AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented March 8, 1963

Title ISOLATION AND DETERMINATION OF THE FREE FATTY ACIDS OF MILK FATS

Abstract approved _____
(Major professor)

The free fatty acids of milk fat are believed to be involved in imparting flavor properties to milk and other dairy products. In the past the free fatty acids have largely been related to quality deterioration and hence the methods for measurement have been devised to determine the changes in the free fatty acid content and to relate these data with quality. No method has been reported to determine the quantities of individual free fatty acids. The purpose of this investigation was to evaluate procedures and adapt methods for isolation and measurement of the free fatty acids of milk fat and then to evaluate adapted methods by quantitative measurement of the individual acids in fresh cream fat samples and in butter made therefrom.

The ion exchange method of Hornstein et al. (50) was modified to isolate and esterify the free fatty acids from milk fat. The free fatty acids were adsorbed on Amberlite IRA-400 resin, the resin

was made fat free and the bound acids were simultaneously esterified and eluted with anhydrous methanol-HCl. The methyl esters were extracted from the reaction mixture with ethyl chloride (b. p. 12. 3° C.). The ethyl chloride was evaporated and the esters weighed. The methyl esters were then separated by gas-liquid chromatography using the thermal conductivity detector. The recovery of each saturated even numbered fatty acid from 4:0 to 18:0 was checked. The percent recoveries obtained were: 4:0, 71. 4; 6:0, 86. 5; 8:0, 66. 6; 10:0, 75. 1; 12:0, 94. 3; 14:0, 100. 2; 16:0, 99. 5 and 18:0, 92. 5.

The ion exchange resin was checked for its fat hydrolysing capacity, for retention of fatty acids when used in successive analyses and for leachings of brown polymers during each analysis. The resin did not show detectable hydrolysis of triglycerides nor did it retain or exchange fatty acids from previous use. It was necessary to pretreat the resin with stearic acid and anhydrous methanol-HCl to avoid interference of a leached polymer with quantitative results. An average of 5. 0 mg of residue leached from the resin during every analysis, but this did not interfere with the quantitative determination of free fatty acids.

Twenty samples of milk fat; ten from pasteurized sweet cream, nine from cultured butter and one from sweet cream butter were analyzed for free fatty acids. The results obtained were compared with the esterified fatty acid content of milk fat. The percent

composition of free fatty acids was similar to that of the esterified fatty acids in milk fat. Also the manufacturing process of butter had little or no effect upon the free fatty acid composition of the fat. The values obtained for volatile fatty acids, especially 4:0, were not consistant. One reason for this probably was that evaporation of the ethyl chloride from the solution of the methyl esters was carried out at room temperature and the evaporation rate was not controlled. It is believed that the results would be more consistant if the evaporation of ethyl chloride were carried out under controlled and standardized conditions and if internal standards are employed for quantitative references rather than weighing the ester mixture.

Samples of autoxidized milk fat, sweet cream fat and rancid cream fat were analyzed for further evaluation of the method.

ISOLATION AND DETERMINATION OF THE FREE FATTY ACIDS OF MILK FATS

by

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A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1963

APPROVED:

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Date thesis is presented March 8, 1963

Typed by Jolene Wuest

ACKNOWLEDGMENT

The author wishes to express his deep sense of gratitude to Dr. Edgar Allan Day for his guidance, criticism, helpful suggestions, unselfish devotion of time and encouragement throughout the research and during the preparation of this manuscript. Thankful appreciation is expressed to Dr. George Arthur Richardson, Professor of Food Science and Technology for his unselfish guidance and encouragement.

Last, but not least, the author wishes to thank his wife Ratna L. Khatri, parents and friends for their encouragement and financial help throughout the period of this work.

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INTRODUCTION

From the standpoint of flavor, milk fat is a very important constituent of milk and other dairy products. It imparts flavor properties to these products that are not duplicated by any other fat. Chemical definition of all of the responsible flavor compounds has not been reported. It is believed, however, that free fatty acids associated with the fat may play a part (58, p. 31). When the concentration of free fatty acids exceeds certain unknown levels, the fat becomes rancid. At levels below the flavor threshold of rancidity, the free acids could well contribute to the rich tasting qualities of the fat.

Up to the present time, the concentration of free fatty acids in milk fat and dairy products has been largely related to quality deterioration. Many procedures have been devised, therefore, to obtain some measurement of changes in the free fatty acid content and to relate these data with quality. These procedures have included titration of the fat, isolation and titration of water insoluble acids, titration of steam distillates from the fats to name a few. All of the procedures have had one main shortcoming; namely, the quantities of individual acids can not be determined. Therefore, the data are

obtained by empirical methods and tell nothing about the amounts of individual acids present or their possible significance. This point is especially important in the case of studies with the natural flavor of milk fat.

