$ -ketoglycerides, when heated under proper conditions, would then give rise to a homologous series of methyl ketones with odd numbers of carbon atoms.

## EXPERIMENTAL

### Preparation of the Milk Fat

Raw sweet cream was obtained from the university dairy two days after milking. The cream was cooled to 10°C and churned. The buttermilk fraction was drained off and the milk fat granules were washed three times with an equal volume of cold distilled water. The fat was tempered at 40°C in a constant temperature water bath for one hour after which it was placed in a separatory funnel and washed three times with an equal volume of 40°C distilled water. This procedure of preparing the milk fat was adequate for removal of the phospholipids (10, p. 586). Clarification of the fat was accomplished by centrifugation for 20 minutes at 30,000 x G in a Servall SS-3 superspeed centrifuge. At the conclusion of centrifugation the temperature of the supernatant fat was 40°C. The clarified fat was transferred from the centrifuge tubes via a hypodermic syringe to heat treatment vials. The vials were made of pyrex glass (1.8 cm i. d. - 2.5 cm o. d. x 16 cm) with a length of thick walled pyrex tubing (0.2 cm i. d. - 1.0 cm o. d. x 10 cm) attached at one end which provided an entrance for the syringe needle. Ten milliliters of fat (9.2221 g) were used for each treatment. The fat containing vials were degassed at two to five microns pressure and maintained at 40°C by a

thermostatically controlled water bath during a degassing period of one hour. In several studies one milliliter of water was added to the fat in the vials in order to evaluate the effect of added water on methyl ketone formation. In this case the vials were degassed with the fat frozen. After degassing, all the evacuated vials (two to five microns) were sealed with an oxygen torch. The samples were quick frozen and stored at  $-18^{\circ}\text{C}$  until subjected to heat treatment.

#### Heat Treatment of Milk Fat

The apparatus used for heat treatment was a pyrex saucepan filled with glycerine, a laboratory hot plate to heat the glycerine and a stirring motor to aid in maintaining a constant temperature during heating. The bath temperature was adjusted and maintained by a Powerstat. The samples to be heated were pre-warmed to  $40^{\circ}\text{C}$  for 30 minutes prior to being placed into the temperature controlled glycerine bath. At the completion of the heat treatment, the samples immediately were quick frozen and stored at  $-18^{\circ}\text{C}$  prior to analysis for methyl ketones.



Preparation of Solvents Used in the Isolation and  
Identification of Methyl Ketones

Hexane and Benzene: High purity grade hexane and reagent grade benzene were treated for removal of carbonyls by the method of Hornstein and Crowe (27), after which they were redistilled.

Chloroform: Reagent grade chloroform was rendered carbonyl-free by the method of Schwartz and Parks (57). The method of Hornstein and Crowe (27) caused decomposition of the chloroform to phosgene and could not be used in these studies.

Ethanol: Ethanol was saturated with DNP-hydrazine and refluxed over trichloroacetic acid for five hours followed by distillation.

Ethylene Chloride: Reagent grade ethylene chloride was distilled over anhydrous potassium carbonate. The distillate was stored over anhydrous potassium carbonate.

Methanol: Reagent grade methanol was purified according to the method of Schwartz et al. (56, p. 37-38).

Nitromethane: Reagent grade nitromethane was distilled over boric acid to remove contaminating impurities.

### Analysis for Methyl Ketones

Formation of the DNP-hydrazones: The reaction column of Schwartz and Parks (57) was used to form the DNP-hydrazones of carbonyls present in the heat treated samples. Ten milliliters of heat treated fat was dissolved in 10 ml of carbonyl-free hexane and allowed to pass into the reaction column by gravity. After the fat-hexane mixture passed into the column, the sides of the chromatographic tube were carefully rinsed with carbonyl-free hexane. Two hundred milliliters of carbonyl-free hexane was then passed over the column and the eluent collected in a 250 ml standard taper flask. The hexane was removed from the DNP-hydrazone-fat mixture by means of a water aspirator with gentle shaking and mild heating. The residue, after removal of hexane, was immediately used in the next step of the analysis.

