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The free fatty acids of Cheddar cheese are important components in the over-all flavor and aroma of the cheese. Although the more volatile fatty acids probably contribute most, there is reason to believe that the higher fatty acids may contribute, to some extent, in imparting typical flavor characteristics. Before the advent of gas-liquid chromatography the quantitative separation of the entire spectrum of fatty acids was difficult to achieve. Earlier workers were forced to resort to methods of distillation and later to methods of partition chromatography in attempting to determine the free fatty acids in cheese and other biological materials. In general, the earlier investigators were only successful in separating some of the more volatile free fatty acids. The purpose of this investigation was to appraise the recent developments in the field of column and gas-liquid chromatography and to utilize these methods for the analysis

of the complete series of free fatty acids in Cheddar cheese.

The more volatile fatty acids were determined by two methods of column chromatography. The celite column of Wiseman and Irvin (107) was adapted to the determination of formic, acetic, and propionic acids. Butyric acid was determined by a modification of the silicic acid column developed by Keeney (59, p. 212-225). In determining butyric acid it was necessary to collect the eluate from the column as ten milliliter fractions and to titrate these separately. The total moles of acids with carbon chains longer than butyric also were determined by titration of the fractions preceding butyric acid from the column.

The determination as individual acids of the free fatty acids with carbon chains longer than butyric acid required both the measurement of the total molar concentration by means of the silicic acid column and the determination of the molar ratio between individual acids by gas-liquid chromatography. The modified method of Hornstein (46) was used to isolate the free fatty acids from a portion of fat obtained by centrifuging whole acidified cheese. The free fatty acids were converted to methyl esters directly from the ion exchange resin used for isolation and then extracted with ethyl chloride. The ethyl chloride extract was concentrated in a special reflux system and the methyl esters analyzed by gas-liquid chromatography. The use of two internal standards resulted in improved precision and

accuracy in the analysis of free fatty acids.

Eight samples of Cheddar cheese were analyzed. Formic and propionic acids were not found in any of the cheeses; the average concentration in mg/kg of other major free fatty acids was as follows: 2:0, 949.6; 4:0, 131.0; 6:0, 43.6; 8:0, 46.4; 10:0, 59.5; 12:0, 89.2; 14:0, 242.5; 16:0, 543.3; 18:0, 189.8; 18:1, 504.6; 18:2, 79.3; 18:3, 45.2.

A METHOD FOR THE DETERMINATION OF THE FREE FATTY ACIDS OF CHEDDAR CHEESE

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INTRODUCTION

Many compounds have been identified which contribute to the over-all flavor of Cheddar cheese. Among these, the volatile free fatty acids have been recognized as some of the more important characteristic compounds of Cheddar cheese aroma and flavor. When present in the yet unknown proper balance with other flavor constituents, the free fatty acids aid in imparting the typical desirable flavor of good Cheddar cheese; at higher levels, however, they produce the undesirable sensation of rancidity.

Like many other compounds responsible for food flavors, the free fatty acids in Cheddar cheese exist in low concentrations. For this reason, a satisfactory means of isolating free fatty acids from the cheese becomes a prerequisite to their analysis. New techniques for isolating free fatty acids from biological materials have recently been developed. In addition, the introduction of gas-liquid and liquid-liquid chromatographic methods affords adequate separation for most of the major fatty acids.

Up to the present time, the quantitative analysis of free fatty acids in Cheddar cheese has failed to separate the acids higher than butyric. Although the total mole concentration of higher fatty acids has been reported, little has been done toward determining individual

acids. This is a rather important point considering the extreme difference in odor between such widely separated members as caproic acid and stearic acid; caproic acid is potently odorous, whereas stearic acid has no odor.

The purpose of this investigation was to evaluate some of the newer procedures for fatty acid analysis with regard to Cheddar cheese. It was especially desirable to find or develop techniques for determining the entire spectrum of major individual free fatty acids including the long-chain acids which have not been previously reported.

REVIEW OF LITERATURE

Origin of Free Fatty Acids in Cheddar Cheese

One can postulate three general sources for the total free fatty acids present in Cheddar cheese at the end of the ripening period. First, a portion of the free fatty acids present in the milk from which the cheese is made will, no doubt, be incorporated into the cheese during the manufacturing process; second, free fatty acids may be produced in the cheese during the manufacturing and ripening process by the enzymatic hydrolysis of milk fat; and third, free fatty acids may be produced from non-lipid material as the result of microbial activity. The extent to which each of these sources contributes to the total free fatty acids of the finished cheese is not well defined in the literature; however, one may gain some insight into the relative importance of each by observing that new-made Cheddar cheese curd has a very bland flavor suggesting a low concentration of free fatty acids and that Cheddar cheese manufactured from skim milk never develops a true Cheddar flavor and lacks the definite fatty acid-like flavor of whole milk cheese. These observations would suggest that the free fatty acids incorporated from the milk represent only a small portion of the free fatty acids in the aged cheese and that milk fat provides a suitable substrate for lipase activity or

microbial attack. The development of free fatty acids during the manufacturing and ripening period has been well substantiated, as will be shown from the literature.

Hydrolysis of Triglycerides by Lipases: The enzymes responsible for lipolysis in Cheddar cheese may be from the milk, from microorganisms, or from enzyme preparations added to the milk.

Normal milk always contains the lipase enzyme (56, p. 194). Although the lipase enzyme of milk has not been completely characterized, there is evidence for the presence of a family of enzymes, rather than a single enzyme, with widely varying pH optima (1; 26; 42; 93, p. 831-841). If natural milk lipase is responsible for the hydrolysis of milk fat in Cheddar cheese during the ripening process, it must be capable of effecting hydrolysis at the pH of the cheese, 5.0-5.5. Crude milk lipase, according to Schwartz et al. (96, p. 1364-1374), exhibited at least three pH optima: 6.5 to 7.0, 7.9, and 8.5 to 9.0. It was pointed out by Chandan and Shahani (11), however, that crude preparations or preparations of unknown purity have been used in all of the studies on milk lipase. These authors postulated that the observed characteristics of the lipase may have been influenced by the presence of impurities in the enzyme sources. The same authors, working with highly purified milk lipase, found that the enzyme catalyzed the hydrolysis of milk fat over the wide pH

range of 5.0 to 10.0 but exhibited a distinct maximum activity at pH 9.0-9.2. Even these authors, however, did not discount the possibility that lipase enzymes other than the one isolated in their laboratory might be responsible for lower pH optima observed by others.

Rice and Markley (92, p. 64-82) first suggested that milk lipase was one of the causes of rancidity in cheese. Lane and Hammer produced typical rancid Cheddar cheese by the addition of lipase sources such as rennet paste and pancreatin (67) and also by the addition of homogenized raw cream to raw skim milk as a means of lipase activation (68). Hood et al. (45) found that rancid Cheddar cheese could be produced under commercial conditions by the vigorous agitation of raw cheese milk prior to the manufacture of the cheese, a known method for activating milk lipase.

Peterson and Johnson (89) observed that milk lipase is inactive at the pH of Cheddar cheese and is absent in the cheese after pressing. They further noted that the addition of rennet during the manufacturing process caused an increase in lipolytic activity which disappeared within approximately 30 minutes. They reported the appearance of lipases in new-made cheese after 5 to 20 days and attributed these lipases to the growth of lipolytic bacteria in the cheese. The bulk of the so-called "cheese lipase", active at pH 5, was produced between the fifth and hundredth day of ripening. The

lipolytic activity in cheese made from pasteurized milk increased more rapidly initially but was less than half that of raw milk cheese after 30 days. They believed bacterial lipases to be at least partially responsible for the more rapid flavor development in raw milk Cheddar cheese as compared to Cheddar cheese made from pasteurized milk.

Albrecht and Jaynes (1), however, reported two additional milk lipase systems active in the pH range 5.0 to 6.6 with optima at pH 5.4 and 6.3. They concluded that at least a portion of the active lipase found in the raw milk cheese by Peterson and Johnson (89) was not of bacterial origin but was a natural part of the milk.

Franklin and Sharpe (27, p. 87-99) isolated and identified various bacteria from Cheddar cheese. Lipolytic organisms were detected by their ability to produce clear zones of lipolysis on tributyrin medium and subsequently on butterfat agar. The organisms capable of hydrolyzing butterfat consisted mainly of Gram-positive cocci, predominantly Staphylococcus saprophyticus. Other organisms occurred less frequently, Streptococcus faecium being the most common lipolytic streptococci. Because the population of lipolytic organisms remained nearly static throughout the ripening period, the authors concluded that the cells were either dormant or multiplying at a rate equal to the death rate. For this reason, the importance of

lipolytic organisms in the development of free fatty acids was considered doubtful.

Metabolic Production of Free Fatty Acids by Microorganisms: Free fatty acids can arise in Cheddar cheese as end products of fermentation or by the deamination of amino acids (25, p. 345). Since formic, acetic, and propionic acid, which have been found in Cheddar cheese (64; 102, p. 431-458), are not present in the milk triglycerides and are present only in exceedingly small amounts in milk, their origin must assuredly be metabolic. The microorganisms involved in Cheddar cheese ripening have been studied but not completely elaborated; therefore, little can be said concerning specific microorganisms or metabolic routes. The task of identifying and assigning roles to the bacterial population of Cheddar cheese becomes formidable when one recalls that the count of viable organisms may reach 10^9 per gram of cheese during ripening (25, p. 369).

Numerous microorganisms have the capacity to carry out glycolysis, a direct pathway to lactic or acetic acid. Streptococcus sp. and Lactobacillus sp. are examples of organisms with this capacity, and both have been isolated from Cheddar cheese at various stages of the ripening period (27, p. 87-99; 81). Since Cheddar cheese contains some lactose initially, this route could be responsible for the formation of some of the lower fatty acids. Fagan et al.

(24) studied the fate of lactose during the early stages of Cheddar cheese ripening. Lactose was rapidly hydrolyzed to glucose and galactose. The glucose then quickly disappeared, whereas the galactose could be found for several weeks, especially in cheeses made from pasteurized milk.

Barnett and Tawab (3) observed that acetic acid is present in Cheddar-like cheese immediately following the manufacturing process and increases significantly throughout the ripening period. In the early stages of ripening, butyric acid could result from the breakdown of lactose via acetic acid as Hillig and Montgomery (43, p. 750-760) suggested may happen in cream.