The purpose of this investigation was to evaluate procedures for isolation and measurement of the free fatty acids of milk fat. Attempts were made to obtain quantitative data on the individual acids in fresh cream fat samples and in butter made therewith.

REVIEW OF LITERATURE

FATTY ACID COMPOSITION OF MILK FAT

Milk fat from mammals differs from other fats and oils because of the relatively abundant quantities of the short chain acids; 4:0, 6:0, 8:0 and 10:0. Bovine milk fat contains approximately 10 mole percent of butyric acid which makes it unique among the more common edible fats and oils.

The fatty acid composition of milk fat is complex and it has maintained the attention of many researchers since the presence of saturated fatty acids was first reported by Chevreul in 1823(91, p. 173). The most significant advances in fatty acid composition studies have been made, however, during the past decade. Developments in analytical procedures, particularly chromatography and spectroscopy, have been largely responsible for the progress. Recent reviews by Hilditch (37, p. 125-134), Ling (67, p. 145), Jack and Smith (54, p. 3-10), Shorland and Hansen (91, p. 167-190), Pont (83, p. 134-138 and 144-146) and Jack (52) provide an excellent coverage of literature through 1959.

The major acids of milk fat all contain an even number of carbon atoms ranging from C_4 through C_{20}. The major unsaturated acids are oleic (18:1), linoleic (18:2), linolenic (18:3) and arachidonic (20:4). In addition to these major acids approximately 50 other acids occur in the fat (47 and 52). These may be classified as saturated even numbered fatty acids from 20:0 through 26:0, mono and poly unsaturated conjugated and unconjugated fatty acids, saturated normal odd numbered fatty acids from 5:0 through 25:0, saturated branched chain iso- and anteiso- as well as multibranched chain fatty acids and cis-trans isomers of unsaturated fatty acids.

The presence of many of these components has been confirmed during the past three to four years. Hansen and Shorland (33 and 34) have established the presence of saturated odd numbered fatty acids from 19:0 to 23:0, saturated even numbered fatty acids from 22:0 through 26:0 and cis - Δ^9-heptadecenoic acid. The presence of 9:0 to 17:0 odd numbered saturated fatty acids was confirmed by Jensen and Gander (59). The monosaturated even numbered fatty acids from 10:1 to 16:1 also have been reported by many investigators ((27; 60; 61, p. 1987; 80 and 93, p. 614-616).

Very recently, Magidman and coworkers (47 and 69) carried out quantitative analysis of fatty acids present in milk fat. They reported the identification of many previously unidentified fatty acids in the

TABLE 1

Fatty Acid Composition of Cow's Milk Fat

Acid	% a, c,	% b, c,
4:0	3.5	2.79
5:0	--	0.01
6:0	1.4	2.34
7:0	--	0.02
8:0	1.7	1.06
9:0	--	0.03
10:0	2.7	3.04
10:1	0.3	0.27 f
11:0	Trace	0.03
12:0	4.5	2.87
12:1	0.2	0.14 g
13:0	0.03	0.06
13:0 br(branched)	0.05 (iso)[d]	0.04
13:0 br	0.01 (anteiso)[d]	--
14:0	14.7	8.94
14:0 br	0.05	0.10
14:1	1.5	0.769
15:0	0.82	0.79
15:0 br	-- (iso)[d]	0.24 (A)[e]
15:0 br	0.43 (anteiso)[d]	0.38 (B)[e]
15:1	--	0.07
16:0	30.0	23.8
16:0 br	--	0.17
16:1	5.7	1.79 h
17:0	Identified	0.70
17:0 br	Trace (iso)[d]	0.35 (A)[e]
17:0 br	0.41 (anteiso)[d]	0.25 (B)[e]
17:1	--	0.27
18:0	10.4	13.2
18:0 br	Trace	Trace
18:0 br (Multi)	Trace	--
18:1	18.7	29.6
18:2	--	2.11
18:2[i] (cis-trans)	--	0.63
18:2[i] (trans-trans)	--	0.09
18:3	--	0.50
18:3[i]	--	0.01
19:0	0.05	0.27
19:1	--	0.06

TABLE 1 (Continued)

Acid	% a, c,	% b, c,
20:0	1.7	0.28
20:0 br	--	Trace
20:1	1.0	0.22
20:2	--	0.05
20:3	--	0.11
20:4	--	0.14
20:5	--	0.04
21:0	0.05	0.04
21:1	--	0.02
22:0	0.07	0.11
22:1	--	0.03
22:2	--	0.01
22:3	--	0.02
22:4	--	0.05
22:5	--	0.06
23:0	0.06	0.03
23:1	--	0.03
24:0	0.05	0.07
24:1	--	0.01
25:0	--	0.01
26:0	0.06	0.07

a. Data of Jack., E. L. (52).
b. Data of Herb et al. (47).
c. Fatty acids expressed as percent weight of total fatty acids.
d. Acids reported as iso- and anteiso-isomers as shown above.
e. Acids reported as A and B being two isomers.
f. Terminal Double bond.
g. Includes cis, trans and terminal double bond isomers.
h. Includes cis and trans isomers.
i. Conjugated double bonds.

milk fat. The total number of fatty acids identified by them in milk fat was at least 60. Their results along with those given by Jack (52) are presented in Table 1.