Removal of the Gross Amount of Fat from the DNP-Hydrazones: The majority of the fat was removed from the hydrazones by modification of the method of Schwartz et al. (55). A ratio of 1:2 of Seasorb 43 to Celite 545 was used instead of 1:1 as previously reported (55). Eight grams of Seasorb 43 and 16 g of Celite 545 were slurried in hexane and poured into a chromatographic tube (2.2 cm i. d. x 40 cm) containing a glass wool plug at the bottom. The slurry

was packed with air pressure. The DNP-hydrazone-fat mixture from the preceding step was dissolved in an equal volume of hexane and applied to the column. After rinsing the sides of the column, the chromatographic tube was filled with carbonyl-free hexane and 1000 ml was allowed to pass over the column. The hexane removed the majority of the milk fat from the deep red band of adsorbed DNP-hydrazones. Although the hydrazones were adsorbed quantitatively on the support the monocarbonyl fraction was adsorbed least strongly and tended to streak; hence a large amount of support was necessary if quantitative results were desired. The DNP-hydrazones were removed from the column with 200 ml of a nitromethane-chloroform solution (1:4). The nitromethane-chloroform solution was evaporated from the DNP-hydrazones by the method used in the preceding step for removal of hexane. The DNP-hydrazone residue was immediately used in the next step of the analysis.

Removal of Excess DNP-Reagent: The DNP-hydrazones obtained from the previous step were dissolved in a minimum volume of methanol-benzene (1:1) and passed over Dowex 50 X 8 ion exchange resin as described by Schwartz et al. (56). The excess DNP-hydrazine was removed by the resin. The DNP-hydrazones passed through the resin with the methanol:benzene solvent. The methanol:benzene solvent was removed from the reagent-free DNP-hydrazones

by a water aspirator. The removal of excess DNP-hydrazine was not a necessity but the chromatographic behavior of the DNP-hydrazones was improved by including the ion exchange procedure.

Removal of Trace Amounts of Fat: The method of Schwartz et al. (55) for removal of the gross amount of fat from the DNP-hydrazones was modified in order to remove the final traces of fat from the DNP-hydrazones. The following alterations in the procedure were made: Seasorb 43 and Celite 545 in a ratio of 3:4 were slurried in carbonyl-free benzene and poured into a chromatographic tube with a glass wool plug at the bottom. The slurry was packed with air pressure. Nine grams of Seasorb 43 and 12 g of Celite 545 were sufficient for this procedure. The reagent-free DNP-hydrazones from the preceding step were applied to the column in a minimum amount of carbonyl-free benzene. After careful rinsing of the flask and the sides of column, the chromatographic tube was filled with carbonyl-free benzene and 1000 ml of this solvent was passed over the column. The DNP-hydrazones were adsorbed, appearing as a brown band at the top of the column. The carbonyl-free benzene removes the final traces of fat from the adsorbed DNP-hydrazones. The DNP-hydrazones then were eluted from the column with 200 ml of nitromethane chloroform (1:4). The solvents were removed from the DNP-hydrazones with a water aspirator. At this point in the analysis

the samples were stored in the dark at room temperature for various lengths of time. Since essentially all the fat was removed from the DNP-hydrazones, the possibility of artifacts arising from autoxidation of the oil residue was eliminated.

Class Separation: The DNP-hydrazones were separated into classes by the procedure of Schwartz et al. (58) with the following modifications: Seasorb 43 was heated at 400°C for 48 hours and Celite 545 was heated at 150°C for 24 hours. Columns were effective in separating the saturated methyl ketones from alk-2-enals, and alk-2,4-dienals when the modifications in heat treating of the supports were employed. The fat free DNP-hydrazones from the preceding step were taken up in a minimum volume of ethylene chloride and applied to the column. The DNP-hydrazones were separated into classes using ethylene chloride as a developing solvent. The initial grey band, containing the methyl ketone derivatives, was collected and evaporated to dryness by means of a water aspirator.

