Fat was separated from commercial samples of 12-month-old Cheddar cheese by centrifugation and then distilled in a wiped-film molecular still. After removal of the free fatty acids by extraction with 1% sodium carbonate, the molecular distillates were separated into three fractions by preparative gas-liquid chromatography (GLC). Each fraction was then analyzed by combined GLC-mass spectrometry. Thirty-three compounds were positively identified by comparison of their mass spectra with reference spectra. Relative retention times obtained by gas-liquid chromatography were used to confirm the mass spectral identifications. Those compounds positively identified included δ-C_{10}, C_{12}, C_{14}, C_{15}, and C_{16} lactones, γ-C_{12}, C_{14}, and C_{16} lactones, ethyl dodecanoate, ethyl tetradecanoate, ethyl hexadecanoate, ethyl octadecanoate, methyl tetradecanoate,
methyl hexadecanoate, methyl octadecanoate, n-C_{16} - C_{25} alkanes, diethyl phthalate, dibutyl phthalate, bis (2-ethylhexyl)phthalate, n-hexadecanal, 3, 5-di-tert-butyl-4-hydroxy toluene (BHT) and benzaldehyde. The following ten compounds were tentatively identified on the basis of mass spectral evidence: 2, 6-di-tert-butyl-4-ethylphenol, 1-octadecanol, dibutyl adipate, ethyl octadecadienoate, ethyl octadecatrienoate, heptadecyl butyrate, octadecyl butyrate, pentadecyl hexanoate and tetracosane (branched). δ-C_{18} lactone was tentatively identified on the basis of GLC retention time.

The lactones constituted approximately 70% by weight of the high boiling compounds in the molecular distillates and experiments were conducted to determine their effect on the flavor of Cheddar cheese. A simulated Cheddar cheese mixture was prepared by blending ketones, aldehydes, acids, and 3-mercaptopropionic acid with safflower oil and washed cottage cheese curds. Informal flavor assessment, by five experienced dairy product judges, indicated that Cheddar cheese aroma of the simulated cheese was enhanced by the addition of δ-dodecalactone and δ-tetradecalactone at 4 and 1.5 parts per million (ppm) respectively.

Alkanes with an even number of carbon atoms were present in the molecular distillates to about the same extent as alkanes with an odd number of carbon atoms. This distribution pattern was more characteristic of petroleum products than milk fat lipids where odd numbered hydrocarbons are generally more prevalent than even
numbered hydrocarbons. Some of the cheeses used in the present investigation were coated with wax while the other cheeses had been wrapped in a plastic film (Parakote). The Parakote film consisted of two sheets of cellophane laminated together and coated on the inner surface with an elastomer-wax mixture. Experiments were conducted to determine if hydrocarbons were diffusing from the packaging materials into the cheese. Molecular distillates of fat from the outer surface and center of cheeses wrapped in Parakote film were analyzed for their hydrocarbon composition by GLC. It was found that there was a slightly higher concentration of hydrocarbons in the outer surface than in the center of the cheeses. However, since the hydrocarbons were present at concentrations of approximately 0.2 ppm (in the cheese fat) in both samples it was difficult to state with any degree of certainty that hydrocarbon compounds had diffused into the cheese from the Parakote film.

More substantial evidence in support of the idea of diffusion was obtained in the case of wax-coated cheeses. It was found that n-alkanes from C$_{21}$ to C$_{25}$ were present at a concentration of 5.00 ppm in the fat from wax-coated cheeses compared to a concentration of 0.24 ppm in the fat from cheeses packaged in Parakote film. Analysis of equal amounts of cheese wax and the elastomer-wax mixture from Parakote film also revealed that n-alkanes were present in cheese wax at levels approximately three times greater than the
corresponding n-alkanes in the elastomer-wax mixture.

Physical contact between the cheese fat and high vacuum greases occurred at three different locations on the molecular still; however, experiments on the molecular distillation of safflower oil indicated that hydrocarbons from the stopcock grease were not contaminating lipid materials in the still.

Although several phthalate esters were identified in the molecular distillates, dibutyl phthalate was the only one present at a significantly high concentration (1.6 ppm of cheese fat). It was found as a plasticizer at a level of 4% in the cellophane sheets of the Parakote film. The film however, cannot be readily implicated as the sole source of the compound, as dibutyl phthalate was also found in wax coated cheeses. There was no evidence to indicate that the compound was derived from experimental procedures and thus it is believed that it may be present in the cheese as a result of the milk coming into contact with plastics such as flexible tubing either at the farm or in the processing plant.

The antioxidant, 3,5-tert-butyl-4-hydroxytoluene (BHT), was found to be present in Cheddar cheese fat at a level of 0.1 ppm. It was also identified in both the cheese wax and the elastomer-wax mixture of the Parakote film at a concentration of 100 ppm. Therefore it would appear that BHT was diffusing into the cheese from the packaging materials. However, BHT is also sometimes used as an
antioxidant in animal feedstuffs and this may also be a source of the compound in Cheddar cheese.

Several species and strains of lactobacilli were added to experimental Cheddar cheeses at the time of manufacture. After a three-month ripening period, when the inoculated cheeses were compared to controls, the former were found to have a more pronounced Cheddar cheese flavor. The lactobacilli, however, were found to have little, if any, influence on the production of neutral high boiling compounds.
Identification of Some Neutral High Boiling Compounds in Molecular Distillates from Cheddar Cheese Fat

by

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IDENTIFICATION OF SOME NEUTRAL HIGH BOILING COMPOUNDS FROM MOLECULAR DISTILLATES OF CHEDDAR CHEESE FAT

INTRODUCTION

The identification of volatile compounds in Cheddar cheese has been of major concern to many investigators. A wide variety of compounds have been identified including free fatty acids, carbonyl compounds, esters, alcohols and sulfur compounds. However, no single compound or combination of compounds can be considered as truly representative of Cheddar cheese flavor.

Although water soluble compounds of low volatility such as sugars, amino acids, peptides and proteins have been well characterized in food products, until recently few studies have been conducted on the analysis of high boiling lipid soluble compounds with molecular weights from 200 to 400. To a large extent this was the result of a lack of suitable techniques for the isolation and adequate characterization of the compounds. High temperatures are necessary to distill compounds of low volatility using conventional distillation apparatus at atmospheric pressure or low vacuum and the high temperatures may result either in the decomposition of certain compounds or in the formation of new compounds not naturally present in the food. This problem can be largely surmounted by the use of laboratory scale wiped-film molecular stills for distillation.
Although volatile compounds are generally considered to be responsible for the aroma properties of foods, there are several instances where compounds having relatively high boiling points have been shown to affect food odor. Patterson (1968) found that the "boar odor" associated with meat from mature or incompletely castrated boars was due to the presence of 4-α-androst-16-ene-3-one in the fat tissue. This compound has a molecular weight of 272.

Other high boiling compounds in foods may possibly be significant from a toxicological viewpoint. Phthalate esters are widely used as plasticizers in synthetic polymers, especially in products made from polyvinyl chloride (PVC). It has recently been shown that fluids perfused through PVC tubing can produce toxic effects in mammalian tissue cultures (DeHaan, 1971).

The object of this investigation was to identify some of the high boiling compounds in Cheddar cheese fat and to determine, if possible, their origin.
REVIEW OF LITERATURE

A considerable amount of work has been done on the characterization of volatile compounds in Cheddar cheese from the standpoint of determining their contribution to flavor. Although various synthetic flavor formulations have been devised, both in academic (Day and Bassette, 1960; Walker, 1961) and industrial laboratories (Henning, 1970; Beatrice Foods, 1971), it has not yet been possible to duplicate a true Cheddar cheese flavor.

Early investigators in this area had hoped to find a single compound which would have a strong Cheddar cheese aroma. Since such a compound has eluded detection, it is currently believed that Cheddar cheese flavor is the result of a blend of many different compounds (Mulder, 1952; Harper, 1959; Kristoffersen and Gould, 1959).

Free Fatty Acids

Fatty acids constitute the major fraction of the volatile compounds found in Cheddar cheese. Bills and Day (1964) obtained quantitative data for the free fatty acids present in 14 different samples of Cheddar cheese. Column and gas-liquid chromatographic (GLC) techniques were used for the analysis of the complete series of fatty acids. Neither formic acid nor propionic acid were detected in
any of the cheeses. Acetic acid was the most abundant acid present, and it also exhibited the greatest variability in concentration. The average concentrations found for the various different acids were as follows (expressed as mg free fatty acid per kg of cheese): 2:0\(^1\), 865; 4:0, 115; 6:0, 38; 8:0, 41; 10:0, 49; 12:0, 81; 14:0, 218; 16:0, 503; 18:0, 172; 18:1, 467; 18:2, 69; 18:3, 40. In the two rancid samples the concentrations of acetic acid were within the expected range, but the other fatty acids were present at levels approximately ten times greater than those found in the 12 normal cheeses.

Patton (1963) used reagents to selectively block the functional groups of free fatty acids and carbonyl compounds in Cheddar cheese slurries and also in distillates from the cheese. He concluded that carbonyl compounds contributed very little to the development of Cheddar cheese flavor, but that the free fatty acids were the most important factor. It was suggested that the more volatile free fatty acids constituted the "backbone" of Cheddar cheese flavor.

Kristoffersen and Gould (1960) analyzed 14 different lots of Cheddar cheese for characteristic flavor, ammonia, hydrogen sulfide, free fatty acids, free amino acids, acidic and neutral carbonyl compounds, pH and total bacterial count. Their results indicated that the ratio between free fatty acids and hydrogen sulfide was important

\(^1\)Number of carbon atoms: number of double bonds.
in the flavor of Cheddar cheese.

Forss and Patton (1963) briefly reviewed the literature on Cheddar cheese flavor, comparing the taste threshold levels of various different compounds with the amounts found by investigators in the cheese. They concluded that the fatty acids made a very important contribution to Cheddar cheese flavor, and that in this group the acetic, butyric and caproic acids had the most significant effects. These acids are present in Cheddar cheese at concentrations greatly exceeding their threshold values in edible oil and in water.

In a more recent study Ohren and Tuckey (1964) demonstrated that typical Cheddar cheese flavor is the result of a balance between the amount of acetic acid and the concentrations of the longer chain free fatty acids. It was also found that typical Cheddar cheese flavor did not develop in cheeses which contained less than 50% fat on a dry weight basis.

Carbonyl Compounds

Both acidic and neutral carbonyl compounds have been identified in Cheddar cheese. The former consist of keto acids while the latter are mainly ketones and aldehydes.

Acidic Carbonyl Compounds

In a study involving four different varieties of cheese, Bassett
and Harper (1958) found the following keto acids in Cheddar cheese: \( \alpha \)-keto glutaric, oxalacetic, pyruvic, \( \alpha \)-acetolactic and \( \alpha \)-keto isovaleric. These compounds were identified by paper chromatography of their 2,4-dinitrophenylhydrazone (DNPH) derivatives. It was found that \( \alpha \)-acetolactic, \( \alpha \)-keto glutaric and pyruvic acids were relatively abundant; \( \alpha \)-keto isovaleric was less abundant and oxalacetic acid occurred in trace amounts.

Kristoffersen and Gould (1959) identified all of the above compounds with the exception of \( \alpha \)-keto isovaleric acid. In addition these workers also found oxalsuccinic, glyoxylic and \( \alpha \)-keto isocaproic acids.

**Neutral Carbonyl Compounds**

Considerable emphasis has been placed on the analysis of these compounds, as they readily form stable chemical derivatives which can be identified by classical techniques.

Patton et al. (1958) converted the carbonyl compounds in a steam distillate from Cheddar cheese into their DNPH derivatives. The derivatives were separated by paper chromatography, and 2-butanone and 3-hydroxybutanone were conclusively identified by comparison of their melting points, \( R_f \) values and UV absorption maxima with the same parameters determined for known compounds. Acetaldehyde, 2-heptanone, acetone and formaldehyde were only
tentatively identified from \( R_f \) and absorption maxima data, as the amounts of derivatives available were insufficient for the determination of melting points. Day et al. (1960) also analyzed the carbonyl compounds in a steam distillate of Cheddar cheese. After reaction with 2, 4-dinitrophenylhydrazine, the DNPH derivatives were separated by column chromatography and identified by comparison of their chromatographic threshold volumes, melting points and absorption maxima with the same parameters determined for known compounds. The following compounds were conclusively identified: acetone, butanone, 2-pentanone, 2-heptanone, 2-nonanone, methanal, ethanal, propanal, 3-methylbutanal and 3-methylthiopropanal (methional). The ketones 2-undecanone, 2-tridecanone, 3-hydroxy-2-butanone (acetoin), and 2, 3-butanedione (diacetyl) were only tentatively identified as their DNPH derivatives were not sufficiently crystalline for melting point determinations.