Peterson and Johnson (87) noted an increase in butyric acid without an appreciable increase in other short-chain acids in Cheddar cheese during the early ripening period. They suggested that the butyric acid produced in this manner was the end product of bacterial fermentation. The same authors also noted a steady increase of acetic acid in both raw and pasteurized cheeses over a period of 300 days. They concluded that acetic acid is probably produced during the ripening period by the decomposition of lactate and protein by bacteria.

The synthesis of higher fatty acids by microorganisms was studied by Bornstein and Barker (8, p. 659-669). Although the

organism which they studied, Clostridium kluyveri, has not been reported in Cheddar cheese, it may serve as an example for a possible route of synthesis. When ethanol and acetate were fermented by the above organism, the authors observed that butyric and caproic acids were produced nearly quantitatively.

Although the development of free fatty acids during the manufacturing and aging of Cheddar cheese has been well demonstrated, the literature provides no conclusive evidence concerning the origin or origins of individual free fatty acids.

Relationship of Free Fatty Acids to Cheddar Cheese Flavor

Cheddar cheese contains many compounds which are responsible for the over-all flavor. Essentially, the flavor compounds arise during the manufacturing and ripening process through the degradation of fat, protein, and lactose to free amino acids, peptides, free fatty acids, carbonyl compounds, alcohols, esters, bases and gaseous compounds such as H_2S and NH_3. Numerous investigators have attempted to relate proteolysis to flavor (2, 15, 16, 17, 50, 66, 98), carbonyl compounds to flavor (20, p. 463-474; 63; 106), and lipolysis to flavor (4; 7; 86; 87; 102, p. 431-458). Patton (84) evaluated the contribution of various classes of compounds to Cheddar cheese aroma by adding reagents to block functional groups in cheese

and cheese distillates. He concluded, from the response of a ten-member technical panel, that acetic, butyric, caproic, and caprylic acids "constitute the backbone of Cheddar cheese aroma".

Peterson et al. (87) found that the fatty acids of intermediate chain length (C_6-C_{10}) were produced during the ripening process and were characteristic of Cheddar cheese. The addition of these acids to a bland base, in concentrations found in aged Cheddar cheese, resulted in an aroma resembling that of Cheddar cheese. The complete flavor was lacking, however, because many other flavor compounds are required to produce a true "balanced" flavor. Several investigators have acknowledged that the total flavor of Cheddar cheese is a "balanced" combination of many constituents (37; 62, p. 201-219; 80; 99).

A high correlation between the ratio of hydrogen sulfide to free fatty acids and the acceptability of the cheese was noted by Kristoffersen and Gould (65, p. 1202-1215). They found that certain cheeses having a fatty acid-like flavor contained normal amounts of fatty acids but lower than normal amounts of hydrogen sulfide, and conversely that certain cheeses having a sulfide-like flavor contained normal amounts of hydrogen sulfide but lower than normal amounts of free fatty acids.

Silverman and Kosikowski (99) added mixtures of free amino

acids and free fatty acids in the proper concentrations to bland curd. The result was a Cheddar-like but incomplete Cheddar flavor. A mixture containing only acetic acid, butyric acid, methionine and butanone is reported to have an odor resembling more closely that of Cheddar cheese (62, p. 201-219).

Walker (105) attempted to simulate a Cheddar-type flavor by the addition of mixtures of methyl ketones and fatty acids to bland cheese curd, but found that the flavors produced were sharp and musty unless thioacetamide, a source of H_2S, was added in which case the flavor was improved.

Although the flavor components of Cheddar cheese have not been completely elucidated, the free fatty acids, no doubt, play an important role.

Methods for the Isolation and Determination of Fatty Acids

Distillation: One of the earliest references to the determination of the short-chain organic acids of Cheddar cheese is that of Suzuki et al. (102, p. 431-458). The short-chain acids were steam distilled from Cheddar cheese suspended in a slurry. Fractions were collected and titrated according to the Duclaux procedure, thus providing an identification for the acids obtained. The acids identified and measured in this manner were formic, acetic, propionic,

butyric, caproic, lactic, and succinic.

Kosikowsky and Dahlberg (61, p. 861-871) reported a rapid method for the determination of volatile fatty acids in Cheddar cheese. A finely ground sulfuric acid-cheese mixture with a low pH was refluxed to drive off CO_2 and directly distilled in the presence of $MgSO_4$. The aqueous distillate was titrated with base and the titration value recorded. Recoveries of known acids varied from acetic acid 98% to myristic acid 17%.

The retention of volatile fatty acids by the protein and fat of cheese during steam distillation was reported by Hiscox and Harrison (44, p. 215-226). They reported that steam distillation does not give a true figure for volatile fatty acids for this reason.

Column Chromatography: Smith (101) first developed a method for separating short-chain organic acids by partition chromatography. Other investigators have expanded this technique and utilized it for various purposes.

Bulen et al. (10) utilized a silicic acid column for the separation of various organic acids from plant tissues. Isherwood (51) developed a silicic acid column for the isolation and determination of the organic acids in fruit.

Marvel and Rands (76) were among the first to use step-wise increases in the polarity of the elution solvent. They used

increasing amounts of n-butanol in chloroform to elute organic acids differing widely in chain length and polarity. The column consisted of silicic acid with water as the stationary phase.

A method for determining acetic, propionic, and butyric acids in cheese by column chromatography was developed by Harper (38). The fatty acids with carbon chains longer than butyric were eluted as one fraction which was titrated to give an estimation of the long-chain acids. Essentially Harper's column consisted of three layers: a bottom layer of 10.0 g silicic acid plus 6.0 ml of pH 6.5 buffer, a middle layer of 3.0 g silicic acid plus 1.5 ml of 10% H_2SO_4, and a top layer (the "cap" material) of 10.0 g silicic acid ground intimately with 5.0 g of cheese acidified to a pH of 2.0 with 20% H_2SO_4. Stepwise elution employing from 1.0 to 20.0% butanol in chloroform separated the acids according to their polarity. The long-chain acids were eluted first followed by the more polar short-chain acids. Fractions of the eluate were collected and titrated with standard base.

Column chromatographic methods utilizing internal indicators have the advantage of allowing the visual observation of the bands as they are developed and eluted. In this manner, one needs to collect only the individual band as a fraction rather than the many small fractions which are required when the progress of the bands cannot be observed. Methods utilizing an internal indicator have been

reported by Elsden (22), Gray (34), and Ramsey and Patterson (91, p. 644-656) among others. Zbinovsky (108) developed a column capable of resolving mono- and dicarboxylic organic acids with varying chain lengths utilizing an internal indicator, bromcresol green. Zbinovsky reported separation of all the saturated fatty acids from 2:0 through 14:0. Successive fractions as small as 1 ml were collected and titrated with 0.05 N base to determine the recovery of known acids. Recoveries generally were found to be between 97 to 101%. Keeney (59, p. 212-225) devised a column capable of separating butyric acid from the higher homologues resulting from the hydrolysis of milk fat. Because milk fat contains more butyric acid than other edible fats, Keeney was able to detect the adulteration of milk fat by other fats and oils by comparing the mole ratio of butyric acid to the longer-chain acids after hydrolysis of the fat, separation on the column, and titration with standard base. Keeney's column consisted of silicic acid with water and ethylene glycol as the stationary phase plus bromcresol green as the internal indicator. Sufficient ammonium hydroxide was added to the packing to produce a deep green color, and butyric acid was visible as a yellow band during the development of the column.

Wiseman and Irvin (107) developed a column employing an internal indicator, alphamine red-R, for the separation of organic

acids in silage. Their method employed Celite as the inert support and acetone-Skellysolve B solutions as eluents. A concentrated sucrose solution was used as the stationary phase to resist the leaching action of the solvents, and a small amount of sulfuric acid was added to the stationary phase to avoid the retention of organic acids by the column. The organic acids, in aqueous solution, were added directly to the column, and by using increasing proportions of acetone in Skellysolve B, they were separated into the following fractions: butyric and higher, propionic, acetic, formic, lactic, and succinic. The fractions were then titrated with standard base. The recovery of known amounts (about 50 microequivalents) of individual acids from a composite test solution was excellent.

McCarthy and Duthie (77) developed an adsorbtion chromatography technique for separating free fatty acids from unsaponified fat. They treated silicic acid with isopropanol-KOH, then washed it with ethyl ether. A glass column was packed with this material, and the triglycerides containing free fatty acids were introduced in ethyl ether solution. Elution of the triglycerides was accomplished with ethyl ether. The fatty acids were then eluted from the column with 50 ml of 2.0% formic acid in ethyl ether followed by 75 to 100 ml of ethyl ether. The results of 15 analyses gave an average recovery of 99.0% for triglycerides and 97.5% for

free fatty acids. The free fatty acids thus obtained were analyzed by gas-liquid chromatography after being converted to methyl esters.

Gas-Liquid Chromatography of Fatty Acids: The development of gas-liquid chromatography (GLC) has provided a powerful tool for the analytical chemist. High resolution of the components of a mixture is achieved by virtue of the large number of theoretical plates obtained with a GLC column, and sensitive detectors permit the analysis of quantities too small to work with by conventional methods. Also, GLC is a versatile technique because the many variables involved in the separation of mixtures can be changed quickly and easily. A survey of the literature pertaining to GLC to 1960 is provided by Dal Nogare (18). This review includes 244 references.

The GLC separation of unesterified fatty acids was first reported by James and Martin (53, p. 679-690), who used a liquid phase of Silicone oil containing ten percent stearic acid. Recently more polar liquid phases have been used. Hunter et al. (49) used columns with a diethyleneglycol adipate liquid phase for the separation of short-chain fatty acids.

Emery and Koerner (23) were able to separate the fatty acids from acetic through valeric from aqueous mixtures containing as little as 0.01% of each acid. This was accomplished through the use of the flame ionization detector which is relatively insensitive to

large amounts of water in the sample. Column packing consisted of 20% Tween 80 on acid-washed 60 to 80 mesh Chromosorb W.

Considerable tailing of the peaks due to the association of the carboxyl groups of free fatty acids results from the use of ordinary columns with polar or nonpolar liquid phases. Metcalfe (78) was able to overcome this difficulty by incorporating 2.0% phosphoric acid into the column packing consisting of 25% diethylene glycol adipate polyester on 60-80 mesh Celite. The phosphoric acid treated polyester column gave excellent separation and symmetrical peaks for the higher fatty acids. In addition, the separation of the unsaturated fatty acids, oleic and linoleic, was quite good due to the polar character of the packing.