The methyl ketone derivatives were applied to a second class separation column to remove the acetone derivative from the other DNP-hydrazones. The Seasorb 43 used in this column was heated at 380°C for 48 hours. The developing solvent, ethylene chloride, resolved the derivatives into two chromatographic bands. Each band was collected separately and evaporated to dryness with a water aspirator.

### Qualitative and Quantitative Identification of the Methyl Ketone

Class: The two bands eluted from the class column were qualitatively checked for class by the chromatographic procedure of Gaddis and Ellis (19) and the alkali fading reaction reported by Jones et al. (32). The concentration of the methyl ketone class was quantitatively determined by measurement of absorbance at the ultraviolet maximum of the DNP-hydrazones in chloroform.

### Separation of the Methyl Ketone Class into Individual Members:

The two chromatographic bands qualitatively and quantitatively identified as methyl ketone derivatives, obtained from the class column, were subjected to the liquid-liquid partition column described by Day and Keeney (9) and further elaborated by Day et al. (8). Columns were prepared according to method B (8, p. 465). The faster of the two bands on the class separation column contained the  $C_5$ ,  $C_7$ ,  $C_9$ ,  $C_{11}$ ,  $C_{13}$ , and  $C_{15}$  methyl ketone derivatives. The slower band from the class column which was applied to a separate partition column contained the  $C_3$  derivative. The DNP-hydrazone of acetone travels very slowly in the partition system so it was advantageous to have it separated from the rest of the methyl ketone derivatives. The  $C_{15}$  and  $C_{13}$  methyl ketone derivatives did not separate well on the initial partition column. Improved separation was achieved when the pooled ( $C_{15}$  and  $C_{13}$ ) bands were applied to a column (1.2 cm i. d. x 70 cm)

containing 16 g of support. The longer column enabled complete separation of these two derivatives.

#### Determination of Methyl Ketone Chain Length and Concentra-

tion: The volume of mobile phase required to move a particular hydrazone derivative through a defined amount of partition column (threshold volume) is reproducible (13); hence, the threshold volume provided an indication of the chain length of the methyl ketone derivative in each chromatographic band. The chain lengths of the ketone derivatives were then confirmed by the paper chromatography procedures of Huelin (20) and Klein and De Jong (34). The concentration of each methyl ketone derivative was calculated from the absorbance taken at 363 millimicrons in chloroform.

#### Estimation of the Water Content of Vacuum Degassed Milk

Fat: The amount of water in vacuum degassed milk fat was determined by near infrared spectrophotometry. The method was first described by Meeker et al. (43) and recently applied to milk fat by Kliman and Pallansch (35). The procedure outlined by Kliman and Pallansch was used.

## RESULTS AND DISCUSSION

### Quantitative Evaluation of the Analytical Procedure

The analytical procedure was evaluated using a standard solution of the  $C_3$ ,  $C_5$ ,  $C_7$ ,  $C_9$ ,  $C_{11}$  and  $C_{13}$  n-alk-2-ones. The ketones were diluted with carbonyl-free hexane so that their amounts would be similar to those detected in the analysis of a heat treated milk fat sample. The diluted standard was analyzed exactly in the same manner as a heat treated sample. The quantitative recovery of total methyl ketones, after each step in the analysis procedure, was calculated from the absorbance of the derivatives in chloroform at 363 millimicrons. The quantitative recovery of each methyl ketone at the completion of the entire analysis procedure was determined and is presented in Table 1.

Formation of the DNP-hydrazones: The method of Schwartz and Parks (57) for the direct reaction of lipid with DNP-hydrazine was excellent in conversion of methyl ketones to their DNP-hydrazones. The main advantage of this method was that it enabled a study of the factors influencing methyl ketone formation. The method of steam stripping or distillation as used by other investigators (3; 38; 44; 46 and 48) causes ketone formation. Large quantities of water also are



required to recover longer chain length ketones which makes recovery of the ketones from the distillate cumbersome. The method used here presents no such difficulties. The quantitative conversion of the methyl ketones to their DNP-hydrazones in the standard mixture was 100.00%.

Removal of the Gross Amount of Fat from the DNP-hydrazones:

The chromatographic procedure for isolation of the DNP-hydrazones from lipid resulted in a 92.74% recovery of the derivatives.