Factors Influencing Fatty Acid Composition of Milk Fat: Hilditch (37, p. 125-134), Jack and Smith (54, p. 3-10), Ling (67, p. 146) and Pont (83, p. 134-138 and 144-146) have reviewed various factors which influence the qualitative and quantitative composition of milk fat. When the environmental temperature exceeds about 85° F, the amount of low molecular weight acids is reduced and the degree of unsaturation is increased. A lowered plane of nutrition also has a similar affect (52 and 53).

Richardson et al. (87) studied the effect of humidity and temperature on the fatty acid composition of milk fat. They also found that at 85° F and 70% relative humidity (R. H.), as compared to the control conditions (65° and 50% R. H.), the production of short chain fatty acids was reduced while that of the long chain fatty acids was increased (30). There was no observed change in the concentration of myristic acid (C_{14}), while oleic (C_{18} monoethenoic) acid was reduced. It should be noted that the decrease in oleic acid at higher temperature is contrary to the observations made by Jack (52 and 53).

TOTAL FREE FATTY ACIDS OF MILK FAT

The free fatty acids of milk fat were early recognized to be of practical importance in quality deterioration problems and considerable work has been published on their measurement in dairy products.

Most of the early methods developed to measure fat hydrolysis in farm gathered cream and butter involved titration of a fat sample dissolved in a solvent, with standard base. Many variations in procedures for isolation and titration of the fat have been published (4, p. 417; 10; 13, p. 477; 65, p. 75; and 96). The main shortcomings of direct titration methods are that they can only be applied to anhydrous milk fat (82) and they do not give a measure of individual acids.

In an effort to extend the titration procedure to fluid dairy products, solvent extraction procedures were developed to isolate the free acids from aqueous systems (23, p. 752; 48; 82 and 95). Frankel and Tarassuk (23, p. 752) were able to achieve 95 to 100% recovery of high molecular weight acids and 52 to 58% recovery of low molecular weight acids from fluid milk by this approach. Perrin and Perrin (82) were able to improve the recoveries by adjustment of pH of milk to 3.0.

The disadvantage of solvent extraction methods is that only partial recovery of water soluble acids is obtained (19, 62 and 76).

This was amply stated by Jack and Smith (54, p. 2):

> Although the solvent extraction procedures reveal a larger proportion of the fatty acids in fresh milk than does the acid degree of the churned fat, these procedures probably still fall short of quantitative recovery of all the fatty acids, especially butanoic (4:0), which is partly miscible with water. These procedures may also extract non-fat acidic materials.

WATER INSOLUBLE FATTY ACIDS (WIA)

In the course of measuring the volatile acids and lactic acid in cream and butter, Hillig and Ahlman (42, p. 742) also determined the total acids. The difference [Total acids - (lactic + volatile acids)] was termed "unidentified acids" and these were later found to be chiefly WIA. Most of the WIA were retained in the butter after churning and only small amounts of WIA were lost in the buttermilk (42, p. 747). Hillig and Montgomery (44, p. 755-756) found that propionic acid was completely lost in the buttermilk while a variable but substantial portion of butyric acid, usually remained in the butter.

On further investigations the method of Hillig (38) for determining WIA was found to give satisfactory results (39) and hence it was adapted as first action by Association of Official Agricultural Chemists (11). The method of Hillig (38) also seemed to agree well with the flavor scores of butter (3, p. 29). This is indicated by the following results:

Average Score of Butter	Average WIA mg/100 g fat
90.1	159
89.0	244
88.8	378

In studying the effect of various factors on the butyric acid and WIA content of the butter, Hillig, Ahlman and Jordan (46) found that the neutralization of cream as practiced in the industry, did not have any affect on the WIA and butyric acid content. The "continuous" process of butter making, also did not seem to affect the WIA content of butter, but approximately two-thirds of the butyric acid present in the cream was lost from the butter made by this process as compared to the churned butter in which most of the butyric acid is lost (43).

In an effort to reduce the time of analysis of WIA in butter and cream, Hillig (40) suggested a "sorting" method. This method avoided the weighing of the WIA; instead the WIA were titrated and the approximate weight was calculated from the titer reading, using 270 as the mean molecular weight. The value 270 was found to be the average of many determinations (40).