A method for determining the short-chain free fatty acids in milk was developed by Hankinson et al. (36). The fatty acids were extracted from acidified milk by a modified Roese-Gottlieb procedure and converted to sodium salts. After removal of the solvent, the salts were converted to free acids with HCl and analyzed by GLC. The column was packed with Celite 545 coated with stearic acid. Acids separated in this manner were formic, acetic, propionic, butyric, valeric, caproic, and caprylic.

Shelley et al. (97) used a column containing 1.3 g of ethylene glycol adipate, 0.26 g of phosphoric acid, and 12.5 g of Anakrom

ABS (Analabs) for the separation of formic, acetic, propionic, and butyric acid. They analyzed fish and liquid egg for the above fatty acids by steam distillation followed by neutralization, evaporation of the water, liberation of the fatty acids with dichloroacetic acid, and GLC employing methyl heptanoate as an internal standard.

Gas-Liquid Chromatography of Methyl Esters: A more generally useful method of fatty acid analysis by GLC involves the conversion of the acids to their methyl esters prior to chromatography. By using esters, rather than free acids, the troublesome tailing of the acids on common GLC columns is eliminated. Also, free acids are sufficiently corrosive to cause harm to the GLC detector over a prolonged period of time. Methyl esters permit the operation of the column at lower temperatures which is especially desirable with heat labile stationary phases such as diethylene glycol succinate polyesters, some of which decompose at temperatures exceeding 200°C.

One year after the publication of the first paper by James and Martin (53, p. 679-690), Cropper and Heywood (56) extended gas-liquid chromatographic separation to the methyl esters of fatty acids. Since the fatty acid composition of many plant and animal triglycerides had been poorly established until that time, many investigators began hydrolyzing various triglycerides, forming the

methyl esters of their fatty acids, then analyzing the methyl esters by GLC. In the course of this work many suitable GLC column packing materials for the separation of methyl esters were reported: Apiezon L, Dow Corning Silicone oil, Silicone grease, and polyethylene alkatheme (5); diethylene glycol succinate and diethylene glycol adipate (14); and poly vinyl acetate polyester (47).

Patton et al. (85) investigated several stationary phases coated on acid-washed Celite-545 for the separation of the methyl esters of the fatty acids of milk fat. These included diethylene glycol succinate (LAC-4R-777) and adipate (LAC-2R-446) polyesters, Reoplex-400 (another polyester), and Apiezon-L (a petroleum product). Diethylene glycol succinate polyester and Apiezon-L were chosen for use in the analyses of methyl esters although the other stationary phases also performed well. Using diethylene glycol succinate polyester, Patton was able to separate the esters of the following fatty acids from milk fat; 4:0, 6:0, 8:0, 9:0, 10:0, 11:0, 12:0, 13:0, 14:0, 15:0, 16:0, 16:1, 17:0, 18:0, 18:1, 18:2, 18:3. As Patton noted in another paper (83) the resolution of the cis and trans isomers of 18:1 is not achieved with this packed column; resolution of the two isomers was achieved, however, using a capillary column coated with Apiezon-L.

Both methyl and butyl esters were used by Gander et al. (30) in

the analysis of milk fat fatty acids. Butyl esters were used to determine butyric, caproic, and caprylic acids, and the less volatile acids were determined as methyl esters. Better recoveries were claimed for the short-chain acids when butyl esters were used. Columns employing both Apiezon-L and diethylene glycol succinate were found to be satisfactory for the separation of the butyl and methyl esters.

Jensen and Sampugna (58) noted that several groups of methyl esters of fatty acids from milk fat cannot be readily separated by the usual gas-liquid chromatographic techniques. They resorted to the use of both gas-liquid and thin-layer chromatography for the resolution of acids which could not be separated easily by GLC. They cited, as an example, an unsaturated acid and the branched-chain acid containing one additional carbon atom.

Methods of Esterification: Theoretically, many of the familiar methods for forming the methyl esters of organic acids should be applicable to the quantitative GLC analysis of fatty acids isolated from various sources. In choosing one of the methods, completeness of esterification, the volatility of the sample, and the number of transfers or extractions should be considered.

The well-known esterification with diazomethane has been investigated with regard to complete reaction with fatty acids and

found to be satisfactory as a means of esterifying micro quantities (95). When used on a small scale with proper precautions, the explosive hazard and side reactions of diazomethane were minimized. Diazomethane esterification also provides a convenient way to prepare micro amounts of methyl-C^{14} esters of fatty acids (94).

Luddy et al. (73) compared acid-catalyzed esterification with the diazomethane reaction. The former esterification was carried out on 10 to 100 mg quantities of fatty acids in a small reflux flask with either 4.0% HCl or 4.0% H_2SO_4 in methanol; the latter method consisted of weighing 10 to 20 mg of fatty acids into a small tube, adding an ether solution of diazomethane, then evaporating off any excess ether and reagent after one hour. Both methods reportedly gave quantitative yields of methyl esters.

Radin et al. (90) described a general method for the preparation of methyl esters of most types of carboxylic acids employing methanol, aqueous HCl, and 2, 2-dimethoxypropane as a water scavenger. This method was reported to be satisfactory for micro amounts of fatty acids.

A procedure for the preparation of esters using a BF_3-methanol reagent was investigated by Metcalfe and Schmitz (79). Although the method had the advantage of being rapid, the recoveries reported were low, and it would appear to have limited value for quantitative

work.

Gehrke and Goerlitz (32) developed an esterification method which involved the conversion of fatty acids first to their silver salts then to methyl esters. After extraction from a biological sample, the aqueous fatty acid solution was made alkaline (pH 10) with KOH and a twofold excess of $AgNO_3$ was added. The water was then evaporated and iodomethane in isopentane added. The reaction mixture was allowed to stand for eight hours prior to gas-liquid chromatographic analysis of the methyl esters. The authors claimed 96.8-99.9% recovery for butyric acid using this method.

Hornstein et al. (46) used a basic ion exchange resin (Amberlite IRA 400) to isolate free fatty acids from the fat of cooked and cured meats. The fat was dissolved in petroleum ether and stirred with the resin. The fat was then washed from the resin with solvent and the fatty acids which had been adsorbed to the resin were directly converted to methyl esters with five to ten percent HCl in methanol. The methyl esters were extracted, concentrated, and analyzed by GLC.

A quantitative comparison of four methods for the preparation of methyl esters of fatty acids for GLC was conducted by Vorbeck et al. (104). The reagents used were: diazomethane, methanol-HCl with sublimation, methanol-HCl on ion exchange resin, and

methanol-boron trifluoride. They reported that the four methods gave comparable results for the higher molecular weight fatty acids, but the diazomethane procedure gave a better recovery for fatty acids of lower molecular weight.

Some of the potential sources of contamination when working with microgram quantities of methyl esters were described by Lindgren et al. (72). They found that common undistilled commercial solvents and filter paper could contribute significant amounts of contaminants during the course of methyl ester analysis. Whatman No. 43 filter paper was extracted with chloroform and the extract then methylated; gas chromatographic separation of the methylated material revealed numerous peaks which could easily be mistaken for the legitimate methyl esters from a sample during the course of an analysis.

Factors Influencing Quantitative Gas-Liquid Chromatography: In order to use GLC for quantitative analysis one must be able to relate peak area or peak height to the weight or mole-concentration of each component being analyzed. Several problems arise due to the nature of the GLC detecting and recording mechanisms and also due to the manipulation of the micro amounts involved in an analysis.

Thermal conductivity detectors (katharometers) have been widely used in the analysis of both fatty acids and methyl esters.

Composition of a mixture of fatty acids or methyl esters has frequently been estimated from peak areas when a thermal conductivity detector is used (13, 41, 54). Some authors have reported that the peak area by thermal conductivity is more closely related to the weight percent of the components than the mole percent (35, 82). Peak area, however, is not a direct function of either weight or mole concentration even when comparing members of an homologous series. Horrocks et al. (48) demonstrated this with mixtures of methyl esters. For the methyl esters of the saturated, even numbered acids 2:0 through 22:0; the following relationship for molar response was found:

$$R = 24.68 + 5.79\ N - 0.075\ N^2$$

where R is the relative molar response compared to methyl palmitate, which was assigned a calibration factor of 100, and N is the number of carbon atoms in the molecule. For unsaturated acids of 18 carbon atoms similar relationships were calculated, but it was noted that cis and trans isomers gave different relative molar responses. When the experimentally found relative response factors were used to analyze methyl ester mixtures of known composition, the accuracy was greatly improved.

Although detectors other than the thermal conductivity detector have been employed for the analysis of fatty acids or methyl

esters (23, 83, 85, 104), the thermal conductivity detector appears to give the widest range of linear response for samples of various sizes and requires the least rigorous operating conditions. It has, therefore, been used by the majority of investigators.

In general, calibration of the detector and recorder system to be used for quantitative analysis is a necessary part of the analysis. This has been accomplished by injecting known mixtures of the components to be analyzed in approximately the concentration anticipated in the unknown mixture. From the data obtained from the known mixtures, appropriate factors, equations, or standard curves have been established for the analysis of unknown mixtures (19, p. 259; 32; 33, 46; 104). Two methods of calibration have been used: the absolute method and the internal standard method (19, p. 256). The absolute method involves the calibration of recorder response so that a given unit of peak area (e.g., mm^2) is equated to a given weight of injected compound. Although this method can be quite accurate for certain mixtures, precise duplication of all operating conditions from run to run is required and the aliquot injected must be precisely measured in order to refer the analysis of the aliquot back to the total sample. The internal standard method involves the addition of a known amount of a compound not native to the mixture to be analyzed at some stage of the analysis, frequently just before

injection. The internal standard peak must be completely resolved from all other peaks; this places a limitation on the mixtures and internal standards which can be combined in this way. The calibration of recorder response has been carried out on known mixtures containing the internal standard so that the resulting peak area per weight of internal standard can be related to the peak area per weight of the components to be analyzed by a factor or standard curve. The internal standard method requires less careful attention to the precise replication of operating conditions from run to run but may be somewhat less accurate due to the error involved in measuring both the internal standard peak and the peak of the component being analyzed (31, 46, 97, 103).