Removal of Excess DNP-hydrazine: The ion exchange procedure caused some loss and destruction of DNP-hydrazones. The recovery was 87.17%

Removal of Trace Amounts of Fat: If this step was not employed, derivative separation and identification in later steps of the analysis was not discrete. The method of Schwartz et al. (56, p. 39) for removal of trace amounts of fat from DNP-hydrazones resulted in decomposition of resin in the organic solvents and inconsistent recoveries of DNP-hydrazones. Modification of the Schwartz et al. (55) procedure, as described in this thesis, was more effective in removing the traces of fat from the DNP-hydrazones and although recoveries were low (62.76%), the loss was justified in that one could obtain better separation of the fat-free derivatives in subsequent steps in the analysis.

Class Separation: Separation of the DNP-hydrazones into classes by the procedures of Schwartz et al. (58 and 59) were not satisfactory for these studies. By modification of the original procedure (58) satisfactory separation was obtained. The recovery for the initial separation of the methyl ketone derivatives from the other DNP-hydrazones was 96.61%. The second class column used to separate acetone from the bulk of the methyl ketones had a recovery of 97.74%.

Separation of the Methyl Ketone Class into Individual Members:

The liquid-liquid partition column of Day et al. (8, p. 465) provided an excellent method for separation of the homologous series of methyl ketones into individual members. The recovery was 98.00%.

The recovery of each n-alkyl methyl ketone at the completion of the analysis procedure is reported in Table 1. The value shown for butanone-2 is an average of that for pentanone-2 and acetone and that shown for pentadecanone-2 is an average of the recoveries for tridecanone-2, undecanone-2 and nonanone-2.

TABLE 1

Recovery of Each Methyl Ketone at Completion  
of the Analytical Procedure

n-alk-2-one chain length	Percent recovery
C <sub>15</sub> <sup>*</sup>	53.37
C <sub>13</sub>	51.12
C <sub>11</sub>	54.32
C <sub>9</sub>	54.68
C <sub>7</sub>	47.77
C <sub>5</sub>	39.02
C <sub>3</sub>	30.91
C <sub>4</sub> <sup>*</sup>	34.94

\* Calculated from average values discussed above.

#### Total Methyl Ketone Formation

##### Determination of Temperature Region Most Favorable to

Ketone Formation: The quantity of methyl ketones formed in fat samples heated for 30 minutes at controlled temperatures is shown in Table 2. The temperature range from 120°C to 140°C appears most favorable for ketone formation using treatment temperatures where degradation and hydrolysis of the fat was minimized (36).

TABLE 2  
Influence of Temperature on Total Methyl Ketone Formation<sup>a</sup>

°C	<u>Total Methyl Ketones</u> Millimoles per kilogram fat
40	0.058
60	0.106
80	0.125
100	0.167
120	0.557
140	0.568

<sup>a</sup> All treated samples were aliquots of a single milk fat sample.

Influence of Water, Air and Temperature on Total Ketone

Formation: The total methyl ketone formation as influenced by water, air and temperature is recorded in Table 3. The addition of water to sample 5 enhanced total methyl ketone formation at 140°C (compare with sample 1) but addition of water to sample 6 which was heated to 200°C (compare with sample 2) did not. In the later sample, the 200°C treatment probably approaches pyrolysis conditions (6) and secondary reactions of the methyl ketones with lipid or hydrolytic lipid products, formed during the heat treatment may have influenced the results. Although acid catalyzed aldol type condensations are not favored with ketone substrates they have been reported (5, p. 291; 51, p. 740). Conditions for such condensations are more drastic than

TABLE 3  
Influence of Water, Air and Temperature on  
Total Ketone Formation<sup>a</sup>