Hillig (41) developed a rapid method for determining WIA in cream and butter. In this method he washed the fat free of water soluble acids then dissolved it in ether and titrated to the phenolphthalein end point. The WIA were calculated using the value 1 ml 0.05 N NaOEt ≡ 13.5 mg WIA. This method was quick and gave satisfactory results, when the WIA content was below 1000 mg/100 g fat. This

method was adopted as first action by the Association of Official Agricultural Chemists (12). Using this method, Hillig and Weiss (45) surveyed the WIA content of commercial cream over a twelve month period. They found that the WIA of cream were slightly higher during July, August and September than during the remainder of the year. They also found that the WIA in sweet or clean sour cream with no objectionable odor, were between 180 mg and 325 mg/100 g fat with an average of 252 mg/100 g fat. After comparing the rapid method (41) with the official method (5, p. 277) they established the following correlation:

WIA (rapid method) = 58.7 + 1.007 x WIA (official method)

In a study of WIA content of butters made from previously graded cream, Freeman and Barkman (24) showed that the "carry-over" of WIA from cream to butter varied without any particular trend; also the WIA content of butter increased with age of cream and was markedly higher in winter than in summer. Similar results were obtained by Parmelee and Babel (78, p. 16) who also found that the WIA content of commercial butter was related to season, breed, or stage of lactation of the cow. The ripening of cream with starter cultures, did not seem to have an affect on the WIA content of cream or butter made therefrom; however, stirring and agitating of milk increased the WIA content of cream and butter (16, p. 972-975).

Crowe (16, p. 970-971) found that the WIA content of butter increased more rapidly when held at 40 - 45° F than when held at 55 - 60° F or 70 - 72° F for 0, 2, 4 or 6 days. Acidification of cream in general, reduced the WIA values and the WIA content of the acidified cream increased when held at 70 - 72° F and not at 40 - 45° F or 55 - 60° F for 2, 4, or 6 days.

Freeman and Lewallen (25) suggested that in order to get consistent results by Hillig's method (41), the milk should be pasteurized by heating to 160° F for 30 minutes immediately after milking. For samples in which cream was heavily neutralized before churning, they found that Ensminger's method (20) and Hillig's method (41) gave lower values of WIA. This was because more of the acids being in the salt form, were washed away; hence Ensminger suggested the use of dilute hydrochloric acid for washing (21, p. 769).

Colorimetric Methods for WIA: Robert, Epple and Horral (88) found that when the indicator alpha-naphtholphthalein is added to milk fat containing free fatty acids, a change in color, which is proportional to the amount of WIA present in the fat, was obtained. Armstrong and Harper (3, p. 30) compared this method (88) with that of Hillig's (38) and observed a direct relationship between the color produced and the WIA content of the fat. Armstrong and Harper (2 and 34) further modified the colorimetric method (3, p. 30) to enable

more accurate measurement of the WIA content of butter and cream. The results obtained by this method compared well with those of Hillig's (38) for samples containing less than 400 mg of WIA per 100 g fat. The results were not affected by the presence of butyric or lactic acid in the fat (2 and 35).

Chromatographic Methods: Ramsay and Patterson (85, p. 647) suggested a chromatographic method to separate and determine the individual volatile acids. They separated and identified water soluble fatty acids from 1:0 to 4:0 on the silicic acid column. They modified their method using silicic acid-methanol-bromocresol column to separate steam volatile and less water soluble fatty acids from 5:0 to 10:0 (86, p. 140). Nijkamp (77) later analyzed the 4:0 to 10:0 saturated fatty acids from milk fat by the above method (86, p. 140).

Van Dame (97, p. 407) modified the silicic acid-hexane-butanol-propylene glycol column chromatography method of Keeney (63, p. 5-9) for separation of total esterified butyric acid and higher fatty acids of milk fat, and applied it to determine free butyric acid and WIA in milk fat. This method gave slightly lower values for butyric acid as compared to those by the official method (5, p. 260 and 273); whereas, the WIA values were comparable in both cases. The explanation given for lower butyric acid values was that in the official method (5, p. 260 and 273) there was no correction made for

volume occupied by fat in the calculation of butyric acid; and since butyric acid is much more soluble in water, a greater portion of it should be present in the aqueous layer than in the fat layer during the first step of the method (98).

GAS-LIQUID CHROMATOGRAPHIC (GLC) ANALYSIS OF MILK FAT

Development of GLC: Since the first publication on GLC for the microestimation of fatty acids by James and Martin (56, p. 679-690), the technique has developed with a tremendous pace. James and Martin (56, p. 685) separated short chain fatty acids from 1:0 to 12:0 by using a Kieselguhr column containing silicone plus 10% stearic acid. The eluted fatty acids were absorbed in an aqueous solution of phenol red indicator and were photoelectrically titrated with standard NaOH. The chromatogram was simultaneously and automatically plotted. Later, Martin and James (70) developed a gas density meter for the detection of the vapors in gas streams. This provided a means for the extension of the GLC technique to high boiling fatty acids. They used this detector in the GLC separation of the methyl esters of 1:0 to 18:0 fatty acids (57). Using the same detector, Beerthuis and Keppler (6) separated the methyl esters of fatty acids from 12:0 to 26:0. They also employed a newly developed Katherometer, which

could withstand temperatures up to 350° C (6).