In addition to several other classes of compounds, Day and Libbey (1964) identified many carbonyl compounds in Cheddar cheese fat. The lipid fraction was obtained from intact Cheddar cheese by centrifugation and the cheese oil was then passed through a wiped-film molecular still. The compounds distilled and present in the liquid nitrogen trap were identified by combined GLC and mass spectrometry. They found all the carbonyl compounds previously identified by Day et al. (1960) with the addition of 2-pentadecanone.
Certain β-keto esters are normal constituents of milk fat glycerides (Ven et al., 1963). After hydrolysis of the esters, the resulting β-keto acids readily decarboxylate to form methyl ketones. Lawrence (1963) concluded that these reactions could be induced by heat during the course of atmospheric steam distillations and that as a result most of the methyl ketones should be considered as artifacts. However, Day and Libbey (1964) considered it unlikely that the mild distillation treatment used in their study would result in degradation of the ketone precursors. They were led to believe that the methyl ketones could arise from β-keto esters as a result of natural lipolysis during the course of cheese ripening. Scarpellino and Kosikowski (1962) postulated that butanone can be formed from acetoin by a series of reactions.

The lower molecular weight aldehydes can readily be formed from the transamination and decarboxylation of amino acids (MacLeod and Morgan, 1958) by bacteria in the cheese. Certain aldehydes can also be formed as a result of the Strecker degradation of amino acids (Kenney and Day, 1957).

**Sulfur Compounds**

Several sulfur compounds have been identified in Cheddar cheese and it is believed that they may make a significant contribution to the
overall cheese flavor as they generally have strong, pungent aromas. Kristoffersen and Gould (1960) suggested that the ratio of free fatty acids to hydrogen sulfide was a significant factor in Cheddar cheese flavor. For the production of a synthetic Cheddar cheese flavor, Walker (1961) found that it was necessary to add thioacetamide as a source of hydrogen sulfide to a mixture of free fatty acids and carbonyl compounds. Forss and Patton (1966) pointed out that although hydrogen sulfide is a necessary adjunct to Cheddar cheese flavor its overall concentration may not be that important as the concentration of the compound remains relatively constant throughout the aging period.

Keeney and Day (1957) noted that methional formed by the Strecker degradation of L-methionine had a strong cheese-like aroma. Later work produced evidence that methional was present in Cheddar cheese at a level of 0.1 parts per million (ppm) and it was incorporated into a synthetic flavor mixture at a level of 1 ppm (Day et al., 1960). However, it is also known that methional is unstable and readily decomposes in the presence of Strecker degradation reagents such as diacetyl to produce acrolein, dimethyl sulfide and methyl mercaptan (Ballance, 1961). The two sulfur compounds formed in this reaction have been identified in Cheddar cheese. Libbey et al. (1963) used a wiped-film molecular still for the isolation of flavor compounds from Cheddar cheese fat. By means of GLC retention data, methyl mercaptan and several other alkyl mercaptans were tentatively identified in distillate
from the liquid nitrogen cold trap. More conclusive evidence (Libbey and Day, 1963) for the presence of methyl mercaptan was obtained by the use of thin-layer chromatography. The volatiles, collected in a liquid nitrogen cold trap after the vacuum distillation of Cheddar cheese fat, were reacted with 2, 4-dinitrofluorobenzene (DNFB). After the DNFB derivatives had been extracted and purified, they were separated by thin-layer chromatography. The C$_1$-dinitrophenylsulfide (DNPS) derivative had an $R_f$ identical to that found for the known C$_1$-derivative. Both derivatives also showed UV absorption maxima at 321 nm. From the intensity of the UV absorption it was estimated that methyl mercaptan was present in Cheddar cheese at a concentration between 3 and 30 parts per billion (ppb). Even at this low level the compound could contribute to Cheddar cheese flavor as Day et al. (1957) found that the flavor threshold of methyl mercaptan was 2 ppb in water.

By means of GLC and mass spectrometry, Patton et al. (1958) identified dimethyl sulfide in a steam distillate from Cheddar cheese. McGugan et al. (1968) examined the volatiles from three Cheddar cheeses each of which had been prepared under different conditions. Aseptic vats were used to prepare two of the cheeses, a regular starter culture being utilized for one cheese and the chemical acidulant 5'-gluconolactone for the other. The third cheese was prepared with a regular starter culture in an open vat. After the fat had been
separated from each cheese by centrifugation, it was then subjected to high-vacuum distillation. The cold trap was modified after each distillation so that vapor samples of the distillate could be obtained. The volatiles were examined by GLC using capillary columns coupled to a mass spectrometer. When the volatiles found in the cheeses prepared with starter bacteria were compared with those identified in the cheese made with δ-gluconolactone, the only differences noted were the higher concentrations of dimethyl sulfide and dimethyl disulfide in the former. However, it was found in the case of each cheese that when the effluents from the chromatographic columns were trapped ("total trapping"), their aromas no longer bore any resemblance to those associated with the original injected samples.

**Alcohols**

Ethanol and 2-butanol are the most prominent alcohols in Cheddar cheese and their presence has been widely reported in the literature (Dacre, 1953; Patton et al., 1958; Scarpellino and Kosikowski, 1962). Other secondary and primary alcohols have also been found in Cheddar cheese (Day and Libbey, 1964; McGugan et al., 1968; Liebich et al., 1970). They could be formed from reduction of the corresponding aldehydes and ketones by alcohol dehydrogenase enzymes of bacterial origin (Day, 1967).

Trace amounts of methanol have recently been found in Cheddar
cheese (McGugan et al., 1968). No known metabolic pathway can account for the presence of this compound in Cheddar cheese (Day, 1967).

**Esters**

Suzuki et al. (1910) suggested that esters of fatty acids might occur in relatively high concentrations in Cheddar cheese, although their presence has only recently been confirmed by several investigators (McGugan and Howsam, 1962; Day and Libbey, 1964; Bills et al., 1965; McGugan et al., 1968; Liebich et al., 1970). Ethyl esters are found in relatively higher concentrations than the corresponding methyl esters. This is as one would expect from a comparison of the previously reported levels of ethanol and methanol.

Reddy et al. (1969) found that the flavor thresholds of ethyl butyrate and ethyl hexanoate in homogenized milk were 0.015 and 0.021 ppm respectively. Bills (1966) estimated from GLC data that the concentrations of ethyl butyrate ranged from 0.7 to 4.7 ppm and ethyl hexanoate from 0.3 to 2.2 ppm in good quality Cheddar cheese. However, in cheeses which had developed the fruity flavor defect, these concentrations were higher by a factor of 4 or 5. It would therefore appear that ethyl esters should make a positive contribution to the flavor of Cheddar cheese at lower concentrations, but in excessive amounts they can be responsible for the
Lactones

The discovery of lactones in dairy products resulted from an investigation to determine the origin of the coconut flavor defect which develops in dried whole milk during storage. Keeney and Patton (1956a, b) demonstrated that this off-odor could principally be attributed to the presence of \( \delta \)-decalactone.

Since that time several other \( \gamma \)- and \( \delta \)-lactones have been found in milk fat (Tharp and Patton, 1960; Boldingh and Taylor, 1962; Parliament et al., 1965; Wyatt et al., 1967a) and butterfat (Boldingh and Taylor, 1962; Jurriens and Oele, 1965; Forss et al., 1966). Recently, trace quantities of an unsaturated lactone (Van der Zijden, 1966) and a branched chain lactone (Honkanen et al., 1968) have been identified in milk fat.

Lactones have not been extensively investigated in cheese products. Day and Libbey (1964) found \( \delta \)-octalactone and \( \delta \)-decalactone in the cold trap isolate after high-vacuum distillation of Cheddar cheese fat. Liebich et al. (1970) reported the presence of \( \delta \)-C\(_{10}\), C\(_{12}\), C\(_{14}\) and C\(_{16}\) lactones in a high-vacuum distillate of Cheddar cheese fat. By utilizing the molecular distillation technique of Libbey et al. (1963), O'Keefe et al. (1969) identified all of the above lactones in the cold finger molecular distillate. In addition,
several other lactones were found; these were the $\delta-C_{15}$ lactone and $\gamma-C_{12}$, $C_{14}$ and $C_{16}$ lactones. The $\delta-C_{18}$ lactone was tentatively identified by its retention time in GLC analysis but no mass spectral evidence was available for conclusive identification.

A considerable amount of work has been done to determine the origin of lactones in milk fat. From the results of low temperature crystallization studies in acetone, Mattick (1959) was led to conclude that lactones were bound as simple esters. However, all the evidence currently available indicates that lactones are formed from monohydroxy-acyl-triglycerides. By utilizing various thin-layer and column chromatographic techniques, several investigators (Parliament et al., 1966; Kinsella et al., 1967; Wyatt et al., 1967b) have isolated polar fractions from milk fat which on heating formed lactones. These compounds had the chromatographic properties of diglycerides, but their molecular weights were in the range one would expect for triglycerides (Parliament et al., 1966). Jurriens and Oele (1965) and Dolendo et al. (1969) have synthesized monohydroxy-acyl-triglycerides and it was found that lactones were formed by heating the triglycerides in the presence of water. This evidence supports the hypothesis that monohydroxy-acyl-triglycerides are the natural precursors of lactones in milk fat.

Lactones exhibit quite a wide range of flavor properties and their odors have been variously described as peachy ($\delta-C_{10}$),
spicy (\(\gamma-C_{11}\)), coconut (\(\delta-C_{12}\)), and musk (\(\delta-C_{15}\)) (Bedoukian, 1967). Siek et al. (1969) found that \(\delta\)- and \(\gamma\)-lactones have low flavor thresholds in butter oil. The lactones were reported to have individual threshold levels ranging from about 1 ppm for \(\gamma-C_{11}\) to 500 ppm for \(\delta-C_{14}\). Lactones in low concentration have been shown to be important in butter flavor (Boldingh and Taylor, 1962; Pardun, 1963) and patents have been filed for the addition of \(\delta\)-decalactone and \(\delta\)-dodecalactone to margarine (Boldingh et al., 1956; Woode and Holm, 1959).

**Other Compounds Isolated by High Vacuum Techniques**

In an investigation of volatiles in Cheddar cheese fat using a molecular still, Day and Libbey (1964) found four aromatic hydrocarbons and one unidentified sesquiterpene. McGugan et al. (1968) identified traces of hydrocarbon compounds, mainly aromatic, in a vapor sample obtained from a cold trap following high-vacuum distillation of cheese oil. In their study of Cheddar cheese volatiles, Liebich et al. (1970) used the following techniques to prepare cheese samples for analysis: (1) centrifugation of cheese with subsequent direct injection of the cheese oil onto a GLC column, (2) vacuum distillation of cheese oil, whole grated cheese and spray dried cheese, and (3) extraction of the cheese oil with methanol. In addition to the normally expected volatiles they found a large variety of alkanes, olefins
and aromatic hydrocarbons together with the terpenes; α-pinene, β-pinene, camphene and limonene. Many of these compounds could have been heat induced as for the most part they were present only in the distillate obtained from spray dried cheese. It is also possible that some of them could have been produced by bacterial metabolism in the cheese.

The Contribution of Non-starter Microorganisms to Cheddar Cheese Flavor

Lactobacilli are the principal microorganisms associated with the ripening of Cheddar cheese (Marth, 1963). Lactobacillus casei and L. plantarum are the most numerous among the species of lactobacilli present in the cheese, and it is believed that they make a positive contribution to the development of Cheddar cheese flavor (Marth, 1963).

Bullock and Irvine (1956) found that the amino acid levels of cheese inoculated with L. casei and control cheese were similar at four months, but at eight and one-half months higher levels of amino acids appeared in the inoculated cheese. Yates et al. (1955) observed that addition of proteolytic strains of L. casei to Cheddar cheese resulted in accelerated flavor development and improved flavor in mature cheese.

Lactobacilli are generally considered to be "weakly lipolytic" during their normal life cycle. Peterson and Johnson (1949), however, found that certain cultures of L. casei demonstrated lipolytic
activity after 60 days growth and subsequent autolysis in a culture medium containing milk fat and adjusted to pH 5 to 6. From this observation it was concluded that the lipase enzymes were intracellular and could be released only by autolytic action.