<u>Sample Number</u>	<u>Temperature</u> ° C	<u>Time</u> hr	<u>Fat Condition</u>	<u>Total Methyl Ketone</u> Millimoles per kilogram fat
1	140	0.5	Degassed at 40°C	0.846
2	200	0.5	Degassed at 40°C	1.145
3	140	3.0	Degassed at 40°C	1.733
4	140	18.0	Degassed at 40°C	1.679
5	140	0.5	1 ml of H <sub>2</sub> O added Degassed frozen	1.364
6	200	0.5	1 ml of H <sub>2</sub> O added Degassed frozen	0.851
7	140	0.5	Treated in air	1.008
8	140	0.5	1 ml H <sub>2</sub> O added Treated in air	0.834
9	40	0.5	Control	0.083
10	140	0.5	Dried over CaH <sub>2</sub> Degassed at 40°C	0.081

<sup>a</sup> All treated samples were aliquots of a single milk fat sample.

would be found in the system studied here; therefore reduction in total methyl ketones shown for sample 6 probably is not related to this type of reaction. Kritchevsky et al. (36) reported that heat effected hydrolysis of triglycerides. They found that the amount of hydrolysis occurring in fat during heating was a function of temperature, duration of heating and water content. The heat treated fat was probably acid in pH and contained an abundance of degraded lipid products.

These degradation products, in conjunction with added water, must have served in a yet unrecognized reaction that caused reduction in the amount of total methyl ketones.

The maximum total methyl ketones formed during a half hour heating period was noted in the sample containing 10% water (v/v) and heated at 140°C (sample 5). Water should enhance hydrolysis of the  $\beta$ -ketoesters in milk fat, and the resulting  $\beta$ -ketoacids would decarboxylate on gentle heating to give methyl ketones.

In the absence of added water, an increase in the amount of total methyl ketones occurred when the temperature was raised to 200°C (compare sample 1 and 2). Since the addition of water to fat will enhance hydrolysis and degradation (36 and 6), samples that were relatively water free should be more stable at higher temperatures than those containing water. Thus the fat heated at 200°C (sample 2) and relatively moisture free should be less susceptible to the secondary reactions discussed in reference to sample 6. In the case of sample 2, it is believed that decomposition of  $\beta$ -ketoesters in the fat was not complicated by secondary reactions; hence the greater total methyl ketones noted.

Samples 7 and 8 were heat treated by refluxing at atmospheric pressure. These treatments enabled a study of the influence of air on total methyl ketone formation. It is evident that heating the fat in air increases total ketone formation (sample 7 and 1). This is

probably due to the effect of oxygen at atmospheric pressure. The total methyl ketones detected in fat treated in air undoubtedly were less than were actually formed. The difference is attributed to the loss to the atmosphere of the more volatile ketones. When water was added to fat heated at atmospheric pressure (sample 8) there was a reduction in total ketones detected. This decrease in concentration may be partially attributed to the same factors accounting for ketone reduction in sample 6. Loss of ketones due to solubility in water and to volatility also were important.

The effect of longer heating periods is shown by comparing samples 3 and 4 with sample 1. When the heating time was increased six-fold or thirty-six-fold the total ketone formation increased approximately two-fold. In these studies maximum ketone formation in milk fat occurred during the first three hours of heat treatment.

Assuming that the precursors of the methyl ketones were  $\beta$ -ketoesters (68), water would be needed in the hydrolysis reaction. If one molecule of water were used per  $\beta$ -ketoester linkage, sample 3 would require 0.0029% water to give the quantity of ketones found. By measurement of the absorbance of a solution of fat in carbon tetrachloride at 1.87 microns, which is the characteristic absorption band for water (35), it was found that a sample which had been degassed at two to five microns pressure for one hour contained 0.27%

water. Therefore sample 3 contained nearly 100 times an excess of water for the hydrolysis reaction. Apparently milk fat binds or tenaciously holds these last traces of water which makes it difficult to prepare a dry sample by conventional methods.

If the fat were completely dry there should be a significant reduction in the total methyl ketones formed during heat treatment. This is illustrated by sample 10, Table 3, where the fat that had been dried over calcium hydride (35) prior to heat treatment shows a great reduction in total ketones (compare to sample 1). Obviously, water is a limiting factor in ketone formation. Nawar et al. (44) reported that their fat was moisture free, but in reality it must have contained water which is required for ketone formation.