Several methods for measuring peak area are currently in use. Electromechanical integrators attach directly to the recorder and provide a convenient and accurate means for obtaining relative peak area. One of the most accurate manual means for computing peak area is the use of the planimeter. Peak areas, measured with the planimeter, are reported to be reproducible to $\pm$ 0.1 cm^2 (19, p. 255; 55; 71). Peak areas also have been determined by cutting out the peak and weighing the paper, but the accuracy of this method depends upon the moisture content and homogeneity of the paper as well as careful cutting (55). A common means of measuring peak areas

consists of multiplying the peak height by either width at one-half peak height or one-half base width after drawing lines to convert the peak to an isosceles triangle. The latter technique is fast and gives reasonable accuracy, providing the peaks are symmetrical, or nearly so (19, p. 255).

EXPERIMENTAL

Determination of Acetic Acid

Early in the course of the literature search and preliminary work it was recognized that no single method would easily permit the isolation and separation of the entire series of major free fatty acids of Cheddar cheese. The outline in this section will be devoted to the methods eventually applied to the analysis of Cheddar cheese samples, and the phases of the preliminary work leading to the selection of these methods will be presented in the discussion.

Acetic acid was determined by the Celite column of Wiseman and Irvin (107). Although this column is also capable of resolving formic and propionic acid, these acids were not observed in the series of cheeses analyzed for this thesis.

Preparation of the Column: Seventy-five grams of Celite 545 (Johns-Manville analytical filter aid) were weighed and added to 720 ml of acetone-hexane (360 ml of each) in a Waring Blendor. The Celite and solvent were mixed briefly and then a mixture consisting of 50 ml of a 66.6% solution of sucrose, 0.5 ml of 0.1 N H_2SO_4, and 12 ml of alphamine red-R solution (400 mg per 100 ml H_2O) was added. The entire mixture was then blended for three minutes at moderate speed.

The glass chromatographic column was constructed to provide a reservoir at the top; the upper 15 cm of the column utilized 35 mm O. D. tubing while the lower 30 cm was made of 20 mm O. D. tubing.

A glass wool plug was placed in the bottom of the column and the packing slurry poured in. The slurry was then packed by means of air pressure and a packing rod. After the column had been uniformly packed, it was rinsed down with one percent acetone in hexane and then stoppered.

Five grams of Cheddar cheese was acidified with 50% H_2SO_4 until the pH was lowered to 1. 8 to 2. 0. Nine grams of silicic acid, Mallinckrodt 2847 dried at 175°C for 18 hours, was ground with the sample in a mortar and pestle until no cheese particles were evident. The prepared sample was added to the top of the column and tamped gently with a packing rod.

Column Development: Elution was initiated with one percent acetone in hexane while sufficient air pressure was applied at the top of the column to provide a flow rate of two or three drops per second. Butyric acid and the higher homologues were eluted with one percent acetone in hexane, the progress of the acids down the column being followed by an indicator change from orange to blue. Successively higher concentrations of acetone in hexane were then added to complete the column development (5%, 15%, 20%, and 30% acetone

in hexane). Propionic acid should have been eluted with the 5% solvent, acetic acid with the 15% solvent, formic acid with the 20% solvent, and lactic acid with the 30% solvent according to the work of Wiseman and Irvin and also the experimental separation of known acids on the column in our laboratory. Of these acids, only a light band for acetic acid and a heavy band representing lactic acid or lactic acid plus other organic acids were observed. Collection flasks were changed when the visible bands were about one inch from the bottom of the column.

Titration of the fractions was accomplished using a five milliliter buret graduated in 0.01 ml. An air stream, freed of CO_2 by bubbling through 20% KOH, prevented the fading of the end points and provided agitation during the titration. Phenolphthalein, 0.5% in ethanol, served as the indicator. The standard base was prepared by dissolving 2.5 g of KOH in 40 ml of isopropanol on a steam bath, decanting the isopropanol-KOH solution from the small amount of aqueous solution clinging to the bottom of the flask, then diluting 12 ml of this stock solution to one liter using equal parts of methanol and isopropanol. The base (approximately 0.01 N) was standardized with potassium acid phthalate.

Determination of Butyric Acid

Butyric acid and the total equivalents of higher fatty acids were determined from a five gram Cheddar cheese sample by a modification of the silicic acid column developed by Keeney (59, p. 212-225).

Silicic acid, Mallinckrodt 2847, was dried in an oven at 175° C for 18 hours, then stored in a tightly sealed container. The glycol stationary phase was prepared by dissolving 700 mg of bromcresol green in 700 ml of ethylene glycol on a steam bath, then adding 40 ml of 0.1 N NH_4OH and enough water to make one liter. Column packing consisted of 100 g of the dried silicic acid and 95 ml of the glycol reagent mixed thoroughly in a mortar and pestle. The elution solvent employed was one percent by volume n-butanol (reagent grade) in n-hexane (high purity grade; Phillips Petroleum Co., Bartlesville, Okla.). The glass chromatographic column was as described in the previous section.

Preparation of the Column: Thirty-five grams of the prepared packing were covered with the elution solvent in a mortar and mixed with a pestle to form a slurry. The lower end of the column was sealed off by means of a 6 mm length of rubber tubing and a pinch clamp. Elution solvent was added until the column reservoir was

half full. A small glass wool plug was then placed in the constricted end of the column and tamped into place with a packing rod. The prepared packing slurry was slowly added at the top of the column by means of a teaspoon, and the last traces of air were removed by allowing it to sift slowly downward through the length of the column. After the packing had all been added to the column, the lower end of the column was opened so that solvent flowed out and the packing settled. The solvent level was never allowed to fall below the top of the packing material at any time during the packing procedure. Final packing was accomplished by carefully applying air pressure at the top of the column and gently tamping the top of the packing with a packing rod. The lower end of the column was then again sealed off.

A Cheddar cheese sample of five grams was acidified by adding 50% H_2SO_4 and mixing in a mortar and pestle until the pH was lowered to 1.8 to 2.0. Nine grams of silicic acid, as described for column packing, were added to the sample and ground intimately with it until no Cheddar cheese particles were visible. The prepared sample (cap material) was added to the column by sifting slowly into a small amount of elution solvent overlaying the packing. The cap material was then gently packed with a packing rod, and a filter paper disk was placed on top of it.

Collection and Titration of Fractions: The finished column was mounted over an LKB RadiRac fraction collector equipped with a ten milliliter siphon. Three small portions of elution solvent of about ten milliliters each were added successively at the top of the column to initiate development, and then the reservoir was filled. The flow rate through the column at this point was approximately three milliliters per min. Forty fractions (400 ml total) were collected in six inch test tubes before column development was stopped. The ten milliliter fractions were then titrated in the test tubes in which they had been collected using a carbon dioxide-free air stream to provide agitation and to prevent the fading of the end points. The base and buret were as previously described, and one drop per tube of 0.5% phenolphthalein in absolute ethanol served as the indicator.

Isolation and Esterification of the Higher Fatty Acids

Materials: Ethanol ---- absolute and 95%, reagent grade.

Methanol ---- reagent grade.

Hexane ---- high purity grade (Phillips Petroleum Co., Bartlesville, Okla.).

Ethyl Chloride ---- U.S.P. (The Matheson Co., Newark, Calif.).

Ion exchange resin ---- Amberlite IRA-400 C.P.

HCl-methanol ---- HCl gas was generated by dropping concentrated sulfuric acid on reagent grade sodium chloride. The HCl gas was then bubbled through methanol until five to ten percent HCl in methanol was obtained as determined by titration.

Isolation of the Fat by Centrifugation: A 100 g portion of cheese was ground in a mortar and pestle with sufficient 50% H_2SO_4 to reduce the pH to 2.0, as determined with a pH meter. The internal standards, 2.5 mg of heptanoic acid and 25 mg of heptadecanoic acid in five milliliters of hexane, were mixed with the acidified cheese. The cheese was packed tightly into 50 ml stainless steel centrifuge tubes and warmed in a 40° C water bath for one-half hour. The tubes were then placed in a Servall SS-3 superspeed centrifuge and centrifuged for 20 min at 30,000 X G. The centrifugation technique was essentially that of Libbey et al. (70). After centrifugation, three layers were present in the tubes: a layer of fat, an aqueous layer and a plug of solid material at the bottom. The fat layer at the top of each tube was drawn off with a pipette, taking care to avoid the removal of any of the aqueous layer. The usual yield of fat per 100 g of cheese was about 25 g.

Preparation of Ion Exchange Resin: Pretreatment of the resin, Amberlite IRA-400, was found to be essential to avoid the leaching

of low molecular weight polymers (6). Proper pretreatment virtually eliminated contamination from this source. The resin, once treated, could be stored for several weeks at refrigerator temperature before being used. The resin was treated as follows:

(a) Twenty-five grams of Amberlite IRA-400 were weighed into a 250 ml Erlenmeyer flask and stirred with 50 ml of one normal sodium hydroxide for five minutes with a magnetic stirrer. The resin was then washed with successive 100 ml portions of distilled water to remove the last traces of alkali, after which it was washed twice with 50 ml portions of 95% ethanol, once with 50 ml of absolute ethanol for removal of the last traces of water and, finally, three times with 50 ml portions of hexane.

(b) Three-hundred milligrams of stearic acid were dissolved in 50 ml of hexane and stirred with the resin for 15 min. The resin was then washed three times with 50 ml portions of hexane, once with 50 ml of absolute ethanol, and twice with 50 ml portions 95% ethanol.

(c) The resin was then stirred 20 min with 40 ml of five to ten percent HCl-methanol and the treatment was repeated with 20 ml of five to ten percent HCl-methanol, stirring for five minutes. The resin was then washed twice with 100

ml portions of distilled water.

(d) The treatment with one normal sodium hydroxide followed by the washings outlined in step (a) was repeated, and a final washing with two 50 ml portions of absolute ethanol was included at this point.

(e) The HCl-methanol treatment outlined in step (c) was repeated.

(f) The treatment with alkali outlined in step (d) was repeated.

(g) The HCl-methanol treatment outlined in step (c) was repeated.

(h) Finally, the resin was stirred five minutes with 50 ml of one normal sodium hydroxide, washed free of alkali with successive 100 ml portions of distilled water, washed once with 50 ml of methanol, and stored under a layer of methanol in the refrigerator.

Isolation of Free Fatty Acids from Fat: After decanting the layer of methanol, the resin was washed twice with 50 ml portions of absolute ethanol and twice with 50 ml portions of hexane. The fat, obtained from the cheese sample by centrifugation, was dissolved in 50 ml of hexane and added to the resin. The solution of fat was stirred with the resin for 20 min and then decanted off. The resin was then washed four times with 50 ml portions of hexane, twice

with 50 ml portions of ethanol, and twice with 50 ml portions of methanol.