Once the basic principles of GLC were laid out by these pioneer workers, subsequent developments in the GLC technique mainly dealt with the use of different types of stationary phases, inert supports, columns and temperature programming procedures.

Separation of Fatty Acids by GLC: The original method of James and Martin (56, p. 685) and Van de Kamer (99) was modified by Hankinson and Harper (31 and 32). They increased the ratio of Kieselguhr (support) to liquid phase (silicone) and by decreasing the percent of stearic acid in the liquid phase they obtained better separation of fatty acids from 1:0 to 8:0 and were able to quantitatively estimate the free fatty acids of milk.

Recently, Metcalfe (71) separated fatty acids from 4:0 to 22:0 (unesterified) by GLC. He used 25% (W/W)DEGA (diethylene glycol adipate) polyester (LAC-2R-446) and 2% phosphoric acid as stationary phase on 60-80 mesh Celite 545 as support.

GLC of Methyl Esters of Fatty Acids: After the gas density meter was developed (70), it became easy to detect vapors of neutral compounds in gas streams. The lower boiling points of methyl esters of fatty acids made these more attractive than the free acids for GLC analysis. Using this detector, James and Martin (57) separated the

methyl esters of fatty acids from 1:0 to 18:0 on columns containing dicotyl phthalate, paraffin wax, or Apiezon M as the stationary phases. Beerthuis and Keppler (6) have suggested many stationary phases which could be used for separating fatty acid methyl esters. These are: "Apiezon L", Dow Corning Silicone oil, Silicone Grease and polyethylene alkatheme.

Liberti, Cartoni and Pallotta (66) analyzed from 2:0 to 22:0 fatty acid methyl esters using Silicone vacuum grease. The separated esters were detected by electronic titration of the CO_2 liberated upon the incineration of the esters. Craig and Murthy (14) employed Silicone grease and DEGS (diethylene glycol succinate) or Diethylene glycol adipate to separate fatty acid methyl esters. They were able to separate the unsaturated fatty acid esters from saturated ones which was a significant break through in GLC analysis. Hornstein, Elliot and Crowe (49) used poly vinyl acetate polyester to separate long chain fatty acid methyl esters including C_{18} unsaturated fatty acids. They were able to estimate these fatty acids quantitatively with an accuracy of 5%.

Patton et al. (81) compared various stationary phases for separating fatty acid methyl esters. They used Diethylene glycol Succinate and LAC-2R-446 and Apiezon-L vacuum grease. They found that DEGS gave very good results in resolving C_{18} unsaturated

acids and it had better capacity for rapid separations. Apiezon-L was also found to give good separation of the monounsaturated fatty acid esters.

Until recently, the columns used in GLC were packed with a stationary phase supported on one of many inert supports such as Celite 545 (6, 30, 57, 71 and 81), powdered glass (66), and fire-brick (14). Capilliary columns with the inside wall coated with a stationary phase (Apiezon L) gave better separation than a packed column for the short chain mono unsaturated fatty acid esters, but it did not give good resolution for the C_{18} acids (81).

To get better separation and to enhance the accuracy of the determination of fatty acids, Van de Kamer and coworkers (99) employed gradually increasing temperatures of the column from 100° C to 150° C. The temperature of the column was maintained by circulating glycerine, and controlling its temperature with a thermostat. This technique of a steady increase in the temperature of the column during the chromatographic separation is now known as "temperature programing". This method was later used by Hankinson and Harper (31) to quantitatively estimate the free fatty acids of milk. Gander, Jensen and Sampugna (27 and 60) found that the Apiezon L column worked satisfactorily while the DEGS column bled excessively when temperature programed.

One of the problems in quantitative GLC analysis of fatty acid methyl esters is the volatility of short chain fatty acid methyl esters. To overcome this problem Jensen and Gander (59) used allyl esters of fatty acids instead of methyl esters and got quantitative results. Oette and Ahrens (64) have used 2-chloroethanol esters of short chain fatty acids.

Gehrke et al. (30) used GLC analysis of methyl esters but they chromatographed the esters at three different temperatures to get good separation between the peaks. To keep the short chain fatty acid methyl esters from volatilizing, Gehrke and Lamkin (29) took special care to get rid of the ethyl ether which was used to extract the esters from the reaction mixture. They evaporated the ether at -50° C and 6 mm Hg and claimed quantitative recoveries. Smith (93, p. 608) suggested the use of ethyl chloride (b. p. 12.3° C), for extracting methyl esters from the reaction mixture. This solvent can be removed at room temperature without loosing much of the volatile methyl esters.