Several investigators have demonstrated that cultures of L. casei (Kristoffersen and Nelson, 1954, 1955; Sharpe and Franklin, 1962) and L. plantarum (Sharpe and Franklin, 1962) are capable of producing hydrogen sulfide.

Although the population of micrococci tends to decrease during the course of ripening, it is believed that they aid in the development of Cheddar cheese flavor during the early stages of the aging process (Marth, 1963). Micrococcus freudenreichii possesses proteolytic enzymes with pH and temperature optima very similar to those found for proteolytic enzymes isolated from one-year-old Cheddar cheese (Baribo and Foster, 1952). From these results it was concluded that micrococci could conceivably contribute to the total proteolytic activity in Cheddar cheese. Micrococci display little lipolytic activity during their life cycle, but it was found that cultures of M. conglomeratus, M. freudenreichii and M. caseolyticus can produce considerable amounts of volatile fatty acids after incubation in a milk fat medium for 60 days (Peterson and Johnson, 1949). From this it was reasoned that the lipase enzymes were intracellular and after autolysis of the bacterial cells, the enzymes might contribute to the
formation of volatile fatty acids in the cheese.

Many studies have been conducted in which the cheese milk was inoculated with cultures of lactobacilli or micrococci prior to the addition of rennet. The results of these investigations were discussed by Marth (1963) in a review of the chemical and microbiological aspects of Cheddar cheese ripening. In many instances there was either an acceleration of flavor development or an improvement in flavor of the mature cheese. Recently a patent has been filed (Beatrice Foods, 1971) for the formation of Cheddar cheese flavor by the addition of a culture of *M. caseolyticus* to an acidified aqueous slurry of fat, protein and a source of lactose.
EXPERIMENTAL

Commercial Samples of Cheddar Cheese

The initial experiments were carried out with 12-month-old 20-lb. wheels of Cheddar cheese which had been coated with wax. The cheeses were of medium flavor intensity and were obtained from a local cheese processing plant. Later studies involved the use of 12-month-old 40-lb blocks of Cheddar cheese which had been wrapped in Parakote, a plastic packaging film consisting of two layers of cellophane bound together by an elastomer-wax mixture and also coated on the interior surface with elastomer-wax.

Extraction of Fat from the Cheese

The method used was essentially that of McGugan and Howsam (1962) and Libbey et al. (1963). The protective wrapping material together with a 1/4-in. surface layer was removed from the cheese which was then cut up into small cubes and packed into Servall centrifuge tubes which were made of either stainless steel or polyethylene. The filled tubes were heated for 10 min in a 40°C water bath in order to facilitate separation of the fat (Liebich et al., 1970). Centrifugation was carried out in a Servall SS-3 centrifuge which was

1 Manufactured by American Can Company, Menasha, Wisconsin.
maintained at 30,000 x g for 20 min. The centrifuge was initially at room temperature but after centrifugation the rotor had reached a temperature of 40°C as a result of air friction.

It was found that 1.20 l of cheese oil were obtained from 6.8 kg of cheese. This was a yield of 60% considering that Cheddar cheese is approximately 30% fat.

Molecular Distillation of Cheese Fat

A Rota-Film Molecular Still² Model 30-2, was used to isolate the high boiling compounds from Cheddar cheese fat. The apparatus is shown in diagrammatic form in Figure 1.

With the exception of the precision ground glass stopcocks B and C which were coated with cheese oil, all the other ground glass joints in the system were sealed with Spectro-Vac³, a low vapor pressure stopcock grease. Approximately 1.20 l of cheese oil were placed in the feed flask A after making sure that the stopcocks B and C were in a closed position. The glass tube N was connected by high vacuum butyl rubber tubing to a diffusion pump which was in turn attached to a mechanical rotary pump. Initially the mechanical pump was turned on until most of the air had been removed from the system

² Manufactured by Arthur F. Smith Company, Pompano Beach, Florida.
³ Prepared by Robert R. Austin, Pasadena, California.
Figure 1. Diagram of molecular distillation apparatus.

A - Feed flask  
B, C - Stopcocks  
D - Wiper blade motor  
E - Cold finger cooled by tap water  
F - Cold finger trap  
G - Residue container  
H, I - Vacuum gauge monitoring sites  
J₁, J₂ - Glass connector joints  
K - Liquid nitrogen safety trap  
L - Liquid nitrogen trap  
M - Distillation chamber  
N - Vacuum pump line
and the vacuum gauges registered $10^{-2}$ mm Hg. The safety trap K and the trap L for isolating volatiles were now immersed in Dewar flasks of liquid nitrogen and the diffusion pump was switched on.

Prior to molecular distillation the cheese oil was degassed. This step involved the removal of entrapped air from the oil by slowly opening stopcock C. During the course of this operation many of the more volatile compounds in the cheese lipid were isolated in trap L. After approximately three hours the cheese oil was degassed and the operating pressure of $1 \times 10^{-3}$ to $6 \times 10^{-4}$ mm Hg was attained. This pressure range was considered adequate for the molecular distillation of high boiling compounds in the cheese fat.

The distillation chamber M was then surrounded by a heating jacket which was maintained at a temperature of 60°C and tap water was circulated through the cold finger E. The stopcock B was slowly opened and the oil was allowed to flow from the feed flask A at a rate of approximately 3 ml/min. The oil was maintained in the form of a thin cylindrical film on the walls of the distillation chamber M by means of spring loaded Teflon wiper blades driven by the electric motor D. The residual oil collected in the flask G. As the oil flowed through the distillation chamber M the high boiling compounds were molecularly distilled onto the cold finger E. At the end of the experiment the isolated high boiling compounds were washed from the cold finger E into the cold finger flask F using reagent grade diethyl ether.
In later experiments, Freon 11 (trichlorofluromethane) was used in place of diethyl ether. Since Freon 11 has a boiling point of 23°C, the molecular distillate could be concentrated using somewhat lower temperatures than when diethyl ether was used.

**Removal of Free Fatty Acids from the High Boiling Extract**

Since the free fatty acid composition of Cheddar cheese fat is well known, and as these compounds could interfere with the analysis of the neutral high boiling compounds, it was considered desirable to remove the acidic compounds from the extract. The cold finger isolate (150 ml, including solvent) was extracted three times with 100 ml portions of a 1% NaHCO₃ solution which had been saturated with NaCl. Although the use of a saturated salt solution prevented the formation of a complete emulsion, there was some flocculent material at the interface between the organic and aqueous phases after the first extraction. This flocculent material was centrifuged for 10 min at 30,000 x g in a Servall centrifuge to yield a clear biphasic solution. After the distillate had been extracted three times it was washed with a saturated NaCl solution and then dried over anhydrous granular Na₂SO₄.

**Concentration of High Boiling Extract**

The extract of neutral high boiling compounds was concentrated
initially by removing the major portion of the solvent with a fractional distillation column. The dimensions of the column were 1 x 60 cm and it was packed with glass helices. An electronic device was used to control the reflux ratio so that two parts were distilled while three parts were refluxed. After the extract had been concentrated to approximately 30 ml, the flask was removed from the distillation apparatus. The remainder of the solvent was then evaporated under a carefully regulated stream of nitrogen; the sample flask being maintained at 40 °C in the case of diethyl ether and at 28 °C in the case of Freon 11.

The molecular distillation of 1.20 l of Cheddar cheese oil yielded a high boiling extract of about 50 μl. On occasion traces of a white solid were found suspended in the extract. This material was centrifuged to the bottom of the concentrating tube and analyzed by thin layer chromatography.

**Thin Layer Chromatography of Solid Material in High Boiling Extracts**

Silica gel G thin layer chromatographic plates of 250 μ thickness were prepared according to the method of Stahl et al. (1956). The sample material and standards (stearic acid, monostearin, distearin, tristearin and cholesterol) were applied to the plates. Initially the plates were developed to a height of 5 cm with a solution of chloroform-methanol-water (60:10:1, v/v). After drying, the plates
were developed an additional 10 cm with petroleum ether-diethyl ether (70:10, v/v). Finally, the plates were dried and then the spots visualized by spraying with a 0.2% ethanolic solution of 2',7' dichlorofluorescein followed by examination under UV light.

Analysis of High Boiling Extract by Gas-Liquid Chromatography

In the early stages of the investigation aliquots of the neutral high boiling extracts were analyzed isothermally with packed gas-liquid chromatographic (GLC) columns containing butanediol succinate (BDS) as a polar liquid phase. Later studies involved the use of packed columns containing the non-polar liquid phase, Apiezon L. These later analyses were carried out with temperature programming.

The operating conditions for the GLC columns were as follows:

BDS Columns

<table>
<thead>
<tr>
<th>Column</th>
<th>3% BDS, 0.05% Igepal Co-880 on 100/120 mesh AW-DMCS(^4) Chromosorb G, 10 ft x 1/8 in. O. D. stainless steel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column temperature</td>
<td>170°C for 3 hr</td>
</tr>
<tr>
<td>Injection port temperature</td>
<td>220°C</td>
</tr>
</tbody>
</table>

\(^4\) The support material had been washed with acid (AW) to remove impurities and then treated with dimethyldichlorosilane (DMCS) to inactivate hydroxyl groups.
Detector temperature: 280°C
Carrier gas: Nitrogen at 25 ml/min

Apiezon L Columns
Column: 20% Apiezon L on 80/100 mesh Celite 545, 10 ft x 1/8 in. O.D. stainless steel
Column temperature: 170°C for 1 hr, 2°C/min to 250°C, held for 1 hr 20 min
Injection port temperature: 230°C
Detector temperature: 310°C
Carrier gas: Nitrogen at 25 ml/min

GLC retention time data were generally referred to authentic standards. In the case of some series of compounds, where certain members of the series were unavailable, evidence for the presence of unknown compounds was obtained from semi-log plots of retention time data versus chain length.

All analyses were carried out with a Varian Aerograph Model 1200 Gas Chromatograph. The instrument was equipped with an effluent splitter which allowed the investigator to make an evaluation of each compound's aroma simultaneously with its detection by a flame ionization detector.

Analysis of High Boiling Extract by Combined Gas-Liquid Chromatography - Mass Spectrometry

Capillary GLC - Mass Spectrometry

The first experiments were carried out using a capillary BDS column connected directly through the Atlas CS-2 inlet into the high
vacuum of the mass spectrometer. The gas chromatograph was an F and M Model 810 and the mass spectrometer (MS) was a single focusing Atlas CH-4 (9 in, \(60^\circ\) sector). The MS was equipped with a double ion source; the 20 eV source served as a chromatographic detector and the 70 eV source provided the fragmentation patterns. The mass spectra were recorded using a Honeywell Visicorder model 1508.

The following operating conditions were used:

**GLC**

- **Column**: BDS, 300 ft x 0.01 in. I.D. stainless steel
- **Sample size**: 1.5 μl
- **Injection split ratio and temperature**: 1:50; \(220^\circ\)C
- **Column temperature**: \(150^\circ\)C for 5 min, \(2^\circ\)C/min to \(220^\circ\)C, held for 3 hr
- **Carrier gas**: Helium at 1 ml/min

**MS**

- **Filament current**: 20 eV source: 50 μA
- **70 eV source**: 25 μA
- **Ion source pressure**: \(1 \times 10^{-6}\) mm Hg
- **Accelerating voltage**: 3.0 KV
- **Electron multiplier voltage**: 1.6 KV
- **Ion source temperature**: 250°C
- **Scan speed**: 6 sec from m/e 25 to 500
Fractionation of Extract by Preparative GLC

A 20 µl portion of the extract was chromatographed on a 10 ft x 1/4 in. I.D. column using 20% Apiezon L as the liquid phase. The effluent from the column was divided so that one part went to the flame ionization detector to provide a chromatographic record, and 20 parts were diverted for collection in glass melting-point capillary tubes cooled by dry ice. The capillary tubes were joined to the effluent splitter by means of a Teflon sleeve. It was necessary to heat that portion of the splitter protruding from the gas chromatograph by means of a heat gun in order to prevent premature condensation of the effluent vapor. The GLC conditions were similar to those previously utilized with the packed Apiezon L columns. The effluent was collected as three fractions at the time intervals indicated below:

Fraction I 0 min to 1 hr
Fraction II 1 hr to 2 hr
Fraction III 2 hr to 3 hr

Analysis of Extract Fractions by Combined Packed Column GLC - Mass Spectrometry

Each of the three fractions obtained from trapping the molecular distillate by preparative GLC was analyzed by combined packed column GLC - mass spectrometry. A packed column containing 5%
Apiezon L as the liquid phase was connected through the EC-1 throttle valve to the ion source of the Atlas CH-4 mass spectrometer. Before entering the EC-1 valve, the effluent from the column passed through a silicone rubber membrane separator which served as a semipermeable barrier between the high vacuum of the mass spectrometer and the atmospheric pressure of the GLC column. The silicone membrane was much more permeable to organic compounds than to inorganic compounds. As a result of this, most of the helium carrier gas, which constituted the major portion of the column effluent, was much less able to pass through and was diverted to the atmosphere. The organic compounds, however, were more soluble in the silicone membrane and had a much greater ability to penetrate the membrane. It was therefore possible to divert 80% of the column effluent toward the mass spectrometer without seriously lowering the vacuum in the system.