Esterification of the Fatty Acids: Forty milliliters of five to ten percent HCl-methanol were stirred with the resin for 20 min, then carefully decanted into a separatory funnel containing 25 ml of cold distilled water. This was repeated with a 20 ml portion of HCl-methanol, stirring for five minutes. The contents of the separatory funnel were cooled to 2^{o} C prior to extraction.

Extraction of the Esters with Ethyl Chloride: Extraction was carried out in a 2^{o} C room. Forty milliliters of ethyl chloride were added to the separatory funnel which was then shaken vigorously for one minute. Ten milliliters of cold water were added to promote the rapid separation of the phases. After ten minutes the aqueous phase was removed and saturated with sodium chloride, and the ethyl chloride was drawn off into a flask. The resin was rinsed with a second 40 ml portion of ethyl chloride which was then used for a second extraction of the aqueous phase. The ethyl chloride from the second extraction was added to that of the first. A third 40 ml portion of ethyl chloride was used for a final extraction of the aqueous phase after which the ethyl chloride extracts were combined and washed twice with ten milliliter portions of cold distilled water. The

ethyl chloride extract was then dried overnight with ten grams of anhydrous sodium sulfate at -20° C.

Regeneration of Resin: After its use as outlined above, the resin was regenerated as soon as possible to the more stable basic form. The resin was washed with two 50 ml portions of methanol and two 100 ml portions of distilled water, then stirred five minutes with 50 ml of one normal sodium hydroxide. The resin was then washed free of alkali with successive 100 ml portions of distilled water, washed with 50 ml of methanol, and stored under a layer of methanol in the refrigerator.

Concentration of the Ethyl Chloride Extract: Concentration of the ethyl chloride extract was accomplished with a reflux system designed to avoid the loss of the methyl esters (see Figure 1). The pear-shaped evaporating flask was immersed in a thermostatically controlled water bath at 20° C while water at 11° C was circulated through the reflux condenser. The temperature of the water entering the reflux condenser was adjusted to 11° C by means of the screw clamp. Water was passed through eight feet of 1/4 inch O. D. aluminum tubing in the 20° C bath and 15 inches of the same tubing in the ice bath. Passing the condenser water through the 20° bath prior to the ice bath compensated for fluctuations in the temperature of the

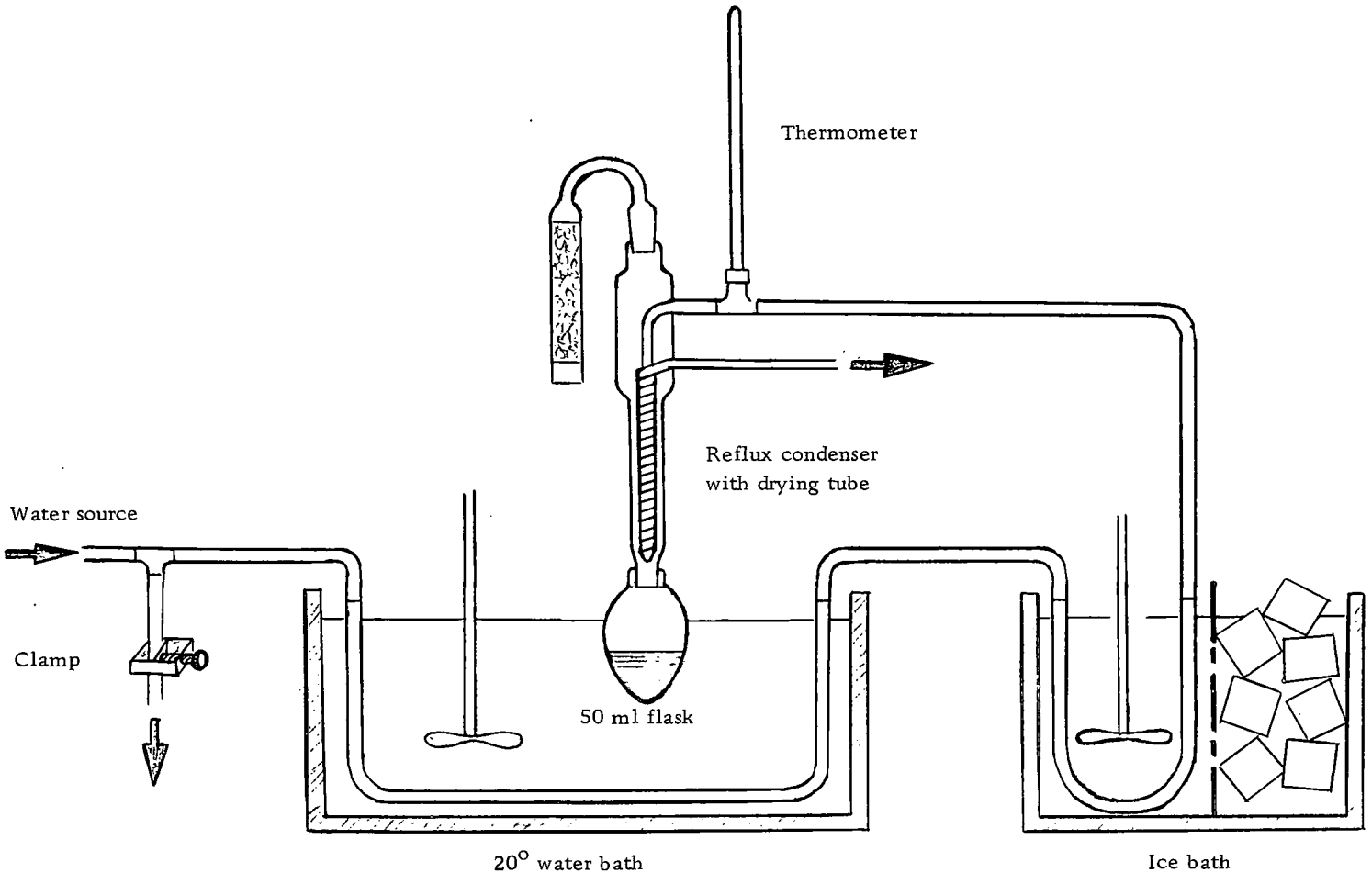

Figure 1. Apparatus for removal of excess ethyl chloride prior to gas chromatography.

tap water and assured an even temperature at the condenser. A reflux rate of about one drop per second was obtained. When the ethyl chloride extract had been reduced to about one milliliter, the pear-shaped flask was removed, stoppered, and stored at -20° C.

Gas Chromatographic Separation of the Methyl Esters: The Aerograph Model A-100 with a thermal conductivity detector was employed. A 1/4 inch O. D. aluminum column, six feet in length, packed with 20% LAC-3R-728 (Diethylene glycol succinate) on 80-100 mesh Celite 545 (acid and alkali treated) was used. Excess ethyl chloride was evaporated from the sample at the chromatograph until introduction into the syringe was possible. Two column temperatures were employed; one injection was made at 94° C and a second injection at 194° C. The methyl esters of the fatty acids 6:0 and 8:0 were analyzed at the lower temperature by means of the 7:0 internal standard while the methyl esters of the fatty acids 10:0-18:3 were analyzed at the higher temperature by means of the 17:0 internal standard. Heptanoic (7:0) and heptadecanoic (17:0) acids were chosen as internal standards because of their very low concentration in the triglycerides of milk fat (74). A mixture containing the homologous series of known methyl esters of even-numbered fatty acids was injected prior to the injection of the sample to be analyzed; this provided a rapid check of operating conditions and also a basis for the

identification of sample components by retention time.

Factors for Relating the Internal Standards to the Free Fatty Acids

Since recorder response is not linear with respect to weight for different esters and recoveries for different esters may vary, it was necessary to establish factors for relating the peak areas of the esters of the internal standards to the peak areas of the esters of free fatty acids.

Weighed amounts of about ten milligrams of each fatty acid, including the internal standards, were added to 25 g of mineral oil. The mineral oil containing the fatty acids was dissolved in hexane and carried through the entire procedure of methyl ester formation and GLC. An appropriate factor for each acid was calculated from the resulting peak areas of the methyl esters by the following equation (using stearic acid as an example):

$$\text{Factor for stearic acid} = \frac{\text{Wt of stearic acid added to the mineral oil}}{\text{Measured area of the methyl stearate peak}} \times \frac{\text{Measured area of the methyl heptadecanoate peak}}{\text{Wt of heptadecanoic acid added to the mineral oil}}$$

The factors obtained in this manner for individual fatty acids are given in Table 1. After the factors had been determined, the same

TABLE 1

Factors for relating the internal standards to the methyl esters of the other individual acids

Acid	Number of trials	Average factor[a]	Standard deviation
6:0	6	1.019	0.0202
7:0	6	1.000[b]	---
8:0	6	1.004	0.0252
10:0	14	0.606	0.0878
12:0	16	0.706	0.0613
14:0	16	0.771	0.0361
16:0	16	0.765	0.0345
17:0	16	1.000[c]	---
18:0	16	0.832	0.0304
18:1	3	0.795	0.0245
18:2	3	0.836	0.0157
18:3	3	0.952	0.0251

[a] Corrected for the purity of the known acids used in the study.

[b, c] Assigned.

equation was used when analyzing fat samples from cheese for fatty acids by solving for the weight of the fatty acid in the following way:

$$\text{Wt of stearic acid in fat sample} = \frac{\text{Factor for stearic acid} \times \text{Measured area of methyl stearate peak} \times \text{Wt of heptadecanoic acid added to the fat sample}}{\text{Measured area of the methyl heptadecanoate peak}}$$

Since the use of the anion exchange resin imposes limitations on the amount and type of solvent which can be used for the

extraction of free fatty acids from cheese, the absolute recovery of the long-chain fatty acids from the samples was not achieved. It was necessary, therefore, to combine the results of the silicic acid column and GLC for the analysis of the fatty acids 6:0 through 18:3. The total moles of higher fatty acids, as determined by titration from the silicic acid column, were distributed according to the molar ratios obtained from the gas-liquid chromatographic analysis. Such a calculation is reasonable if one assumes that each member of the series of higher fatty acids 6:0 - 18:3 partitions equally into the fat during centrifugation. This assumption is supported by noting that the relative solubilities of the series of acids is similar in various organic solvents and that all are nearly insoluble in water (69). Also, the experimental addition of known amounts of caproic, caprylic, and stearic acids to cheese prior to centrifugation indicated that the recovery of each was nearly uniform, as determined by titrating a portion of the fat layer after centrifuging. When the total weight of each acid added to 40 g cheese was divided by the weight of the acid recovered in one gram of fat, the following ratios were obtained: 6:0, 0.0777; 8:0, 0.0859; 18:0, 0.0809. Furthermore, by adding the internal standards directly to the cheese, the slight water-solubility of caproic and caprylic acids would be partially compensated for by the heptanoic acid internal standard which is also slightly water soluble.