METHYLATION AND SEPARATION OF FREE FATTY ACIDS

Methods of Methylation: The GLC of methyl esters of fatty acids has been preferred by many workers over the GLC of free acids. The main factor influencing the trend is that the methyl esters are more

volatile, less polar and many types of stationary phases are suitable for their effective separation.

Many methods have been used to esterify the fatty acids even before the advent of GLC. Interesterification or ester exchange is one of the methods which has been used (37, p. 571). The method consists in refluxing the fat with methanol in the presence of strong alkali or acid as catalyst. This method has been used by many workers to prepare fatty acid methyl esters from milk fat (15, 81 and 93, p. 609). The methyl esters obtained in the above case were extracted from the reaction mixture by various solvents and the solvents evaporated.

James (55) esterified the fatty acids by refluxing them with 0.8 N HCl in dry methanol. This reaction was further studied and modified in order to reduce the time of esterification and to get quantitative results (9, p. 806 and 92).

Mitchell, Smith and Bryant (75) developed a method to quantitatively esterify carboxylic acids. The reagent used was methanol-borontrifluoride made by bubbling BF_3 gas through methanol (74, p. 300-301). Metcalfe and Schmitz (72) modified the original method by refluxing the fatty acids with methanol - BF_3 reagent over a steam bath and the methyl esters were then extracted from the mixture using an appropriate solvent. The method gave 90 percent recovery

of the fatty acids.

Diazomethane gas has been used for the methylation of fatty acids. Since it is explosive and poisonous, it is not stored but is prepared just before it is used. DeBoer and Backer (17 and 18) obtained diazomethane as a result of alkaline decomposition of p-tolylsulfonyl methyl-nitrosoamide. Diazomethane obtained by this method was successfully used by Hudy (51) to make methyl esters of fatty acids. Roper and Ma (89, p. 247-248) reported a method for microsynthesis of fatty acid methyl esters using diazomethane in which they divised a microsynthesis apparatus. Diazomethane was generated by the action of alkali on N-nitroso methyl urea. The methyl esters were formed by bubbling diazomethane through an ether solution of fatty acids. Schlenk and Gellarman(90) obtained satisfactory results using diazomethane as the methylating agent. They suggested that when precautions were taken and small amounts of reagents used, the hazards of toxicity and explosion could be deterred. Apart from the toxicity and explosiveness of diazomethane gas, it has been found to give low yields of the methyl esters and some additional products by addition at double bonds in unsaturated acids (94).

Lorette and Brown (68) prepared methyl esters by using 2, 2-dimethoxypropane (DMP). This method was later modified by Radin, Hajra and Akahori (84) and it consisted of leaving a mixture of fatty

acids, methanol, concentrated HCl and DMP in a stoppered container for one hour. The esters were separated from this reaction mixture by evaporating the excess solvents and reagents under vacuum. The method gave quantitative results.

Gehrke et al. (30) used a silver salt-alkylhalide esterification method to prepare fatty acid methyl esters. The fatty acids were first converted to silver salts by reacting potassium salts of fatty acids with silver nitrate. The silver soaps thus obtained were reacted with methyl iodide solution under anhydrous conditions to give fatty acid methyl esters. The method seemed to give quantitative results (28).

Vorbeck et al. (100) compared four methods of methylation for their quantitative results. The methods compared were: diazomethane method of Roper and Ma (89, p. 247), methanol-hydrochloric acid method with micro-sublimation (94), ion-exchange resin and methanol-hydrochloric acid method of Hornstein et al. (50) and methanol-borontrifluoride method (72). The results showed that in the methanol-hydrochloric acid method of Stoffel, Chu and Ahrens (94), the lower fatty acids were all lost during the sublimation process. The other three methods compared well as far as recoveries of long chain fatty acids were concerned. The Hornstein et al. method (50) gave low recoveries of butyric acid. The diazomethane

method (89, p. 247) gave highest recoveries of all the methods compared and no significant loss of polyunsaturated fatty acids was found to occur by this method.

Methylation and Separation of Free Fatty Acids of Milk Fat: All of the forementioned methods of methylation are good when the fatty acids are not contained with esters such as glycerides of fat. No satisfactory method is available, however, to separate the free fatty acids in milk fat and then esterify them. Methods described earlier in this review for separation of free fatty acids utilized steam distillation of short chain fatty acids (37, p. 571 and 86, p. 140), column chromatography of short chain fatty acids (77; 85, p. 647 and 86, p. 140) or the extraction of WIA (38). None of these procedures provided a means of measuring individually, the complete spectrum of free fatty acids in milk fat.

Recently, Hornstein et al. (50) reported a new method to separate and esterify naturally occurring free fatty acids from fat. The free fatty acids were adsorbed on a strong anion exchange resin, Amberlite IRA-400 and subsequently eluted from the resin and converted to their methyl esters in one step by anhydrous methanol-hydrochloric acid reagent. The fatty acid methyl esters were extracted from the reaction mixture with ethyl ether; the solvent was then evaporated and the methyl esters chromatographed. The results

were reported to be quantitative.