The other 20% of the column effluent was diverted to a flame ionization detector to provide a chromatographic record of the analysis. As discussed previously, the 20 eV unit of the double ion source was used as an auxiliary GLC detector. However, in some instances the 70 eV source alone was used. When this was done the course of the analysis was monitored by measuring the total ionization produced by each eluting compound. The necessity for noting the maximum ionization after introduction of the effluent into the mass spectrometer became apparent when it was found that for compounds in Fractions II and III
there was a time lag between detection by the flame ionization detector and detection by the mass spectrometer ion source.

Some compounds were present in quite large concentrations in Fractions II and III and it was found that this had the effect of increasing the background ionization to the extent where it seriously interfered with interpretation of the spectra. The introduction of a Carle valve\(^5\) between the GLC column exit and the molecular separator served to overcome this problem. The valve operated in two modes and could be switched almost instantaneously from one position to another without interfering with the course of the analysis. In the normal operating mode the column effluent flowed straight through to the separator. In the alternate mode the column effluent was diverted to the atmosphere and a make up stream of carrier gas, flowing at the same rate as the column effluent, was allowed to impinge on the silicone membrane of the separator.

The following conditions were used during the course of analysis of each fraction:

**Fraction I**

**GLC**

<table>
<thead>
<tr>
<th>Column</th>
<th>5% Apiezon L on Gas Chrom Q</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60/80 mesh, 8 ft x 1/8 in. O. D.</td>
</tr>
<tr>
<td></td>
<td>stainless steel</td>
</tr>
</tbody>
</table>

\(^5\) Manufactured by Carle Instruments, Inc., Fullerton, California.
Column temperature: 170°C (isothermal) for 1 hr
Injection port temperature: 218°C
Detector temperature: 240°C
Carrier gas: Helium at 25 ml/min

**MS**

Filament current: 70 eV source: 20 µA; 20 eV source: 45 µA
Ion source pressure: 1 x 10^{-6} mm Hg
Accelerating voltage: 3.0 KV
Electron multiplier voltage: 1.6 KV
Ion source temperature: 250°C
Carle valve temperature: 212°C
Molecular separator temperature: 190°C
Scan speed: 6 sec from m/e 25 to 500

**Fraction II**

**GLC**

Column temperature: 200°C (isothermal) for 2 hr
Injection port temperature: 230°C
Detector temperature: 250°C

Other conditions were as described for Fraction I.
Carle valve temperature  \( 226^\circ C \)

Molecular separator temperature  \( 208^\circ C \)

Other conditions were as described for Fraction I.

**Fraction III**

**GLC**

Column temperature  \( 220^\circ C \) (isothermal) for 3 hr

Injection port temperature  \( 240^\circ C \)

Detector temperature  \( 262^\circ C \)

Other conditions were as described for Fraction I.

**MS**

As described for Fraction II.

**Phthalate Esters**

*Extraction and Analysis of Phthalate Esters in Plastic Cheese Wrapping Material*

Approximately 10 g of Parakote were cut up into small pieces and extracted with 300 ml of redistilled diethyl ether for 30 min by agitating the mixture with a magnetic stirrer. On adding 30 ml of 95% ethanol most of the waxy material extracted from the film was precipitated out of solution. The precipitate was removed by filtering.
the solution through Whatman No. 1 filter paper under suction. The major portion of the diethyl ether solvent was now removed by means of a rotary evaporator, causing further solid material to precipitate out of solution. The alcoholic solution was kept overnight at 5°C and it was then placed for 30 min in an acetone-dry ice mixture to remove final traces of the wax. The precipitate was filtered off, and the ethanol was removed under vacuum in a rotary evaporator. The residue was dissolved in diethyl ether. When the ether was evaporated under a stream of nitrogen in a Concentratube⁶ (sample concentration tube) approximately 2 ml of a viscous biphasic solution were obtained. Each phase was examined by packed column GLC-mass spectrometry under the conditions previously described for the analysis of Fraction II.

Qualitative Tests for Phthalate Esters

Two color reaction tests were developed by Haslam et al. (1951) to indicate the presence of phthalate esters in extracts from plastic films. Approximately 50 mg of resorcinol were added to a 10 x 1 cm test tube and the same amount of phenol was placed in another test tube. A third test tube served as a control. To each tube three drops of plasticizer extract and one drop of concentrated H₂SO₄

⁶ Supplied by Laboratory Research Co., Los Angeles, California.
were added and the tubes were then heated for 3 min in an oil bath at 160°C. After cooling, 2 ml of distilled water and 2 ml of 10% NaOH were added to each tube. Finally, the contents of each test tube were thoroughly stirred. A green fluorescence in the resorcinol tube and a red color in the phenol tube were considered as positive evidence for phthalate esters. The above tests were used in conjunction with retention time data and mass spectral evidence.

Inoculation of Experimental Cheddar Cheese with Lactobacilli

Preparation of Lactobacilli Cultures

The following cultures, which had previously been isolated from Cheddar cheese, were obtained in a freeze-dried form from a university laboratory:

L. casei C 46  L. plantarum K 50/c
L. casei B 144/C  L. plantarum C a 106/c

The freeze-dried cultures were inoculated into tubes of lactic broth and incubated at 30°C for 24 hr. At this time all tubes exhibited bacterial growth as evidenced by turbidity and microscopic examination. Inoculations were now made from the tubes of lactic broth into tubes of reconstituted non-fat milk solids. After incubation at 30°C for 48 hr, the two strains of L. plantarum had coagulated the

7 Dr. Elizabeth Sharpe, N. I. R. D., University of Reading, England.
milk medium, but there was no appearance of coagulation in the tubes containing the two strains of _L. casei_. The _L. plantarum_ cultures were placed under refrigeration while the _L. casei_ cultures were kept at 30 °C for an additional 24 hr. There was slight evidence of coagulation in the tubes containing the _L. casei_ cultures at this time.

In preparation for cheesemaking the tubes of coagulated milk medium were added aseptically to 1-liter culture flasks containing similar milk medium and incubated for 48 hr. At this stage the culture flasks containing the two different strains of _L. plantarum_ exhibited noticeable signs of coagulation, but very little coagulation was noted for the two strains of _L. casei_. The acidity developed by the cultures was measured by titration with 0.1 N NaOH.

**Manufacture of Experimental Cheddar Cheese**

The Cheddar cheese was manufactured in three 75 gal stainless steel vats in the Dairy Products Laboratory at Oregon State University. For cheesemaking each vat was filled with 50 gal of pasteurized milk. When the milk had reached a temperature of 75 °F (25 °C), 4.5 lb of coagulated milk containing a mixed strain starter culture (Klenzade 18)⁸ were added to each vat. The cheese milk was then allowed to develop acidity for a period of 1 hr before adding rennet. Just prior

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⁸Prepared by Moseley Laboratories, Inc., Indianapolis, Indiana.
to the addition of rennet extract the first vat was inoculated with the previously prepared cultures of the two strains of *L. plantarum* and the second vat was inoculated with the two strains of *L. casei*. The third vat served as a control. Normal commercial practice was then followed in performing the remaining steps of the manufacturing process (Kosikowski, 1966). Two blocks of curd, each weighing approximately 20 lb, were obtained from each of the three vats. After holding overnight at a pressure of 30 psi, the blocks were wrapped with Parakote and held for three months at 45°F (8°C).

**Microbiological Examination of Experimental Cheddar Cheese**

Samples were removed aseptically from the cheeses just prior to packaging and also after maturing for three months. The samples were diluted and plated out on Elliker's agar following standard procedures for the microbiological examination of cheese (Frazier et al., 1968). Elliker's agar is considered to be a selective medium for the growth of lactobacilli.

**Analysis of High Boiling Compounds Obtained from Experimental Cheddar Cheese Fat**

The fat was obtained and analyzed according to the procedures previously developed for the examination of commercial cheese.
Synthetic Cheddar Cheese Flavor

Preparation of a Synthetic Cheddar Cheese Flavor Mixture

The mixture used was essentially a modification of one previously developed by Day et al. (1960). A simulated Cheddar cheese system was prepared by blending 100 ml of safflower oil with 450 g of washed cottage cheese curd. The safflower oil was of bland flavor and had been purged with nitrogen by the processor and had been stored at the laboratory under nitrogen at -10°C. The following compounds were added to the simulated Cheddar curd in the amounts indicated (ppm in simulated cheese):

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>960.0</td>
</tr>
<tr>
<td>2-Tridecanone</td>
<td>1.23</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>180.0</td>
</tr>
<tr>
<td>2-Undecanone</td>
<td>0.84</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>60.0</td>
</tr>
<tr>
<td>2-Nonanone</td>
<td>0.42</td>
</tr>
<tr>
<td>Caprylic acid</td>
<td>166.0</td>
</tr>
<tr>
<td>2-Heptanone</td>
<td>0.48</td>
</tr>
<tr>
<td>Capric acid</td>
<td>60.0</td>
</tr>
<tr>
<td>2-Pentanone</td>
<td>0.25</td>
</tr>
<tr>
<td>3-Mercaptopropionic</td>
<td>1.2</td>
</tr>
<tr>
<td>acid</td>
<td></td>
</tr>
<tr>
<td>3-Methylbutanal</td>
<td>0.04</td>
</tr>
<tr>
<td>Propanal</td>
<td>0.60</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>7.8</td>
</tr>
<tr>
<td>3-Methylthiopropanal</td>
<td>0.06</td>
</tr>
<tr>
<td>2-Propanone</td>
<td>4.8</td>
</tr>
<tr>
<td>2,3-Butanedione</td>
<td>0.43</td>
</tr>
<tr>
<td>Ethanol</td>
<td>4.5</td>
</tr>
</tbody>
</table>

9 Obtained from Pacific Vegetable Oil Corporation, Richmond, California.
Addition of Lactones to a Synthetic Cheddar Cheese Flavor Mixture

To a portion of the simulated Cheddar cheese, containing the flavor compounds indicated above, four lactones were added at the following concentrations (ppm in simulated cheese):

- γ-Decalactone 2.4
- δ-Decalactone 4.8
- δ-Dodecalactone 6
- δ-Tetradecalactone 12

To another portion of the simulated Cheddar cheese mixture, containing the flavor compounds indicated earlier, only two lactones were added at the specified concentrations (ppm in simulated cheese):

- δ-Dodecalactone 4
- δ-Tetradecalactone 1.5
RESULTS AND DISCUSSION

Identification of Neutral High Boiling Compounds from Cheddar Cheese Fat

The fat was separated from samples of Cheddar cheese by centrifugation and then subjected to molecular distillation. After removal of free fatty acids, the solvent was evaporated from the molecular distillates. About 25-30 ml of a viscous liquid with a "coconut" aroma were obtained from each distillation of 1 liter of cheese oil. In most instances, some solid material precipitated out of the molecular distillates as they were concentrated.

Analysis of Solid Material in the Molecular Distillates by Thin-Layer Chromatography

It was found that the solid material could be separated into several constituents on a thin-layer chromatographic (TLC) plate, using solvent systems commonly employed for the fractionation of lipids. The results of the TLC separation are shown in Table 1. A comparison of the $R_f$ values of the sample components with those found for known compounds indicated that the solid material probably consisted to a great extent of diglycerides together with some cholesterol esters. The exact composition of these two classes of compounds was not determined.
Table 1. TLC analysis of solid material in molecular distillates of Cheddar cheese fat.