RESULTS AND DISCUSSION

Preliminary Work

Column Chromatography: Work by Harper (38) suggested that a free-flowing mixture of whole cheese acidified to pH 2.0 and ground with silicic acid might serve as the cap material for any number of columns suitable for fatty acid separation. By acidification to pH 2.0, the fatty acids could be assumed to exist nearly exclusively in the form of acids rather than as salts; and by grinding the cheese intimately with silicic acid, a maximum surface area would be exposed to the elution solvent.

The column of Wiseman and Irvin (107) was selected for trial and found to be satisfactory for the separation of formic, acetic, and propionic acids when all three were present simultaneously. The recovery of a known amount of acetic acid added to a cheese sample, after previously determining the acetic acid indigenous to the cheese, was 100%. Although propionic acid was never observed in any cheese, formic acid was observed in some of the cheeses used for the preliminary work but was not observed in any of the cheeses analyzed for the thesis. Acetic acid was found in all cheese samples. The observation that formic and propionic acids are encountered infrequently in Cheddar cheese is supported by the

recent qualitative work of Patton (84) who observed that even when formic and propionic acids were present they occur at much lower levels than acetic or butyric acids. The collection of fractions from the Wiseman column depends upon the visual observation of the bands which are created by the color change in the pH sensitive indicator as an acid moves down the column. For this reason, one would fail to observe and collect acids which exist in very small concentrations and, therefore, do not produce perceptible bands.

Butyric acid, because of its intermediate solubility properties, is difficult to separate from the higher homologues by partition chromatography. The silicic acid column of Keeney (59, p. 212-225) appeared to offer a means of separating butyric acid as well as providing a titration value for the higher fatty acids. Preliminary separation of known acids indicated that the column provided adequate resolution of butyric acid and satisfactory recovery of butyric and other acids. However, when cheese samples were acidified, ground with silicic acid, and added as cap material to the column it became apparent that the concentration of butyric acid in the cheese was insufficient to produce a visible color change in the column indicator. Since there are limitations to the amount of cap material which can be added to the top of a column, it was decided to collect small fractions and titrate

them individually without attempting to observe and collect visible bands. The column was mounted above a fraction collector and successive ten milliliter fractions were collected; this appeared to provide a reproducible means of separating and determining butyric acid. The typical separation of the free fatty acids of Cheddar cheese on the silicic acid column is illustrated in Figure 2.

Isolation of Higher Fatty Acids from Cheese: The work of Hornstein (46) and Khatri (60, p. 1-71) indicated that the use of an anion exchange resin for the isolation of fatty acids followed by esterification, extraction, concentration, and GLC might prove satisfactory for the analysis of the higher fatty acids. One of the major problems which had to be solved in order to apply this technique to Cheddar cheese was the preparation of the sample prior to collecting the free fatty acids on the resin. Since Cheddar cheese contains protein and organic acids (other than fatty acids) as well as inorganic salts, it was necessary to perform an isolation procedure to eliminate these contaminants before collecting the fatty acids on the anion exchange resin. Several unsatisfactory extraction techniques were tried.

In one case, 50 g of cheese was acidified to pH 2.0 then ground with enough silicic acid to form a free-flowing mixture. The entire mixture was then added to a large column 50 mm in diameter and extraction was initiated with an acetone-hexane mixture (equal parts

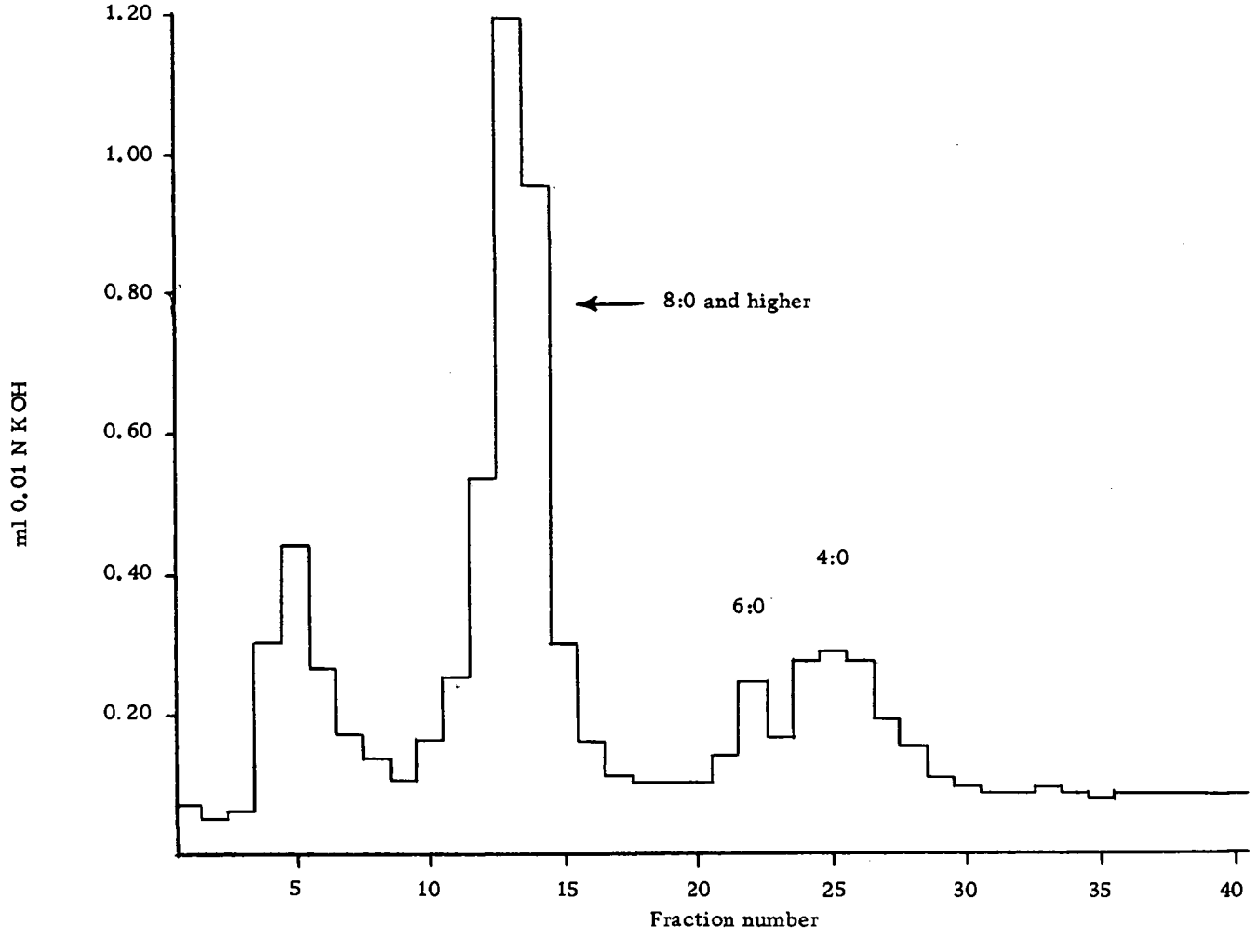

Figure 2. Typical separation obtained with the silicic acid column.

by volume). The eluent was collected in a flask containing 25 g of ion exchange resin stirred by a magnetic stirrer. When the flask was nearly full, the column flow was stopped, and the extract reacted with the resin for 20 min. The elution solvent was then decanted onto the top of the column and flow through the column was again initiated. After repeating this cycle several times, the methyl esters were formed, extracted, concentrated, and subjected to GLC. The chromatogram obtained indicated that the polar solvent had eluted lactic and other organic acids whose methyl esters masked the esters of the fatty acids.

A somewhat more refined extraction technique appeared promising at first, but later proved to give incomplete extraction. Cheddar cheese was acidified to pH 2.0 then centrifuged at 30,000 X G. The fat was drawn off and saved in a separate container while the solid residue was finely ground and added to a modified flow-through Soxhlet extractor. Iso-pentane was then refluxed through the extractor for four hours. The combined extract and fat were then treated on the resin and the methyl esters were formed; GLC revealed an excellent pattern of fatty acid methyl esters. Recoveries of added caproic, caprylic, and stearic acids were found to be approximately 100% for the extraction step. However, when the total moles of caproic-and-higher acids determined by GLC were

compared with the total moles of the same acids determined with the silicic acid column, it was found that the extraction procedure provided only 30-40% recovery. It is difficult to explain the quantitative recovery of added fatty acids while the natural fatty acids were recovered in such poor yield. Since the added fatty acids were introduced in rather high concentration (about one gram of each per 200 g of cheese) to permit the determination of recovery by titration, it is quite possible that they never attained the equilibrium condition within the cheese which caused the retention of the natural acids during extraction. The results of this work point to the fact that one cannot assume that compounds added to a complex mixture will be recovered in the same manner as the same compounds already present in the mixture. It has been noted in the case of steam distillation that a considerable portion of free fatty acids is retained by the protein of cheese (44, p. 215-226).

When ethyl ether was used as the extraction solvent in the above mentioned apparatus, the problem of eluting the more polar organic acids, such as lactic acid, again appeared. Because some of the polar organic acids are in much higher concentration than fatty acids and their methyl esters have GLC retention times equal to those of several fatty acid methyl esters, the sample must be free of these acids when it is added to the ion exchange resin.

At this point it was decided to determine the higher fatty acids, 6:0 through 18;3, by the combined technique outlined under experimental methods.