Gaedke (26) combined and modified the methods of Hornstein et al. (50) and Gehrke et al. (30) to separate and prepare methyl esters of free fatty acids. The free fatty acids were separated from the milk fat by adsorbing on the ion exchange resin Amberlite IRA-402 or 401. The fatty acids were eluted from the resin with 10% nitric or sulfuric acid in ethyl ether. The acids were successfully converted to potassium soaps, to silver soaps and finally to methyl esters as described by Gehrke et al. (30).

McCarthy and Duthie (73) found that the use of anion exchange resins to separate the free fatty acids from milk fat did not give quantitative results. They reported that only after the same resin was used four or five times, being recharged after every use, did quantitative recovery of free fatty acids become possible. GLC analysis of the eluted acids showed, however, that the fatty acids from previous use were exchanging with the free fatty acids of the sample. They suggested the use of a silicic acid column containing KOH for isolation of the free fatty acids from the lipid mixture.

EXPERIMENTAL

ISOLATION AND ESTERIFICATION OF FREE FATTY ACIDS

In an attempt to develop or adapt methods suitable for free fatty acid analysis of milk fat, a number of isolation procedures were evaluated. These included solvent extraction, column chromatography procedures, and ion exchange resins. The procedure found to be most satisfactory was essentially that of Hornstein et al. (50) wherein the fat, in a non-polar solvent, is mixed with a basic anion exchange resin and the free acids are exchanged with the -OH ions of the resin. The resin is subsequently freed of triglycerides and the resin-retained acids are simultaneously esterified and released from the resin by treatment with methanol-HCl. The esters are then isolated by solvent extraction, the solvent is removed and the esters are analyzed by GLC.

The most satisfactory results were obtained after the original method of Hornstein et al. (50) was modified. Details of the modified procedure follow.

Preparation of Anhydrous Methanol-HCl Reagent: The anhydrous methanol-HCl was prepared by passing dry HCl gas through reagent grade methanol. The HCl gas was generated by dropping concentrated

sulfuric acid over reagent grade sodium chloride in a gently heated reaction bottle. The HCl vapors were dried by passing through a drying tube containing anhydrous calcium chloride. The dry HCl gas was then bubbled into a measured amount of methanol until it reached the concentration of 17-18 percent HCl (W/V) in methanol. The concentration of HCl was measured by titrating a known volume of the methanol - HCl with a standard alkali. The reagent was diluted with methanol to 1/2 the original concentration of HCl before use. The reagent was stored in the refrigerator.

Pretreatment of Ion Exchange Resin: Twenty-five grams of Amberlite IRA-400, a strongly basic, quaternary ammonium resin, was changed to the hydroxide form by stirring, with a magnetic stirrer in 50 ml of an aqueous solution of approximately 1N sodium hydroxide for five minutes. The resin was then washed with successive 100 ml portions of distilled water to remove the last traces of alkali. The resin then was washed twice with 50 ml portions of 95 percent ethanol; once with 50 ml of absolute ethanol, to remove last traces of water; finally, thrice with 50 ml portions of isopentane, to remove the traces of ethanol.

Three-tenths of a gram of stearic acid was weighed, dissolved into 50 ml of isopentane, and stirred with the resin, for 15 minutes by means of a magnetic stirrer. The resin was allowed to settle and

the supernatant liquid poured off. The resin was washed successively; once with 50 ml isopentane, and thrice with 95 percent ethanol. Then the resin was changed to the hydroxide form as described before. The purpose of the stearic acid treatment was to eliminate excessive leaching of a brown polymer from the resin (7).

The resin was checked to assure that the pretreatment had reduced polymer leaching to a minimum by stirring the resin with 50 ml of isopentane for 15 minutes and draining off the isopentane into a tared dish. The isopentane was evaporated first on a steam bath and then at room temperature to complete dryness. The residue was weighed and if found to exceed 0.3 mg, the resin was again treated with stearic acid until the residue weighed less than 0.3 mg.

The resin, after preparing as described above, was stirred with 40 ml of anhydrous methanol-HCl for 20 minutes. It was then allowed to settle and the supernatant liquid poured off. The resin was then put back into the hydroxide form. This process was repeated twice with fresh 40 ml anhydrous methanol-HCl each time. After the second treatment, the methanol-HCl was decanted off into a 250 ml separatory funnel and 25 ml of distilled water was added. The mixture was cooled to about 40° F in a cold room maintained at 34° F and extracted with three 50 ml portions of ethyl chloride. After the first extraction, the aqueous layer was saturated with sodium chloride

(reagent grade) and then extraction was continued. The extracts were combined, dried over anhydrous sodium sulfate, and evaporated at room temperature. The residue was weighed and if the weight exceeded 5 mg, the resin was again treated with methanol-HCl until the residue did not exceed 5 mg.