#### Quantitative Determination of Individual Methyl Ketones in Treated Milk Fat Samples

The methyl ketones from each of the treatments reported in Table 3 were separated into an homologous series of odd numbered carbon n-alk-2-ones and those found are reported in Tables 4, 5 and 6. Sample numbers used in the Tables correspond to samples and treatments recorded in Table 3.

The composition of the methyl ketone mixture isolated from heat treated fat samples is reported in micromoles per kilogram of fat in Table 4. The concentrations of the ketones formed varied according to treatment. The ketone produced in maximum concentration in samples 3, 6, 7 and 8 was heptanone-2 and in samples 1, 2, 4 and 5 acetone was in highest concentration.



TABLE 4

Composition of the Methyl Ketone Mixture Isolated from Heated Milk

Sample Number	Fat Samples <sup>a</sup>							
	n-alk-2-one Chain Length							
	C <sub>15</sub>	C <sub>13</sub>	C <sub>11</sub>	C <sub>9</sub>	C <sub>7</sub>	C <sub>5</sub>	C <sub>3</sub>	C <sub>4</sub>
1	153.00	75.47	63.33	71.56	190.63	92.28	199.30	---
2	215.79	122.21	69.07	87.07	247.88	119.82	283.45	---
3	287.67	229.99	119.82	124.59	331.81	235.85	239.97	182.93
4	198.33	122.64	79.81	93.58	304.59	218.93	477.11	183.58
5	196.27	129.58	82.84	107.13	285.51	183.15	378.87	---
6	153.65	99.11	74.06	70.37	210.26	121.99	121.34	---
7	213.73	117.98	72.22	82.84	222.18	126.44	172.20	---
8	135.11	86.31	47.49	65.28	208.85	126.44	164.50	---
9	---	---	21.36	19.08	30.15	12.47	---	---

<sup>a</sup>Expressed as micromoles per kilogram of fat.

TABLE 5

Percent Composition of the Methyl Ketone Mixture Isolated from

Sample Number	Heated Milk Fat Samples							
	n-alk-2-one Chain Length							
	C <sub>15</sub>	C <sub>13</sub>	C <sub>11</sub>	C <sub>9</sub>	C <sub>7</sub>	C <sub>5</sub>	C <sub>3</sub>	C <sub>4</sub>
1	18.04	8.93	7.49	8.46	22.54	10.91	24.57	---
2	18.84	10.67	6.03	7.60	21.64	10.46	24.75	---
3	16.41	13.12	6.84	7.11	18.93	13.46	13.69	10.44
4	11.81	7.31	4.76	5.58	18.15	13.04	28.42	10.94
5	14.40	9.50	6.08	7.88	20.94	13.43	27.79	---
6	18.06	11.65	8.71	8.27	24.71	14.34	14.26	---
7	21.21	11.71	7.17	8.22	22.05	12.55	17.09	---
8	16.20	10.35	5.69	7.83	25.04	15.16	19.72	---
9	---	---	25.72	22.98	36.29	15.01	---	---

TABLE 6

Concentration of Methyl Ketones Isolated from Heated Milk

Sample Number	Fat Samples <sup>a</sup>							
	n-alk-2-one Chain Length							
	C <sub>15</sub>	C <sub>13</sub>	C <sub>11</sub>	C <sub>9</sub>	C <sub>7</sub>	C <sub>5</sub>	C <sub>3</sub>	C <sub>4</sub>
1	35	15	11	10	22	8	12	--
2	49	22	12	12	28	10	17	--
3	65	46	20	18	38	20	14	13
4	45	24	13	13	35	19	28	13
5	44	24	14	15	33	16	22	--
6	34	18	13	10	24	11	7	--
7	48	22	12	12	25	11	10	--
8	31	16	8	9	24	11	10	--
9	--	--	4	3	3	1	--	--

<sup>a</sup>Expressed as parts per million.