Gas-Liquid Chromatography: Several modifications in the ion exchange resin technique appeared to give more consistent results and better precision between duplicate GLC runs on a given sample. GLC injections were made at two temperatures to provide better separation of the components and to obtain more diffuse peaks for the lower fatty acids (see Figure 3). Two internal standards, heptanoic acid and heptadecanoic acid, were added to the sample initially; and their methyl esters served as references, one for the low temperature run and one for the high temperature run. In determining the factors for relating the internal standards to the free fatty acids of a sample, the purity of the known acids was determined by neutralization equivalents and by GLC. With the exception of heptadecanoic acid, the purity of the known fatty acids ranged from 96 to 99+ percent; the purity of heptanoic acid was found to be about 94%. The factors obtained for relating the higher fatty acids to heptadecanoic acid (see Table 1) suggest that the purity of heptadecanoic acid may have been less than determined. However, since the same lot of heptadecanoic acid was used for determining the factors and in the analyses, and no peaks having the retention

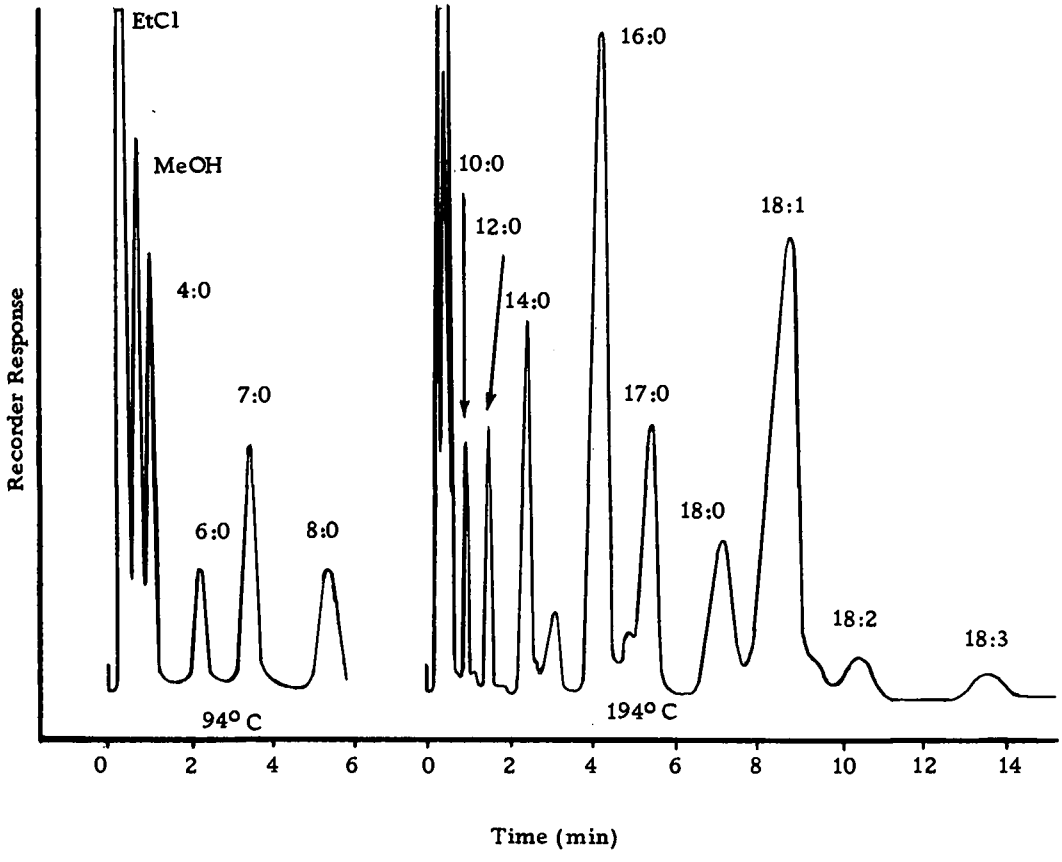

Figure 3. Chromatograms of the methyl esters of the free fatty acids of Cheddar cheese.

time of other major methyl esters were observed when heptadecanoic acid was esterified by itself, the purity would have no effect on the accuracy of the determinations. When using a new lot of heptadecanoic acid, however, new factors should be determined if necessary.

The controlled removal of the ethyl chloride during the concentration step was introduced to avoid the inconsistent loss of the more volatile methyl esters. When known methyl esters of butyric, caproic, and caprylic acids were carried through the extraction and concentration procedures, their relative proportions were unaltered as determined by GLC.

Free Fatty Acids Determined in Cheddar Cheese Samples

The samples analyzed for this manuscript were selected from 36 cheeses entered in the 1963 Oregon Dairy Industries contest. The cheeses were scored under contest conditions by three official judges. After judging, a section of each cheese was cut, waxed, and stored at 0° C until the analysis could be conducted. Cheeses for analysis were selected to represent the following categories: cheeses manufactured from raw and pasteurized milk, cheeses ripened for seven to eight months (aged) and three to four months (medium aged), and cheeses scored high, medium, and low in the

contest. A summary of the cheeses selected for analysis is presented in Table 2.

TABLE 2

Cheeses selected for analysis

Cheese	Age[a]	pH	Score	Criticism
1	Aged[b]	5.26	93.5	No comment
2	Aged[b]	5.19	92.0	Acid
3	Aged[b]	5.35	89.0	Fermented
4	Medium[b]	5.27	93.5	Acid
5	Aged[c]	5.47	95.0	Flat
6	Aged[c]	5.32	90.0	Fermented
7	Aged[c]	5.33	89.0	Bitter, Unclean
8	Medium[c]	5.35	95.5	No comment

[a] Aged cheeses ripened 7-8 months, medium aged cheeses ripened 3-4 months.

[b] Manufactured from raw milk.

[c] Manufactured from pasteurized milk.

The levels of individual free fatty acids found in eight samples of Cheddar cheese and the percent deviation obtained between duplicate analyses and their mean are given in Table 3.

TABLE 3

Averages of duplicate analyses of free fatty acids in Cheddar cheese

Acid	Cheese 1		2		3		4	
	mg/kg cheese	% deviation	mg/kg cheese	% deviation	mg/kg cheese	% deviation	mg/kg cheese	% deviation
2:0	275.3	10.1	1103.0	1.5	1315.7	1.0	1134.2	2.2
4:0	93.9	0.6	177.6	0.5	206.8	3.8	102.1	2.4
6:0	34.9	0.5	78.5	2.2	60.5	0.5	37.1	0.1
8:0	34.2	2.0	74.2	0.1	55.0	0.4	36.2	9.7
10:0	44.8	2.5	74.7	0.4	61.7	2.4	50.2	4.8
12:0	59.7	2.0	109.0	0.6	93.0	1.0	68.9	3.8
14:0	176.1	1.6	308.9	0.6	263.8	0.4	214.7	2.0
16:0	443.0	1.2	689.4	0.1	547.5	0.1	521.6	0.1
18:0	160.4	2.3	236.7	1.3	246.1	0.8	144.1	0.3
18:1	359.7	0.8	590.0	0.7	501.5	1.0	360.6	1.8
18:2	47.6	0.8	87.3	0.9	90.8	1.9	46.4	0.9
18:3	33.4	0.0	57.1	1.4	52.1	0.4	25.9	0.0
Total....	1763.0		3586.4		3494.5		2742.0	

TABLE 3 (Continued)

Acid	Cheese 5		6		7		8	
	mg/kg cheese	% deviation	mg/kg cheese	% deviation	mg/kg cheese	% deviation	mg/kg cheese	% deviation
2:0	836.7	2.0	1325.2	1.0	1170.1	0.4	436.7	1.1
4:0	127.1	0.6	145.2	3.2	119.2	4.6	76.2	0.8
6:0	33.0	1.1	48.2	0.0	27.4	0.7	29.0	1.0
8:0	42.7	2.1	43.6	0.5	49.6	1.2	35.6	2.0
10:0	52.7	0.4	56.8	1.1	79.8	2.3	54.9	1.0
12:0	83.0	1.3	90.0	1.2	122.7	0.7	87.0	0.6
14:0	236.3	0.1	234.5	0.1	314.5	3.2	191.3	0.4
16:0	510.4	0.0	498.1	0.5	620.1	2.1	516.4	0.3
18:0	192.3	0.5	218.5	0.1	216.4	1.6	103.7	0.4
18:1	457.7	0.9	629.9	0.0	818.7	0.2	318.8	0.9
18:2	80.6	0.5	93.2	0.8	125.0	3.2	63.4	1.7
18:3	42.9	0.7	62.2	2.4	59.9	0.0	27.7	4.7
Total....	2695.4		3445.4		3723.4		1940.7	

The data presented in Table 3 is summarized in Table 4. The percent deviation obtained in titrating the caproic-and-higher fraction from the silicic acid column is not listed in the tables but was found to range from 0.7% to 3.1% with 1.66% as the mean; this would have a small effect on the accuracy of the results reported for the fatty acids 6:0 through 18:3, where the average of duplicate titrations from the silicic acid column was used to determine the total moles of these acids.

TABLE 4

Summary of free fatty acids determined in Cheddar cheese

Acid	Average percent deviation[a]	Average concentration in mg/kg cheese	Range in mg/kg cheese
2:0	2.41	949.6	247.4 - 1338.1
4:0	2.06	131.0	76.2 - 206.8
6:0	0.76	43.6	27.4 - 78.5
8:0	2.25	46.4	35.6 - 74.2
10:0	1.86	59.5	44.8 - 79.8
12:0	1.40	89.2	59.7 - 122.7
14:0	1.05	242.5	176.1 - 314.5
16:0	0.55	543.3	443.0 - 689.4
18:0	0.91	189.8	103.7 - 246.1
18:1	0.96	504.6	318.8 - 818.7
18:2	1.34	79.3	46.4 - 125.0
18:3	1.20	45.2	25.9 - 62.2

[a] Between all duplicate analyses and their mean.

In the GLC analysis of the higher fatty acids, only the peak areas of the major methyl esters were measured, although peaks representing several of the minor fatty acids of milk fat were present (see Figure 3). As was noted by Patton (83), the methyl esters of certain branched-chain and unsaturated fatty acids cannot be separated from the methyl esters of other saturated, unbranched acids by the usual packed GLC column. However, the contribution of these minor components to the peak areas of the major components, where retention time is equal, should be nearly negligible.

The differences observed in the concentration of free fatty acids from cheese to cheese are not as striking as one might expect considering the wide differences in score, age, and manufacturing conditions of the cheeses. Considering the cheeses which scored highest in the contest, one finds a range in the concentration of free fatty acids nearly as wide as that of all the samples; this again supports the "balanced" composition theory of Cheddar cheese flavor (37; 62, p. 201-219; 80; 99).