<table>
<thead>
<tr>
<th>Sample</th>
<th>R_f value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular distillate</td>
<td></td>
</tr>
<tr>
<td>Spot #1</td>
<td>0.96</td>
</tr>
<tr>
<td>Spot #2</td>
<td>0.52</td>
</tr>
<tr>
<td>Cholesterol acetate</td>
<td>0.97</td>
</tr>
<tr>
<td>Tristearin</td>
<td>0.72</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>0.63</td>
</tr>
<tr>
<td>Distearin</td>
<td>0.53</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.48</td>
</tr>
<tr>
<td>Monostearin</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Packed Column GLC Analysis of Molecular Distillates

Initially, the molecular distillates obtained from the cheese fat were examined by means of packed BDS columns and compounds were tentatively identified from retention time data and observation of their odors as they eluted from an effluent splitter at the end of the column. The δ- and γ-lactones listed in Table 2 were found and also some other unidentified compounds which were eluted from the column at the beginning of the analysis.
Table 2. Lactones identified in Cheddar cheese fat.

<table>
<thead>
<tr>
<th>Lactone</th>
<th>tr/tr&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Prominent ion in mass spectrum (m/e)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>extract</td>
<td>authentic</td>
</tr>
<tr>
<td>δ-decalactone</td>
<td>0.51</td>
<td>0.52</td>
</tr>
<tr>
<td>γ'-dodecalactone</td>
<td>0.75</td>
<td>0.78</td>
</tr>
<tr>
<td>δ-dodecalactone</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>γ-tetradecalactone</td>
<td>1.32</td>
<td>1.38</td>
</tr>
<tr>
<td>δ-tetradecalactone</td>
<td>2.00</td>
<td>2.05</td>
</tr>
<tr>
<td>γ-hexadecalactone</td>
<td>2.78</td>
<td>2.76</td>
</tr>
<tr>
<td>δ-pentadecalactone</td>
<td>2.90</td>
<td>2.93</td>
</tr>
<tr>
<td>δ-hexadecalactone</td>
<td>3.96</td>
<td>3.84</td>
</tr>
<tr>
<td>δ-octadecalactone</td>
<td>7.37</td>
<td>7.35</td>
</tr>
</tbody>
</table>

<sup>a</sup>3% BDS column at 170°C; relative to δ-dodecalactone.

Capillary GLC-Mass Spectrometry

Confirmatory evidence for the δ- and γ-lactones, tentatively identified in the molecular distillates by packed column GLC, was obtained by using a capillary BDS column coupled to a mass spectrometer. All the lactones listed in Table 2 were identified with the exception of δ-octadecalactone which was present in such a low concentration that it was impossible to obtain mass spectral evidence.

Lactones can usually be readily identified from the presence of certain characteristic fragment ions in their mass spectra. The base peak is formed as the result of loss of the alkyl side chain, giving rise to ions of m/e 99 and m/e 85 for the δ- and γ-lactone rings,
respectively (Table 2). The parent ion (P) is usually weak, but it can be determined from the stronger peaks at P-18 and P-36, arising, respectively, from the loss of one and two molecules of water.

Several compounds, eluted from the column in the early stages of the analysis, were incompletely resolved but their mass spectra indicated that they were probably long chain hydrocarbons.

**Fractionation of Molecular Distillates by Preparative GLC**

Packed columns, containing various other liquid phases instead of BDS, were tried in order to obtain a better separation of the unidentified compounds in the molecular distillates. The best results were obtained by using the non-polar liquid phase Apiezon L in a 1/8 in. O. D. packed column which was used with temperature programming. It was found that a 1 µl aliquot of distillate could be resolved into 50 components with this method of analysis. There were, however, some problems associated with the analysis of distillates by combined packed column GLC-mass spectrometry utilizing the Apiezon L liquid phase. When a 4 µl sample from a molecular distillate was run on a column using temperature programming, it was found that compounds eluting very early in the analysis were present only in trace quantities, whereas those coming off the column in the later stages of the analysis were present at such high concentrations that they tended to overload
the mass spectrometer. The molecular distillates also contained approximately 50% solvent and this was difficult to remove without losing some of the more volatile compounds.

In order to overcome these problems, it was decided to pre-fractionate the high boiling extract prior to analysis by packed column GLC-mass spectrometry. For this purpose the complete extract (20 µl) from one distillation was separated into three fractions on a 1/4 in. I.D. Apiezon L column following the temperature program and other conditions previously developed for use with the Apiezon L packed columns. The quantity of material isolated and the time period during which the fractions were collected are shown in Table 3.

Table 3. Fractions from preparative GLC of molecular distillates.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Collection period&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amount (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1-60</td>
<td>0.8</td>
</tr>
<tr>
<td>II</td>
<td>60-120</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>120-180</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed in min from time of injection.

Analysis of Distillate Fractions by Packed Column GLC-Mass Spectrometry

Each of the three fractions obtained by preparative GLC of a molecular distillate was examined by packed column GLC-mass spectrometry using 5% Apiezon L as the liquid phase. The results of
the analyses are shown in Tables 4, 5, and 6 and Figures 2, 3, and 4. Several of the components were incompletely resolved, but in most of these instances the mass spectra provided sufficient evidence for identification. Wherever possible, the retention time values found for the high boiling compounds were compared with those found for known standard compounds, thereby providing confirmatory evidence for the mass spectral identifications. Evidence for the identity of certain compounds could only be obtained by mass spectrometry and these compounds were considered to have been only tentatively identified.

**Effect of Lactobacilli on High Boiling Compounds**

Lactobacilli are the most predominant group of bacteria present in ripened Cheddar cheese (Reiter et al., 1967). It is believed that they make a contribution to the flavor of Cheddar cheese (Marth, 1963; Reiter et al., 1966), and it was decided to study their effects, if any, on the production of high boiling compounds in the molecular distillates from Cheddar cheese fat.

**Inoculation and Subsequent Growth of Lactobacilli in Experimental Cheddar Cheese**

When the lactobacilli cultures were ready for cheesemaking, the acidity of each culture was determined and the results are given in Table 7.
Table 4. GLC$^a$-MS identification of compounds in Fraction I from Cheddar cheese molecular distillate.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Compound</th>
<th>tr/tr, Ethyl decanoate</th>
<th>MS identification</th>
<th>MS reference</th>
<th>Aroma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Distillate</td>
<td>Authentic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Benzaldehyde</td>
<td>0.10</td>
<td>0.10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>δ-Decalactone</td>
<td>0.64</td>
<td>0.63</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>3, 5-Di-tert-butyl-4-hydroxytoluene</td>
<td>0.75</td>
<td>0.77</td>
<td>+</td>
<td>Cornu and Massot (1966)</td>
</tr>
<tr>
<td>4</td>
<td>Diethyl phthalate</td>
<td>0.88</td>
<td>0.86</td>
<td>+</td>
<td>McFadden et al. (1965)</td>
</tr>
<tr>
<td>5</td>
<td>Ethyl dodecanoate</td>
<td>1.00</td>
<td>1.00</td>
<td>+</td>
<td>ibid.</td>
</tr>
<tr>
<td>6</td>
<td>Dibutyl adipate</td>
<td>1.22</td>
<td>1.21</td>
<td>tentative</td>
<td>ibid.</td>
</tr>
<tr>
<td>7</td>
<td>Y-Dodecalactone</td>
<td>1.37</td>
<td>1.35</td>
<td>+</td>
<td>McFadden et al. (1965)</td>
</tr>
<tr>
<td>8</td>
<td>n-Hexadecane</td>
<td>1.53</td>
<td>1.54</td>
<td>+</td>
<td>Cornu and Massot (1966)</td>
</tr>
<tr>
<td>9</td>
<td>δ-Dodecalactone</td>
<td>1.88</td>
<td>1.87</td>
<td>+</td>
<td>McFadden et al. (1965)</td>
</tr>
<tr>
<td>10</td>
<td>Methyl tetradecanoate</td>
<td>2.04</td>
<td>2.04</td>
<td>+</td>
<td>Cornu and Massot (1966)</td>
</tr>
<tr>
<td>11</td>
<td>2-Pentadecanone</td>
<td>2.32</td>
<td>2.33$^b$</td>
<td>+</td>
<td>ibid. (1967)</td>
</tr>
</tbody>
</table>

$^a$5% Apiezon L on Gas Chrom Q, 60/80 mesh, in a 1/8 in. O.D. x 8 ft column, at 170°C.

$^b$Estimated by extrapolation.

$^c$For chromatogram see Figure 2.
Table 5. GLC<sup>a</sup>-MS identification of compounds in Fraction II of Cheddar cheese molecular distillate.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Compound</th>
<th>tr/tr&lt;sub&gt;MS&lt;/sub&gt;</th>
<th>Hexadecanal</th>
<th>MS identification</th>
<th>MS reference</th>
<th>Aroma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Distillate</td>
<td>Authentic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6-Dodecalactone</td>
<td>0.58</td>
<td>0.58</td>
<td>+</td>
<td>McFadden et al. (1965)</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>n-Heptadecane</td>
<td>0.71</td>
<td>0.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>Cornu and Massot (1966)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2, 6-Di-tert-butyl-4-ethylphenol</td>
<td>0.75</td>
<td>tentative</td>
<td>ibid.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Ethyl tetradecanoate</td>
<td>0.83</td>
<td>0.81</td>
<td>+</td>
<td>ibid.</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>1-Octadecanol C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;24&lt;/sub&gt;</td>
<td>0.93</td>
<td>tentative</td>
<td>ibid.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Hexadecanal</td>
<td>1.00</td>
<td>1.00</td>
<td>+</td>
<td>ibid.</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Ethyl pentadecanoate</td>
<td>1.07</td>
<td>1.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>ibid.</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>n-Octadecane</td>
<td>1.07</td>
<td>1.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>ibid.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Unknown, M.W. 280</td>
<td>1.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Unknown, M.W. 298</td>
<td>1.27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Dibutyl phthalate</td>
<td>1.44</td>
<td>1.42</td>
<td>+</td>
<td>ibid.</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6-Tetradecalactone</td>
<td>1.44</td>
<td>1.42</td>
<td>+</td>
<td>McFadden et al. (1965)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Methyl hexadecanoate</td>
<td>1.44</td>
<td>1.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>Cornu and Massot (1966)</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Ethyl hexadecanoate</td>
<td>2.02</td>
<td>2.01</td>
<td>+</td>
<td>ibid. (1967)</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Unknown, M.W. 266</td>
<td>2.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Unknown, M.W. 254</td>
<td>2.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Eicosane</td>
<td>2.64</td>
<td>2.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>ibid. (1966)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Alcohol, M.W. 298</td>
<td>2.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Unknown, M.W. 278</td>
<td>3.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>6-Hexadecalactone</td>
<td>3.44</td>
<td>3.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>McFadden et al. (1965)</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>5% Apiezon L on Gas Chrom Q, 60/80 mesh, in a 1/8 in. O.D. x 8 ft column, at 200°C.

<sup>b</sup>Estimated by extrapolation.

<sup>c</sup>For chromatogram see Figure 3.
Table 6. GLC\(^a\)-MS identification of compounds in Fraction III of Cheddar cheese molecular distillate.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Compound</th>
<th>(\text{tr/tr}_{\text{Distillate}})</th>
<th>(\text{tr/tr}_{\text{Authentic}})</th>
<th>MS identification</th>
<th>MS reference</th>
<th>Aroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methyl octadecanoate</td>
<td>0.41</td>
<td>0.40(^b)</td>
<td>+</td>
<td>Comu and Massot (1966)</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Ethyl octadecatrienoate</td>
<td>0.42</td>
<td></td>
<td>tentative</td>
<td>\textit{ibid.} (1967)</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Heneicosane</td>
<td>0.45</td>
<td>0.44(^b)</td>
<td>tentative</td>
<td>\textit{ibid.} (1966)</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Ethyl octadecanoate</td>
<td>0.47</td>
<td>0.47</td>
<td>+</td>
<td>\textit{ibid.} (1967)</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Docosane</td>
<td>0.67</td>
<td>0.69(^b)</td>
<td>+</td>
<td>\textit{ibid.} (1966)</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Heptadecyl butyrate</td>
<td>0.90</td>
<td>0.80(^b)</td>
<td>tentative</td>
<td>\textit{ibid.} (1967)</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Tricosane</td>
<td>1.00</td>
<td>1.00</td>
<td>+</td>
<td>\textit{ibid.} (1966)</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Octadecyl butyrate</td>
<td>1.22</td>
<td>1.14(^b)</td>
<td>tentative</td>
<td>\textit{ibid.} (1967)</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>C(_{24}) alkane, branched</td>
<td>1.28</td>
<td></td>
<td>tentative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Tetracosane</td>
<td>1.45</td>
<td>1.47(^b)</td>
<td>+</td>
<td>\textit{ibid.} (1966)</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Bis(2-ethylhexyl) phthalate</td>
<td>1.51</td>
<td>1.53</td>
<td>+</td>
<td>\textit{ibid.} (1966)</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Unknown, M. W. 354</td>
<td>1.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Pentadecyl hexanoate</td>
<td>1.83</td>
<td></td>
<td>tentative</td>
<td>\textit{ibid.} (1967)</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Pentacosane</td>
<td>2.12</td>
<td>2.17(^b)</td>
<td>+</td>
<td>\textit{ibid.} (1966)</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\)5% Apiezon L on Gas Chrom Q, 60/80 mesh, in a 1/8 in. O. D. x 8 ft column, at 220\(^\circ\)C.