The percentage composition of the methyl ketone mixture isolated from heated milk fat samples is presented in Table 5. If the biosynthesis of fatty acids up to palmitic was accomplished by one enzyme complex (69, p. 390-391) and each ketone arose from the corresponding  $\beta$ -ketoacid, a correlation might be expected between the percentages in Table 5 and the percentage fatty acid composition of milk fat. For example: octanoic acid occurs in relatively low concentration in milk triglyceride (29), yet heptanone-2, which would arise from the eight carbon  $\beta$ -ketoacid occurs in highest concentration in a number of the treatments studied. Obviously, no simple correlation exists. If several enzyme systems for fatty acid biosynthesis are operating in the mammary gland a large pool of octanoic acid might accumulate which could be used in synthesis of longer chain fatty acids. Under these circumstances the amount of  $\beta$ -keto-octanoic acid formed, due to incomplete biosynthesis, would be larger. Should the majority of the  $\beta$ -keto-octanoic preferentially go into triglyceride synthesis, a larger percent of heptanone-2 appearing on heat treatment of milk fat would be expected in relation to its octanoic content. Although there is no direct relation between the other methyl ketones and the respective fatty acid in milk triglyceride, the differences in percentage composition are not so great as is noted with octanoic acid. Data in the literature indicate that fatty acids are

synthesized with keto groups in their chain (33; 39; 69, p. 391) but the scheme, enzyme system or systems, and the intermediates are not completely understood (12, p. 41). Thus, although the methyl ketone precursor is synthesized from acetate in the mammary gland (39), its relationship to the fatty acid composition of milk fat is not obvious.

The parts per million concentration of the methyl ketone mixture isolated from the sample treatments is given in Table 6. Considerable data is available concerning the flavor significance of aldehydes in foods (18, p. 98; 2; 11; 15 and 40) but the significance of methyl ketones has not been thoroughly investigated. Wong et al. (70, p. 1703), using the spray technique (47), reported a flavor threshold in water of 0.6 ppm for heptanone-2 and 7.0 ppm for pentanone-2. A liter of four percent fluid milk containing fat from sample 1, Table 6, would have a heptanone-2 concentration of 0.88 ppm. These data suggest that heptanone-2 would make a significant flavor contribution to dairy products containing heated milk fat. It is also possible that an additive interaction of the methyl ketones, as observed by Nawar and Fagerson (45, p. 109), would result in a perceptible flavor from the ketones. Methyl ketones at concentrations reported in Table 6 could present serious problems to those investigators who are seeking to find new uses for milk fat.

Lawrence (38, p. 167) and Boldingh and Taylor (3, p. 913) used exhaustive steam distillation to determine the maximum concentration of methyl ketones obtainable from milk fat. Their data are shown in Table 7. Comparing these data with that reported for sample 3, Table 6, it is apparent that reaction of fat directly is more sensitive in determination of the long chain length methyl ketones.

TABLE 7

Maximum Concentration of Methyl Ketones by Exhaustive Steam  
Distillation of Milk Fat<sup>a</sup>

	n-alk-2-one Chain Length						
	C <sub>15</sub>	C <sub>13</sub>	C <sub>11</sub>	C <sub>9</sub>	C <sub>7</sub>	C <sub>5</sub>	C <sub>3</sub>
Data of Lawrence (38, p. 167)	46	24	14	16	25	13	42
Data of Boldingh and Taylor (3, p. 913)	42	22	13	9	12	--	--

<sup>a</sup>Expressed as parts per million.

It is of interest that butanone-2, which appeared after three hours of heat treatment and represents a significant portion of the total ketones for samples 3 and 4, was not evident in any other sample treatments. After 18 hours of heat treatment, the concentration of butanone-2 was approximately equal to that of the sample receiving three hours of treatment. The ketone was mentioned as a trace constituent in heated milk fat by Nawar et al. (44), Täufel et al. (63) and Lawrence and Hawke (39); however, none of these investigators found quantities as large as reported here. In these studies, time of