Kristoffersen et al. (64) analyzed 25 samples of Cheddar cheese for acetic, propionic, butyric, and higher acids by the method of Harper (38). The cheeses ranged in age from one to 22 months. The averages of the results of Kristoffersen are compared to the averages of the results of the thesis work below:

	Kristoffersen	Thesis
2:0	1, 357 mg/kg cheese	949. 6 mg/kg cheese
3:0	36 mg/kg cheese	0 mg/kg cheese
4:0	176 mg/kg cheese	131 mg/kg cheese
6:0 and higher	8. 6 millimoles/kg cheese	7. 6 millimoles/kg cheese

With the exception of propionic acid, the results agree fairly well. Patton (84), as previously mentioned, found propionic acid only sporadically and in quite low concentrations in Cheddar cheese. The manner in which Kristoffersen collected fractions from the column leaves some doubt as to the correctness of his values for propionic acid; large fractions of 130, 75, 40, and 175 ml were collected for long-chain, butyric, propionic, and acetic acids respectively. Since Kristoffersen's column employed no internal indicator and since the threshold volume for a given acid will vary somewhat from column to column, it is difficult to see how propionic acid or the other acids could have been consistently separated into discrete fractions.

Peterson et al. (87) separated fatty acids from Cheddar cheese by a method of partition chromatography. Separation was claimed for all of the even numbered acids from acetic through capric. Amounts of various acids at the end of a 360 day ripening period were reported as follows: acetic 280-, butyric 420-, caproic 130-, caprylic 280-, and capric 160 mg/kg of cheese. With the exception of acetic acid which is lower, the concentrations of free fatty acids determined by these authors was somewhat higher than in

Kristoffersen's work or the work of this thesis.

In Table 5 the percentages by weight of individual free fatty acids determined in Cheddar cheese are compared to the percentages by weight of individual esterified fatty acids in milk fat. The data concerning the fatty acids of milk fat was reported by Herb et al. (40) and Jack (52). The percentages by weight of the individual free fatty acids, 6:0 through 18:3, are very close to the values reported for the same esterified acids of milk fat. This suggests that the fatty acids 6:0 through 18:3 of Cheddar cheese may be hydrolyzed from milk fat in a nonselective manner by milk or bacterial lipase or lipases. Free butyric acid, however, was always present in about twice the amount of the reported percentage by weight of butyric acid in the triglycerides of milk fat. This suggests that butyric acid is hydrolyzed selectively from the milk fat or is produced by bacterial metabolism from non-fat material. Peterson and Johnson (87) noted an increase in butyric acid without a corresponding increase in other fatty acids during the early period of Cheddar cheese ripening. Harwalkar and Calbert (39) determined the relative amounts of lauric-and-higher, capric, caprylic, caproic, and butyric acids released from milk fat by the action of milk lipases. As lipolysis progressed the mole percent of the lauric-and-higher fraction gradually decreased, whereas butyric acid increased. The authors concluded

TABLE 5

Free fatty acids found in Cheddar cheese compared to the esterified fatty acids of milk fat

Acid	Cheese[a]									Milk fat[b]	
	1	2	3	4	5	6	7	8	Average	c	d
4:0	6.3	7.2	9.5	6.4	6.8	6.8	4.7	5.1	6.6	2.8	3.5
6:0	2.3	3.2	2.8	2.3	1.8	2.3	1.1	1.9	2.2	2.3	1.4
8:0	2.3	3.0	2.5	2.3	2.3	2.1	1.9	2.4	2.4	1.1	1.7
10:0	3.0	3.0	2.8	3.1	2.8	2.7	3.1	3.7	2.7	3.0	2.7
12:0	4.0	4.4	4.3	4.3	4.5	4.2	4.8	5.8	4.5	2.9	4.5
14:0	11.8	12.4	12.1	13.4	12.7	11.1	12.3	12.7	12.3	8.9	14.7
16:0	29.8	27.8	25.1	32.4	27.5	23.5	24.3	34.3	28.1	23.8	30.0
18:0	10.8	9.5	11.3	9.0	10.3	10.3	8.5	6.9	9.6	13.2	10.4
18:1	24.2	23.8	23.0	22.4	24.6	29.7	32.1	21.2	25.1	29.6	18.7
18:2	3.2	3.5	4.2	2.9	4.3	4.4	5.0	4.2	4.0	2.1	--
18:3	2.2	2.3	2.4	1.6	2.3	2.9	2.3	1.8	2.2	0.5	--

[a] Free fatty acids found in Cheddar cheese expressed as percent weight of total free fatty acids (4:0-18:3).

[b] Fatty acids of milk triglycerides expressed as percent weight of total fatty acids.

[c] Data of Herb et al. (40).

[d] Data of Jack (52).

that a slight degree of selective lipolysis of the short-chain acids by milk lipase took place under certain conditions. In a sample of milk activated by homogenization and held for 24 hours at 40° F, the mole percent of lauric-and-higher fraction dropped from 88 to 73 while butyric acid rose from 3.5 to 12.0. Other investigators have demonstrated that milk lipase is somewhat specific for the 1- position of milk triglycerides (29, 57), and it has been observed that 75% of the butyric acid is present in the 1- and 3- positions which are equivalent (12).

The State of Free Fatty Acids in Cheddar Cheese

An important point concerning the contribution of fatty acids to Cheddar cheese flavor which is not discussed in the literature is the ability of the fatty acids to exist as either acids or as salts. Taking into account the pH of normal Cheddar cheese (5.0-5.5) and the pKa's of the fatty acids (3.75-4.90), it is conceivable that a considerable portion of the total free fatty acids exist as salts rather than as acids.

Consideration of the potent odor of, for example, butyric acid in contrast to its odorless salt leads to the conclusion that lower pH's may play a considerable role in intensifying the flavor of Cheddar cheese by effecting the ratio between acid and salt. On the other

hand the salts of fatty acids, even though odorless, may contribute to the flavor of the cheese. This is supported by the observation that cheese which is slightly rancid often has an unbearable "soapy" flavor in conjunction with the rancidity. The "soapy" flavor may very well be caused by the salts of the higher fatty acids which are, indeed, soaps. At lower concentration these soaps may provide a background flavor which contributes to the normal flavor of the cheese.

Whether the ratio of free fatty acids to their respective salts conforms closely to the Henderson-Hasselbalch equation is doubtful since cheese is a complex system. If the ratio were in accordance with the equation, the average ratio of salt to acid for the cheeses analyzed would be 3.31:1.00 respectively (calculated from the average pH of the cheeses, 5.32, and the average pKa of the acids, about 4.80). Brief experiments to determine to what extent a given acid exists as its salt have been inconclusive. Attempts to isolate the free acid while leaving its salt behind by column chromatography apparently cause a shift in the equilibrium between acid and salt. As a consequence, the acid in question is not eluted as a sharp band but tails as elution progresses and the equilibrium in the cap material yields additional free acid from the salt.

Since the cheese samples used in this study were acidified to

pH 2.0 prior to column chromatography or centrifugation for GLC, it is reasonable to assume that the free fatty acids were nearly completely in the acid form. This is partially confirmed by noting that fatty acids eluted from either column came off sharply with little indication of tailing (see Figure 2). Thus, the data accompanying this manuscript is for the total free fatty acids with no discrimination being made between the fatty acids which exist as acids and those which exist as their corresponding salts.

SUMMARY AND CONCLUSIONS

The determination of the major free fatty acids of Cheddar cheese was accomplished by modifying three existing methods of fatty acid analysis. Acetic acid was determined by employing cheese which had been acidified to pH 2.0 and ground with silicic acid as the cap material for the column of Wiseman and Irvin (107). This column would also have provided a means of determining formic and propionic acids had they been present in significant concentrations in any of the cheeses analyzed. Butyric acid and the total moles of higher fatty acids, 6:0-18:3, were determined using the column developed by Keeney (59, p. 212-225). The cheese sample was acidified and ground with silicic acid as described above. Fractions of ten milliliters each were collected and titrated individually.

The higher fatty acids, 6:0 to 18:3, were determined individually by the combined techniques of column chromatography and GLC. The method of Hornstein (46) employing Amberlite IRA 400, a basic ion exchange resin, was modified to provide a means of isolating free fatty acids from the fat obtained from Cheddar cheese by centrifugation. The isolated fatty acids were converted to methyl esters and analyzed by GLC. Since absolute recovery was not attained in the centrifugation step, it was necessary to calculate the concentration of individual free fatty acids indirectly: the total moles of

caproic-and-higher acids, determined by titration, were distributed according to the molar ratios of these acids found by GLC.

Several modifications in the original technique of Hornstein permitted better precision and accuracy in the analysis of free fatty acids isolated from Cheddar cheese. Ethyl chloride was used as the solvent for extraction of the methyl esters, and the ethyl chloride (boiling point 12.3° C) was then removed from the ester mixture under controlled evaporation conditions to standardize the recovery of methyl esters. Two column temperatures were employed; one injection was made at 94° C and another at 194° C. The methyl esters of the acids 6:0 and 8:0 were analyzed at the lower temperature and the methyl esters of the acids 10:0 through 18:3 were analyzed at the higher temperature. Two internal standard acids, 7:0 and 17:0, were added to the cheese prior to centrifugation and provided a means of quantitating the peak areas of the methyl esters in the GLC analysis.

Eight Cheddar cheese samples of various age and score were analyzed for free fatty acids using the above methods. The cheeses included six samples of aged cheese, three manufactured from pasteurized milk and three from raw milk, and two samples of medium aged cheese, one manufactured from pasteurized milk and one from raw milk.

The following conclusions were drawn from the findings of the investigation:

1. The controlled evaporation of ethyl chloride provided more consistent recoveries for the volatile methyl esters.
2. The use of two internal standards and two injection temperatures improved the accuracy and precision of the analysis.
3. Formic and propionic acids were either absent or in concentrations too low to be detected in the Cheddar cheese samples analyzed.
4. The variation in the concentration of acetic acid from cheese to cheese was greater than for any other fatty acid.
5. The higher concentration of butyric acid compared to the concentration of the acids with carbon chains longer than butyric acid indicated that butyric acid is preferentially hydrolyzed from milk fat and/or produced through the metabolic activity of microorganisms.
6. The percent composition of the free fatty acids caproic through linolenic was nearly the same as that reported for the esterified fatty acids of milk fat.
7. The variation in concentration of free fatty acids in the cheeses scoring highest in the contest indicates that over

a yet unknown range the balance between free fatty acids and other flavor constituents is more important to the acceptability of the cheese than the concentration of free fatty acids.

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