\(^b\)Estimated by extrapolation.

\(^c\)For chromatogram see Figure 4.
Figure 2. Gas chromatogram of the neutral high boiling compounds in Fraction I of a Cheddar cheese fat molecular distillate, using an Apiezon L column at 190°C.
Figure 3. Gas chromatogram of the neutral high boiling compounds in Fraction II of a Cheddar cheese fat molecular distillate, using an Apiezon L column at 200°C.
Figure 4. Gas chromatogram of the neutral high boiling compounds in Fraction III of a Cheddar cheese fat molecular distillate, using an Apiezon L column at 220°C.
Table 7. Acidity of lactobacilli cultures.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Aciditya (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. plantarum Cα106/c</td>
<td>0.53</td>
</tr>
<tr>
<td>L. plantarum K 50/c</td>
<td>0.36</td>
</tr>
<tr>
<td>L. casei C 46</td>
<td>0.27</td>
</tr>
<tr>
<td>L. casei B 144/C</td>
<td>0.32</td>
</tr>
</tbody>
</table>

aFrom titration with 0.1 N NaOH, calculated as lactic acid.

These acidity readings showed that the two strains of *L. plantarum* were more active than the two strains of *L. casei*. The data supported earlier findings on the relative abilities of the cultures to coagulate milk.

Prior to the addition of rennet, one vat of cheese milk was inoculated with the *L. plantarum* cultures and another vat with the cultures of *L. casei*. A third vat served as a control. The cheese was examined for lactobacilli immediately after it had been manufactured and also when it was ready for analysis after a three-month ripening period. These results are given in Table 8.

As expected, it was found that the bacterial count of lactobacilli in the raw cheese curd was higher in the case of the inoculated cheese than in the case of the control cheese. During the course of ripening, it was found that the lactobacilli increased in number both in the inoculated cheeses and in the control cheese. It has, in fact, been
Table 8. Numbers of lactobacilli present in fresh Cheddar cheese curd and after a three-month ripening period.

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Raw curd&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ripened cheese&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$1 \times 10^7$</td>
<td>$1 \times 10^8$</td>
</tr>
<tr>
<td>Control + \textit{L. casei}</td>
<td>$1.5 \times 10^8$</td>
<td>$2 \times 10^9$</td>
</tr>
<tr>
<td>Control + \textit{L. plantarum}</td>
<td>$2 \times 10^8$</td>
<td>$3 \times 10^9$</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as organisms/g.

well documented that lactobacilli are the only major group of microorganisms which increase in number during the course of Cheddar cheese ripening (Reiter et al., 1968; Fryer et al., 1966).

**Flavor of Experimental Cheddar Cheese**

After ripening for three months, the experimental cheeses were organoleptically evaluated by several staff members and graduate students in the Dairy Products section of the Department of Food Science and Technology at Oregon State University. All of the ten people who tasted the cheese were unanimous in their opinion that the inoculated cheeses had a good, mild flavor whereas the control cheeses were considered to be rather bland. When the two different types of inoculated cheese were compared with one another, six people thought that the cheeses inoculated with \textit{L. plantarum} had a more intense flavor than those inoculated with \textit{L. casei}. However, these
same people also thought that the cheeses containing *L. plantarum* were slightly overacid. These results agree with the earlier data which showed that the *L. plantarum* cultures were more active than the *L. casei* cultures.

**GLC Analysis of Molecular Distillates from Experimental Cheddar Cheese Fat**

Molecular distillates from the fat of the two inoculated cheeses and the control cheese were analyzed by GLC using a 5% Apiezon L packed column under conditions previously described for the analysis of commercial cheeses. Since a long period of time (approximately 3-1/2 hr) was required for the completion of each GLC analysis, it was not feasible to reduce the chromatograms to a size suitable for incorporation into the thesis. However, a visual comparison of the chromatograms showed that there were no apparent qualitative or quantitative differences between the high boiling compounds present in the different molecular distillates. Therefore it would appear that lactobacilli have no appreciable effect on the production of high boiling compounds in Cheddar cheese fat. It would also seem that the high boiling compounds are not directly related to Cheddar cheese flavor since there were definite differences with regard to flavor between the control and inoculated cheeses. However, it is also possible that some high boiling compound or compounds of flavor significance were
not detected perhaps because they were not adequately resolved, or were decomposed by the GLC conditions used.

**Origin of High Boiling Compounds in Cheddar Cheese Fat**

**Lactones**

Lactones are generally present in milk fat in both the free form and also as the hydroxy acid precursor esterified to the 1 or 3 position of the triglycerides. Jurriens and Oele (1965) analyzed the lactones present in butterfat in the free and bound forms. They used thin-layer and column chromatographic methods and these techniques did not involve the application of heat; this is an important consideration as lactones are readily hydrolyzed from their precursors by heating in the presence of traces of moisture. Although the molecular distillation step used in the present study cannot be considered as a truly quantitative procedure, it was nevertheless considered useful to compare the concentrations of lactones found in Cheddar cheese fat with the corresponding values found by Jurriens and Oele (1965) for lactones in butterfat. This information is presented in Table 9.

From an examination of Table 9 it is readily apparent that, with the possible exception of the $\delta$-C$_{14}$ lactone, all of the other free lactones found in Cheddar cheese fat are present in lower concentrations than the free lactones found in butterfat. This would lead one to
Table 9. Comparison of amounts of certain lactones in butterfat and Cheddar cheese fat.

<table>
<thead>
<tr>
<th>Lactone</th>
<th>Butterfat&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Molecular distillate from Cheddar cheese fat&lt;sup&gt;b, c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bound lactones&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Free lactones&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>γ-C&lt;sub&gt;10&lt;/sub&gt;</td>
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<td></td>
</tr>
<tr>
<td>δ-C&lt;sub&gt;10&lt;/sub&gt;</td>
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<td>9.0</td>
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<tr>
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<tr>
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<td>10.8</td>
<td>28.5</td>
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<tr>
<td>γ-C&lt;sub&gt;14&lt;/sub&gt;</td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>δ-C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>7.4</td>
<td>12.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Jurriens and Oele (1965)

<sup>b</sup>Concentration in ppm of fat

<sup>c</sup>Calculated from peak areas following GLC
believe that the conditions used during the course of molecular distillation were mild enough so that little, if any, of the lactones were hydrolyzed from their precursor glycerides. In fact, the cheese fat was exposed to a temperature of 60°C for only a few seconds as it passed through the distillation chamber. It would also seem that during the course of ripening very little lipolytic enzyme activity developed for the hydrolysis of hydroxy fatty acid esters.

Hydrocarbons

Recent studies have indicated that hydrocarbons are ubiquitous among living things. They are generally found in the unsaponifiable fractions of most lipid materials, although they are often present in only trace amounts. They have been identified in bacteria such as *Vibrio marinus* and *Sarcina lutea* (Albro and Dittmer, 1969); in plant materials such as spotted bur clover *Medicago arabica* (Oró et al., 1965a, b), wheat flour (Young and Gilles, 1970) and edible seed oils (Lefort and Sorba, 1956; Kuksis, 1964). Hydrocarbons have also been found in human serum lipoproteins (Skipski et al., 1967) and in various other animal products: for example, in sheeps wool (Simmonds et al., 1968), in rat liver (Skipski et al., 1965), in the livers of pigs and basking sharks (Gershbein and Singh, 1969), in bovine liver (Nagy et al., 1969) and lard (Rutkowski and Korzeniowski, 1967).

The identification of the hydrocarbon components of milk fat has
been the subject of several investigations. Ristow and Werner (1968) showed that n-alkanes with chain lengths from $C_{14}$ to $C_{35}$ were present in milk fat. The concentration of these n-alkanes varied from 20 to 60 ppm, with the highest concentration being found in the summer. Various branched chain monoolefins were also identified, with the same seasonal variation in concentration. Earlier work by McCarthy et al. (1964) revealed that normal, iso- and possibly 1-cyclohexyl alkanes from $C_{17}$ to $C_{48}$ were present in molecular distillates from butter oil. It was postulated that the shoulders on certain peaks of the gas chromatograms represented unsaturated hydrocarbons but squalene was the only one that could be identified with any degree of certainty. In a recent study of Cheddar cheese volatiles, Liebich et al. (1970) found a number of alkanes and olefins in spray dried Cheddar cheese powder with chain lengths from $C_8$ to $C_{16}$. In the present investigation a series of normal alkanes with chain lengths from $C_{16}$ to $C_{25}$ were identified. There were also a number of unidentified compounds some of which appeared to be branched chain alkanes and other compounds that had molecular weights corresponding to olefins.

The hydrocarbons found in plants and surface soils show a predominance of hydrocarbons with an odd number of carbon atoms over hydrocarbons with an even number of carbon atoms (Oró et al., 1965a). However, fatty acids which contain even numbers of carbon
atoms are generally more abundant in plants than the odd-numbered acids (White et al., 1964). It has therefore been postulated that the n-alkanes are formed in plants by the decarboxylation of fatty acids (Nagy et al., 1969). This high odd-to-even ratio was also found to exist for the n-alkanes in sheeps wool (Simmonds et al., 1968), and milk fat lipids (McCarthy et al., 1964; Ristow and Werner, 1968). However the hydrocarbons of human serum lipoproteins (Skipski et al., 1967) and pig liver (Gershbein and Singh, 1969) did not show any predominance of odd-numbered hydrocarbons over even-numbered hydrocarbons. This would seem to indicate that the hydrocarbon composition of lipids in ruminant animals is influenced to some extent by the hydrocarbon composition of the diet. Additional evidence in support of this theory was obtained by Nagy et al. (1969). These authors found that the two isoprenoid hydrocarbons, phytane and pristane, were present in the unsaponifiable fraction of the lipids from bovine liver. Ultimately the isoprenoids are thought to be derived from chlorophyll (Bendoraitis et al., 1962); thus their presence in the livers of ruminant animals would not be unexpected.

All cheeses used in the present investigation were manufactured between the months of June and August, so the hydrocarbon composition of the cheese fat would be expected to reflect the odd numbered distribution pattern of the hydrocarbon compounds present in pasture plants. However, it was found that n-alkanes with an even
number of carbon atoms were present in the molecular distillates at approximately the same concentration as n-alkanes with an odd number of carbon atoms. This type of hydrocarbon distribution pattern has also been found in older geologic sediments containing petroleum products (Oro et al., 1965a).

In view of these results, experiments were conducted to determine if the hydrocarbons were artifacts derived from sources external to the cheese itself. There appeared to be two ways in which this could occur: (1) from the cheese packaging material, or (2) from the molecular distillation step.

The initial portion of the experiment was carried out using 20 lb wheels of Cheddar cheese which had been coated with wax. Later studies were conducted with 40 lb blocks of Cheddar cheese which were packaged in a two-ply cellophane film. This packaging film (Parakote) was coated on the interior with an elastomer-wax mixture. In either case hydrocarbons might migrate from the packaging material into the cheese. If this occurred, one would expect to find a higher concentration of hydrocarbons near the surface of the cheese than in the interior of the cheese. This hypothesis was tested by obtaining the fat from the outer 1/4 in. layer of a 40 lb block of Parakote wrapped Cheddar cheese and also from the center of the cheese. After going through the centrifugation and distillation procedures, the molecular distillates were fractionated by temperature
programmed preparative GLC using a 1/4 in. I.D. Apiezon L column. When the material trapped as Fraction III from each molecular distillate was re-chromatographed on a packed Apiezon L column, it was found from examination of the peak areas that the hydrocarbon compounds in the surface layer of the cheese were present at concentrations approximately 1.5 times greater than the corresponding hydrocarbons from the center of cheese. It would, therefore, appear that hydrocarbon compounds from the wax-elastomer mixture may have diffused to some extent into the cheese fat. However, in view of the low levels found and the small differences between the hydrocarbon contents from each location in the cheese, the possibility must also be considered that the hydrocarbon compounds are natural constituents of the cheese fat.