treatment was the major factor influencing its appearance. If it arose by the same mechanism as the other methyl ketones its presence should have been detected in the other treatments (see Table 4). There is little difference in the chemical properties of butanone-2 and pentanone-2 and the corresponding  $\beta$ -ketoacids from which they would be formed. Thus, it is difficult to explain the heating-time effect for butanone-2. van der Ven et al. (68) did not isolate a pyrazolone that would correspond to a five carbon  $\beta$ -ketoacid in milk fat. Butanone-2 must therefore arise via a different mechanism. Keeney et al. (33) found that milk fat contains 5 to 15 micromoles per gram of ketoacid moieties esterified in the glycerides. If a long chain ketoacid with a carbonyl group on the third carbon from the methyl terminal end of the fatty acid and  $\alpha$ ,  $\beta$ -unsaturated at the fourth and fifth carbons were present in milk fat, perhaps the "retrograde aldol condensation" type reaction mentioned by Royals (51, p. 752) could be responsible for formation of butanone-2. This reaction is a reversal of an aldol condensation which would give rise to butanone-2 and a semialdehyde. The effect of heat treatment on the fat and the period of the time it was exposed to heat might have been sufficient to foster such a reaction. This type of mechanism, nevertheless, is speculative since the respective  $\alpha$ ,  $\beta$ -unsaturated keto-acid has not been reported in milk fat.

## SUMMARY AND CONCLUSIONS

This investigation was concerned with the qualitative and quantitative methyl ketone composition of milk fat as effected by temperature during heat treatment, time of heat treatment, presence of oxygen and water.

Milk fat, free from phospholipids, centrifuged at 30,000 x G for 20 minutes, degassed at two to five microns pressure for one hour, was heat treated at controlled temperatures and time periods in the presence and absence of air, with and without added water.

The methyl ketones were isolated from heat treated samples by passing the fat over Celite impregnated with DNP-hydrazine reagent, phosphoric acid, and water which served to form the DNP-hydrazones of the carbonyls in the sample. The fat was separated from the hydrazones by column chromatography, excess reagent was removed by ion-exchange chromatography and the final traces of fat were removed by column chromatography. The hydrazone derivatives were separated into classes by column chromatography. The methyl ketone class was further purified and separated into its individual members by liquid-liquid partition chromatography. Spectrophotometric and paper chromatographic methods were used to confirm the identity of the derivatives.

The findings of this investigation were as follows:

1. An homologous series of odd number carbon n-alk-2-ones from acetone to pentadecanone-2 were isolated from heat treated fats and identified.
2. Butanone-2 appears after three hours of heat treatment and probably arises via different mechanism than the other ketones.
3. The ketones formed in large amounts were acetone, heptanone-2 and pentandecanone-2. Considering the flavor properties of heptanone-2, it probably has a significant influence on the flavor of dairy products that contain heated milk fat.
4. Oxygen was not required for the formation of methyl ketones in heat treated milk fat.
5. The temperature during heating, for a constant time, influences total methyl ketone production. A plateau in ketone formation was approached in the temperature range from 120°C to 140°C when the heat treatment period was 30 minutes.
6. The addition of water to the fat enhanced total methyl ketone production at 140°C but not at 200°C when the treatment time was 30 minutes.
7. Heat treating the fat in the presence of air does not hinder methyl ketone formation in the fat.

8. Maximum ketone formation (1.733 millimoles per kilogram fat) was noted after three hours of treatment at 140°C. Increasing the treatment time to 18 hours did not increase the total methyl ketones formed; rather, a slight decrease in total ketone formation was noted.

9. Milk fat centrifuged at 30,000 x G and vacuum degassed contained enough moisture for methyl ketone formation, if it is assumed that the precursors are those described by van der Ven et al. (68). Hence, conventional methods of removing water from milk fat are not adequate to make it moisture free.

10. Total ketone formation was significantly reduced (0.846 to 0.081 millimoles per kilogram fat) when milk fat, prior to vacuum degassing and heat treatment at 140°C for 30 minutes, was dried over calcium hydride for 18 hours. Thus, water appears to be a limiting factor in ketone formation.

11. The maximum concentration of methyl ketones determined by exhaustive steam distillation of fat (38, p. 167; 3, p. 913) was in fair agreement with the data obtained in this thesis where isolation and identification was studied by entirely different techniques.



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