Although the same experiment was not attempted with 20 lb wheels of cheese which had been packaged in wax, it was found that when chromatograms obtained from the distillates of cheeses which had been coated with wax were compared with chromatograms of distillates obtained from cheeses packaged in Parakote, that the former had considerably higher concentrations of hydrocarbons from C<sub>21</sub> to C<sub>25</sub> (Figure 5). A relationship between peak area and hydrocarbon concentration was obtained by injecting a known amount of n-C<sub>16</sub> alkane onto the GLC column. On applying this relationship to the peak areas of chromatograms A and B in Figure 5, it was found
Figure 5. Normal alkanes present in Cheddar cheese which had been packaged in wax (A) and Parakote film (B). Gas chromatograms were obtained from Fractions III of Cheddar cheese fat molecular distillates using an Apiezon L column at 220\(^\circ\)C. Peaks representing other compounds have been deleted.
that the hydrocarbon concentrations of the molecular distillates from wax coated cheeses were approximately 5.00 ppm of cheese fat, whereas the hydrocarbon concentrations of the molecular distillates from cheese packaged in Parakote were 0.24 ppm of cheese fat. It was therefore concluded that some migration of hydrocarbons from the wax into the cheese had occurred during maturation.

Additional evidence in support of this theory was obtained when equal amounts of cheese wax and elastomer coating were separately dissolved in benzene and examined by gas-liquid chromatography. It was found that the hydrocarbons were present in the cheese wax extract at concentrations approximately three times greater than those found for the elastomer-wax extracts from the plastic film (Figure 6). When the GLC chromatograms designated by A in Figures 5 and 6 are compared, it is evident that there is a remarkable similarity between the hydrocarbon composition of the molecular distillates from the fat of wax-coated cheeses and the hydrocarbon composition of the cheese wax itself. These results are also in close agreement with the findings obtained by Levy et al. (1961) and O'Connor et al. (1962) for the analysis of various commercial paraffin waxes.

In addition to the packaging material, another possible source of contamination was the high vacuum stopcock grease (Spectro-Vac) used in the molecular still. It was found by GLC analysis that this material contained a range of hydrocarbons from $C_{18}$ to at least $C_{25}$. 
Figure 6. Gas chromatograms of extracts from cheese wax (A) and wax-elastomer coating on Parakote film (B), using an Apiezon L column at 220°C. In each case 1 µl injections were made from 0.8% benzene solutions.
Although these hydrocarbons were present at relative concentrations somewhat different from those found in molecular distillates from the cheese fat, it was still necessary to consider Spectro-Vac as a possible source of contamination in view of the fact that there was unavoidable physical contact between the cheese fat and the stopcock grease at three locations on the molecular still. These were the two joints on the glass tubing connecting the feed flask to the distillation chamber and also the exterior part of the stopcock regulating the flow of cheese oil out of the feed flask. They are marked $J_1$, $J_2$ and B, respectively, in Figure 1. As the stopcocks on the feed flask were precision ground, it was possible to coat the interior parts with the cheese oil which was being distilled but it was still necessary to apply a coating of stopcock grease to the exterior part in order to maintain the high vacuum necessary for molecular distillation. In a further effort to avoid using stopcock grease, the ground glass joints on the molecular still were fitted with Teflon sleeves.\footnote{Asco Quorn sleeves supplied by Arthur F. Smith Co., Inc., Pompano Beach, Florida.} These sleeves, however, proved to be inadequate gas barriers and leaked air under high vacuum.

Further experiments were conducted to determine if the stopcock grease was indeed acting as a source of contamination. In a study of the hydrocarbon composition of edible seed oils, Kuksis (1964)
found that safflower oil contained hydrocarbon compounds at a very low concentration (0.01%). It was therefore decided to use safflower oil as a medium to pick up possible hydrocarbon contamination from the stopcock grease during molecular distillation.

The still was modified to avoid contact between the stopcock grease and the safflower oil at two of the three positions mentioned previously. This was done by obtaining a feed flask with a drip tube installed at the exit and then connecting the flask directly to the distillation chamber rather than through an intermediate piece of glass tubing. Although this placed the feed flask at an awkward angle, it was still possible to carry out the distillation. The modified feed flask is shown in diagrammatic form in Figure 7. After distilling a liter of safflower oil, about 20 mg of a wax-like substance was obtained from the cold finger. Traces of this substance were soluble in benzene but it was virtually insoluble in other organic solvents. Another distillation was conducted using safflower oil, but the outer edges of the stopcocks in the feed flask were coated with Apiezon W, a black semi-solid wax, rather than the Spectro-Vac grease. Once again about 20 mg of the wax-like material described above were found on the cold finger after the distillation had been completed. When benzene extracts of the substances obtained from each of the above distillations

\[11\] Distributed by James G. Biddle Co., Plymouth Meeting, Pa. 19462.
Figure 7. Modified feed flask arrangement on the molecular still. For legend refer to Figure 1.
were compared by GLC they were found to be similar (Figure 8).

The stopcock B on the feed flask was the only possible place where the stopcock grease could contaminate the safflower oil. If contamination had occurred, one would have expected to find differences between the chromatograms from the two distillations as Spectro-Vac and Apiezon W are of quite different composition. The two sealants had previously been analyzed by GLC, using a packed 5% Apiezon L column held at 220°C for 2 hr. The Spectro-Vac was found to contain a range of hydrocarbons from C_{19} to C_{24}, whereas no peaks appeared on the gas chromatograms when the Apiezon W was analyzed, indicating that the latter material was very likely composed of longer chain hydrocarbons. As a final experiment, the molecular still was maintained at the usual vacuum for molecular distillation for a period of nine hours without passing any oil through the system. No traces of material were found on the cold finger, demonstrating that the hydrocarbons in the Cheddar cheese fat molecular distillates were not derived from direct volatilization of the stopcock grease in the ground glass joints of the still.

The above results indicate that the hydrocarbon compounds found in mature Cheddar cheese are largely the result of migration from packaging materials which contain wax. Migration occurs to a greater extent in cheeses packaged in paraffin wax than in cheeses packaged in Parakote laminate, and this is in agreement with the
Figure 8. Gas chromatograms of molecular distillates from safflower oil where the stopcocks on the molecular still were coated with Apiezon W (A) and Spectro-Vac stopcock grease (B). An Apiezon L column was used at 220°C.
considerably greater hydrocarbon content of the former packaging material. The fact that hydrocarbon compounds in a wax coating can migrate into foods of high lipid content is supported by the work of Cieleszky and Soos (1969). After waxed cheeses had been stored for one month they found that polycyclic hydrocarbon contaminants in the wax had penetrated into the cheese to a depth of 1 cm.

Phthalate Esters

Like the hydrocarbons mentioned previously, the phthalates must be regarded as possible artifacts, since one would not normally expect to find them in a food. They are widely used as plasticizers to impart pliability to plastic materials, especially in products made from polyvinyl chloride (PVC) such as Tygon tubing (Doolittle, 1953). It has been reported that phthalate esters can migrate from lacquered aluminum foil into cheese products during storage at 25°C (Pfab, 1967).

In the present study diethyl, dibutyl and bis(2-ethylhexyl) phthalates were found at a total level of 2.0 ppm of cheese fat, together with traces of dibutyl adipate. Dibutyl phthalate was the only one of these compounds which was present to any significant extent (1.6 ppm of cheese fat). When the Parakote cheese wrapping material was extracted with ether, it was found to contain about 4% of dibutyl phthalate. Under the food additives amendment of the Food, Drug and
Cosmetic Act, dibutyl phthalate can be added to cellophane packaging materials such as Parakote, up to a level of 5% (Code of Federal Regulations, 1969a). However, little difference was found between the actual amounts of dibutyl phthalate in distillates obtained from cheese which had been packaged in plastic film and in the distillates from wax-coated cheeses. This would seem to rule out the possibility that the phthalate esters were migrating from the packaging material into the cheese. This conclusion would appear to contradict the results obtained by Pfab (1967) who found that dibutyl phthalate migrated from plastic films into cheese. However, his experiments were conducted on cheese and lard stored for one month at 25°C while in the present investigation the cheese was ripened by storing at 7°C for 10 months. Diffusion of the plasticizers would be expected to occur more readily at higher temperatures if Fick's law of diffusion were followed:

\[ s_{ix} = -D_i \frac{dc_i}{dx} \]

where

\[ s_{ix} = \text{net flow of material } i \text{ through unit cross section in unit time} \]

\[ \frac{dc_i}{dx} = \text{concentration gradient in the direction in which material } i \text{ is diffusing.} \]

The proportionality factor \( D_i \) is called the diffusion coefficient and is directly dependent on temperature. It is also apparent that in the case
of Parakote the plasticizer has to migrate from the cellophane film through an elastomer-wax coating before entering the cheese, whereas in the work reported by Pfab (1967) the polymer film containing the plasticizer was in direct contact with the cheese surface.

All solvents used in this study were generally redistilled, although on occasion reagent grade diethyl ether was used to dilute viscous distillates prior to injection into a gas chromatograph. Parodi and Dunstan (1968) found that the presence of dibutyl phthalate in the unsaponifiable fraction of butterfat could be traced to the use of undistilled reagent grade diethyl ether in their extraction procedure. In the present study, however, only about 0.2 ml of reagent grade diethyl ether were used on each occasion and it was found by GLC analysis that this amount of ether contained only minute traces of dibutyl phthalate. The Neoprene gaskets on the closures for the Servall centrifuge tubes were also extracted with redistilled diethyl ether and were found to be free of phthalate esters.

Recently, Feofanov et al. (1971) has also found dibutyl phthalate in cheese. It was found that this phthalate ester could be extracted by the cheese milk from plastic containers which the milk happened to contact in the dairy plant. In the United States milk is generally not stored in plastic containers until it reaches the final stage of being packaged for the consumer market. However, various types of plastic and rubber tubing are frequently used to convey fluid milk at
dairy farms and in dairy processing plants where flexible connections are needed (F. W. Bodyfelt, personal communication, 1971). Teat cup liners on milking machines are generally made of flexible rubber such as Delaxtex and Tygon tubing is used to convey milk from bulk farm tanks to tank trucks. In the present study both of these materials were extracted with redistilled diethyl ether but were found to be free of dibutyl phthalate. However, bis(2-ethylhexyl) phthalate was found to be present in Tygon tubing at a relatively high concentration (20%), and indeed, it is one of the compounds most frequently employed as a plasticizer in PVC products (Brydson, 1969).

In an earlier study Cerbulis and Ard (1967) identified bis(2-ethylhexyl) phthalate in milk fat. A careful examination revealed that the phthalate ester was not present in any of their solvents nor was it arising from contaminated laboratory equipment. It was concluded that bis(2-ethylhexyl) phthalate could be present as a result of contamination at the farm or in the processing plant. Peereboom (1966) examined the plasticizers in eight different commercial samples of PVC milk tubing used in dairy manufacturing plants in Holland. Bis(2-ethylhexyl) phthalate was present in six of these eight samples, and in three of them it was found in combination with dibutyl phthalate. PVC milk tubing from only one commercial source was available for

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analysis in the present investigation, but it is possible that PVC tubing from other sources might contain dibutyl phthalate in addition to bis(2-ethylhexyl) phthalate, and this might account for the presence of the two phthalate esters found in Cheddar cheese fat.

Studies with experimental animals indicate that phthalate esters have a low order of toxicity when taken orally. The \( \text{LD}_{50} \) of dibutyl phthalate for rats was found to be 8 g/kg body weight (Smith, 1953) and the \( \text{LD}_{50} \) of bis(2-ethylhexyl) phthalate for rats and rabbits was found to be 30 g/kg body weight (Shaffer et al., 1945). Single doses of 5 g of bis(2-ethylhexyl) phthalate, given orally to humans, did not produce any discernible adverse effects, although ingestion of 10 g produced mild gastric disturbances and moderate catharsis (Shaffer et al., 1945).

However, in view of more recent investigations, using mammalian tissue cultures, it would appear that phthalate esters and other additives in PVC plastics may produce subtle toxic effects which might not be obvious from the examination of animal tissues at autopsy. Solutions containing blood serum, after perfusion through PVC tubing, were found to be highly toxic to tissue cultures of chick embryo heart cells (De Haan, 1971). Jaeger and Rubin (1970a) found bis(2-ethylhexyl) phthalate in the livers, lungs, spleens and abdominal fat of two hospital patients who had received blood transfusions. Another study by Jaeger and Rubin (1970b), on the perfusion of isolated rat...
livers, indicated that bis(2-ethylhexyl) phthalate can accumulate unchanged in the liver. Bis(2-ethylhexyl) phthalate was isolated by Nagy et al. (1971) from the mitochondria of heart muscle cells in cows, dogs, rabbits and cats. The phthalate ester formed approximately 60% of the total esterified fraction derived from the mitochondria of bovine heart muscle tissue. The subtle toxic effects of phthalate esters on rats have recently been reported by Piekacz (1971). Bis(2-ethylhexyl) phthalate, when ingested at a concentration of 3.5 g/kg of food, was found to increase liver and kidney weights (of female rats) and also to increase the activity of glutamic-pyruvic and glutamic-oxaloacetic transaminases. These changes showed no correlation with the results of histological examination of the viscera of the experimental animals.

Although Guess and Haberman (1968) found from mammalian tissue culture studies that octyltin stabilizers, rather than the phthalate ester plasticizers, were the major toxic additives in PVC, they stated that the presence of a plasticizer was necessary to enable the octyltin stabilizer to diffuse through the plastic material. Finally, it should be emphasized that phthalate esters of longer chain alcohols, such as 2-ethylhexanol, are relatively insoluble in water. Therefore, it is possible that the fat content of the food containing the phthalate esters might have a considerable influence on the absorption of the phthalates.
Methyl and ethyl esters of short chain fatty acids were identified by Day and Libbey (1964) in a study of Cheddar cheese volatiles, and one might also then expect to find the higher molecular weight analogs. In the present investigation, ethyl and methyl esters with chain lengths from \( \text{C}_{12} \) to \( \text{C}_{18} \) were found in the molecular distillates from Cheddar cheese fat. Ethanol has been identified as a component of Cheddar cheese by several investigators (Bills et al., 1967; Day and Libbey, 1964; Patton et al., 1958) and methanol has recently been found in trace quantities (McGugan et al., 1968). Acetic acid can be formed by several metabolic pathways and the other fatty acids from \( \text{C}_4 \) to \( \text{C}_{18} \) probably arise as a result of the hydrolysis of triglycerides during the course of cheese ripening (Bills, 1966). Sato et al. (1967) demonstrated that lactic acid bacteria were capable of lipolytic activity in butterfat emulsions.

With regard to the esters identified in the molecular distillates from Cheddar cheese fat, it was found that the methyl esters were present at concentrations amounting to only about 10% of the corresponding ethyl esters. This was contrary to the results of Liebich et al. (1970) who found higher concentrations of methyl esters than ethyl esters. However, these workers had obtained their cheese fat using a methanol extraction procedure. As stated in their paper the use of methanol may have resulted in the formation of methyl esters.
In the present investigation two esters of butyric acid, heptadecyl butyrate and octadecyl butyrate, together with pentadecyl hexanoate, were also tentatively identified by mass spectral evidence. McGugan and Howsam (1962) found several butyric acid esters of short chain alcohols in their study of the neutral volatiles from Cheddar cheese fat. It is not known at the present time whether the esters found in Cheddar cheese are formed by enzymatic catalysis or by mass action.

**Miscellaneous Compounds**

The compound, 3, 5-di-tert-butyl-4-hydroxytoluene (BHT), was identified in Fraction I of the molecular distillates at a concentration of 0.03 ppm in the cheese. It is commonly added to certain food products as an antioxidant and is also used for the same purpose in various plastics.

In the standards of identity, established under the Food, Drug and Cosmetic Act, there is no provision for the addition of BHT to Cheddar cheese (Code of Federal Regulations, 1969b). Therefore, it would appear that BHT is present in the Cheddar cheese fat as a result of contamination. Libbey and Waldradt (1968) found BHT as an undeclared additive in three out of four commercial brands of reagent grade anhydrous diethyl ether. However, the fourth brand of reagent grade diethyl ether (Mallinckrodt), which did not contain any BHT, was used in the present study so this solvent cannot be readily implicated
as a source of BHT. Several polyethylene tubes which were used in the centrifugation of cheese fat were pulverized and extracted with redistilled diethyl ether. No trace of BHT was found when the extracts were analyzed by GLC.

When benzene extracts from cheese wax and the elastomer-wax coating on the Parakote film were analyzed by GLC and mass spectrometry, both of the packaging materials were found to contain BHT at an approximate concentration of 0.01% (100 ppm). Under the food additives amendment to the Federal Food, Drug, and Cosmetic Act, BHT can be added to food packaging materials as an antioxidant provided that it does not migrate into the food to a level in excess of 0.005% (Code of Federal Regulations, 1969c).

It is also possible that the antioxidant could be present in cow's milk as a result of providing the animals with feed containing the compound as an allowed additive. In a review of the properties and toxicity of phenolic antioxidants, Hathway (1966) pointed out that rats fed a diet containing 0.5% of BHT had depot fat concentrations of the antioxidant of almost 0.004%. BHT was also identified by Herz (1968) in an investigation of the volatile compounds from boiled beef. However, no reports were found in the literature concerning the analysis of milk from lactating ruminants which had been fed fodders containing known amounts of phenolic antioxidants such as BHT.

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Benzaldehyde, 2-pentadecanone and hexadecanal were the remaining compounds found in the molecular distillates. Benzaldehyde had not previously been identified in Cheddar cheese but it was found by Scanlan et al. (1968) in steam distillates from heated milk. Day and Libbey (1964) found odd numbered methyl ketones from C_5 to C_{15} during the course of an investigation of the volatile compounds in Cheddar cheese fat. Although Lawrence (1963) has suggested that methyl ketones found in Cheddar cheese steam distillates are produced as a result of the thermal degradation of β-keto glycerides, it is doubtful if the mild conditions used by Day and Libbey (1964) would result in the formation of methyl ketones from their precursors. These authors suggested that the methyl ketones could be formed just as readily by the hydrolysis and subsequent decarboxylation of the β-keto esters during the normal course of cheese ripening. This explanation could account for the 2-pentadecanone found in the present study, as the distillation conditions were very similar to those employed by Day and Libbey (1964).

Lipid autoxidation can account for many of the aldehydes formed in dairy products (Day, 1966). However, the maximum chain length possible for an alkanal formed in this manner is 11 carbon atoms. It is known that longer chain aldehydes are bound to glycerides in the form of enol-ether linkages; these compounds are called plasmalogens, and are labile to acid (White et al., 1964). Evidence is also available
to suggest that the linkages can be cleaved by enzymes and metal complexes (Van Duin, 1958). Parks et al. (1961) examined the bound aldehydes in the plasmalogens of butter serum and in the glycerides of butter oil. Aldehydes with chain lengths from C₉ to C₁₈ were found together with branched chain aldehydes, enals and dienals. In a later study, Parks et al. (1963) attributed the C₁₁ to C₁₆ aldehydes in milk that had undergone spontaneous oxidation to hydrolysis of lipid-bound aldehydes during pasteurization of the milk. In the light of these results, it would seem probable that the n-hexadecanal found in the present study was originally bound to a lipid fraction and then released by hydrolysis, either at the time of milk pasteurization or during the course of cheese ripening.

The Effect of Lactones on Cheddar Cheese Flavor

A "coconut" aroma, commonly associated with lactone compounds, was the predominant flavor note in the molecular distillates obtained from Cheddar cheese fat. Therefore, it was decided to determine if the lactones identified in the distillates had any effect on the flavor of a synthetic Cheddar cheese. In earlier work, using analytical data obtained from steam distillates of Cheddar cheese, Day et al. (1960) combined various ketones, aldehydes, and fatty acids together with 3-mercaptopropionic acid to produce a Cheddar cheese flavor. The flavor mixture was blended with cream and washed
cottage cheese curd to produce a simulated Cheddar cheese containing the same concentrations of fat and solids not fat that one would expect to find in a natural Cheddar cheese.

Essentially, the procedure of Day et al. (1960) was followed in the present study to prepare a simulated Cheddar cheese except that safflower oil was substituted for cream. This was done in order to avoid the presence of milk lipids which are known to contain lactones and their precursors (Tharp and Patton, 1960; Boldingh and Taylor, 1962). An aliquot of the mixture was used as a control, and to another part various lactones were added, using quantitative data obtained from gas-liquid chromatography of molecular distillates from Cheddar cheese fat. The initial mixture consisted of \( \delta-C_{10} \), \( \delta-C_{12} \), \( \delta-C_{14} \) and \( \gamma-C_{10} \) lactones at levels of 4.8, 6, 12, and 2.4 ppm, respectively. The two samples were submitted to five experienced flavor judges at Oregon State University for informal flavor assessment. Four of the five people thought that the control was more cheese-like than the sample containing added lactones which was described as having "a heavy ketone" aroma. This result was attributed to the use of a high concentration (2.4 ppm) of \( \gamma \)-decalactone in order to compensate for the absence of other \( \gamma \)-lactones which were difficult to obtain. Based on the above finding, another sample of simulated Cheddar cheese was prepared which contained only \( \delta \)-dodecalactone and \( \delta \)-tetradecalactone at levels of 4 and 1.5 ppm,
respectively. When the sample, containing the modified lactone mixture and the control sample, was again submitted to the five judges, there was unanimous agreement that the sample containing the two added lactones was more cheese-like than the control. It was described as having "a more rounded character" and thus the effect of the lactones may be one of toning down some of the sharper notes of the short chain fatty acids.
SUMMARY AND CONCLUSIONS

This study was concerned with the identification of neutral high boiling compounds in the molecular distillates from Cheddar cheese fat. Thirty-three compounds were positively identified and ten additional compounds were tentatively identified by means of gas-liquid chromatography and coupled gas-liquid chromatography - mass spectrometry. The phthalate esters and hydrocarbons appeared to be contaminants or artifacts and efforts were made to determine their mode of entry into the cheese. The principal class of compounds identified were the $\gamma$- and $\delta$-lactones and some of these were evaluated for their contribution to flavor by incorporating them into synthetic Cheddar cheese flavor mixtures. Experiments were also conducted to determine if the production of the neutral high boiling compounds was influenced by the lactobacilli present in ripened cheese.

Preliminary flavor panel evidence indicated that the lactones made a contribution to Cheddar cheese flavor. A series of saturated alkanes was identified in cheese fat and evidence was presented suggesting that migration from the wax coating may have been partly responsible for their presence in waxed cheeses. The hydrocarbon compounds were present at a concentration of 5.00 ppm in the fat from waxed cheese, but were found at a considerably lower level (0.24 ppm
of fat) in cheese which had been packaged in plastic film (Parakote); this probably reflects the lower concentration of hydrocarbons in the wax-elastomer coating of the Parakote film. Samples of fat from the surface of these cheeses had slightly higher concentrations of hydrocarbons than samples from the center. However, the hydrocarbons were present in such low concentrations in each case that it was difficult to state, with any degree of certainty, that migration had occurred from the wax-elastomer coating into the cheese and the possibility that the hydrocarbons were largely endogeneous compounds of the cheese fat must also be considered. Experimental evidence minimized the possibility that the hydrocarbons were artifacts derived from the stopcock grease during the course of molecular distillation.

Several phthalate esters were identified but the only one present at a significant concentration was dibutyl phthalate (1.6 ppm of cheese fat). This compound was also found at a relatively high concentration (4%) in the plastic packaging film. However, since dibutyl phthalate was also present at approximately the same concentration in wax coated cheeses, as well as cheeses packaged in plastic film, the latter material cannot readily be implicated as the sole source of the compound. There was no evidence to indicate that the dibutyl phthalate was derived from experimental procedures and it is believed that it may be present in the cheese as a result of the milk coming in contact with plastic materials at the farm or in the processing plant.
Several fatty acid esters, benzaldehyde, 2-pentadecanone, and n-hexadecanal were also found in the molecular distillates. The antioxidant, 3,5-di-tert-butyl-4-hydroxytoluene (BHT), was identified in trace amounts (0.03 ppm of cheese) and it was also found in the cheese wax and in the elastomer-wax coating on the Parakote film. Therefore, it would appear that BHT was diffusing into the cheese fat from the packaging material. However, BHT is also used as an antioxidant in animal feedstuffs and it is possible that BHT might be present in milk and thus find its way into cheese.

Several species and strains of lactobacilli were added to experimental Cheddar cheese at the time of manufacture. Although the microorganisms enhanced the flavor of the cheese after a three-month ripening period, they were found to have little influence on the production of the neutral high boiling compounds.
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