Regeneration of the Ion Exchange Resin: The resin was washed twice with 50 ml isopentane, once with 50 ml of absolute ethanol, twice with 50 ml of 95 percent ethanol, and finally with 100 ml of water. Fifty ml of approximately 1N sodium hydroxide was added to the resin and stirred with a magnetic stirrer for 5 minutes. The supernatant alkali was poured off and the resin was washed successively, first with 100 ml portions of water until the traces of alkali were washed off, and then twice with 50 ml of 95 percent ethanol and once with 50 ml absolute ethanol. Finally, the resin was washed with three 50 ml portions of isopentane and it was then ready to be used for analysis of another sample of milk fat. If the resin had to be stored for a long time, it was stored under a layer of ethanol.

Separation and Esterification of the Free Fatty Acids: Milk fat from butter was obtained by melting it at 45° - 50° C, centrifuging at 1000 R. P. M. for 5 minutes, and separating the oil from the serum. The butter oil was clarified by filtering it through Whatman No. 4 filter

paper at 50° C. For isolation of free acids from cream, the cream was churned in a Waring Blender and the fat was washed with cold distilled water for removal of serum. The butter oil was then prepared in the same manner as described for butter.

Twenty to twenty-five grams of the milk fat, accurately weighed and which normally contained 75 to 150 mg of free fatty acids, was dissolved in 20 ml of isopentane. This solution was transferred into a 250 ml beaker containing the pretreated Amberlite IRA-400 resin (25 g). The container used for weighing the fat was washed with three 10 ml portions of isopentane, and the washings were added to the beaker containing the resin-isopentane- milk fat. The contents in the beaker were stirred, with a magnetic stirrer, for 20 minutes and the resin was then allowed to settle. The fat solution was decanted off and the resin was made fat-free by washing 5 times with 25 ml portions of isopentane; each washing for 2-3 minutes.

The free fatty acids retained on the resin were simultaneously esterified and released by stirring the resin with 40 ml of anhydrous methanol-HCl for 20 minutes. The resin was allowed to settle and the liquid layer (reaction mixture), containing methanol-HCl and methyl esters of fatty acids, was carefully poured into a 250 ml separatory funnel. The resin in the beaker was washed twice with 10 ml portions of fresh anhydrous methanol-HCl. Each washing was stirred for

five minutes and the washings were combined with the reaction mixture in the separatory funnel. To this 25 ml of distilled water was added.

Extraction of the Methyl Esters of Fatty Acids from the Reaction Mixture: Ethyl chloride was used as the extracting solvent for isolation of methyl esters from the reaction mixture. The low boiling point of ethyl chloride (12.3° C) made it necessary to conduct the extraction in a cold room. Before the extraction process 25 ml distilled water was added to the reaction mixture in the separatory funnel and the mixture was cooled together with the washed resin contained in the 250 ml beaker to about 40° F. The resin in the beaker was then washed three times with 20 ml portions of ethyl chloride and the washings were added to the separatory funnel. The methyl esters were extracted from the reaction mixture by shaking the separatory funnel, first slowly and releasing the pressure every few seconds, and then vigorously for about 1 1/2 minutes. The two layers were allowed to separate and the lower aqueous layer was drawn off into a second 250 ml separatory funnel. The ethyl chloride layer was transferred into a 250 ml Erlenmeyer flask. The aqueous layer was saturated with sodium chloride (reagent grade) and extracted twice with 50 ml of fresh ethyl chloride as described above. The extracts were combined and dried over anhydrous sodium sulfate. The dried ethyl

chloride layer was decanted off in a 250 ml Erlenmeyer flask. The sodium sulfate residue was washed thrice with 10 ml of ethyl chloride and the washings were combined with the methyl ester solution.

The clear ethyl chloride solution containing the extracted methyl esters , was evaporated at room temperature to a volume of 3 - 4 ml. This was carefully transferred to a small 20 ml beaker and the Erlenmeyer was washed three times with 3 ml portions of ethyl chloride. The washings were combined with the solution in the beaker. The ethyl chloride was again allowed to evaporate at the room temperature until a few drops were left in the beaker. Care was taken not to evaporate all the ethyl chloride. The concentrated solution of methyl esters was then transferred from the 20 ml beaker to a tared 1 g vial. The beaker was washed thrice with ethyl chloride, and the contents added to the vial. The ethyl chloride in the vial was evaporated until a constant weight was obtained. Care was taken not to keep the vial at room temperature for a long time after all the ethyl chloride had evaporated. The differences in weight of the vial and methyl esters was noted and the vial was stoppered tightly and stored in the freezer compartment of a refrigerator for further analysis. An outline of a complete procedure for isolation, methylation and extraction of free fatty acids is given in Figure 1.

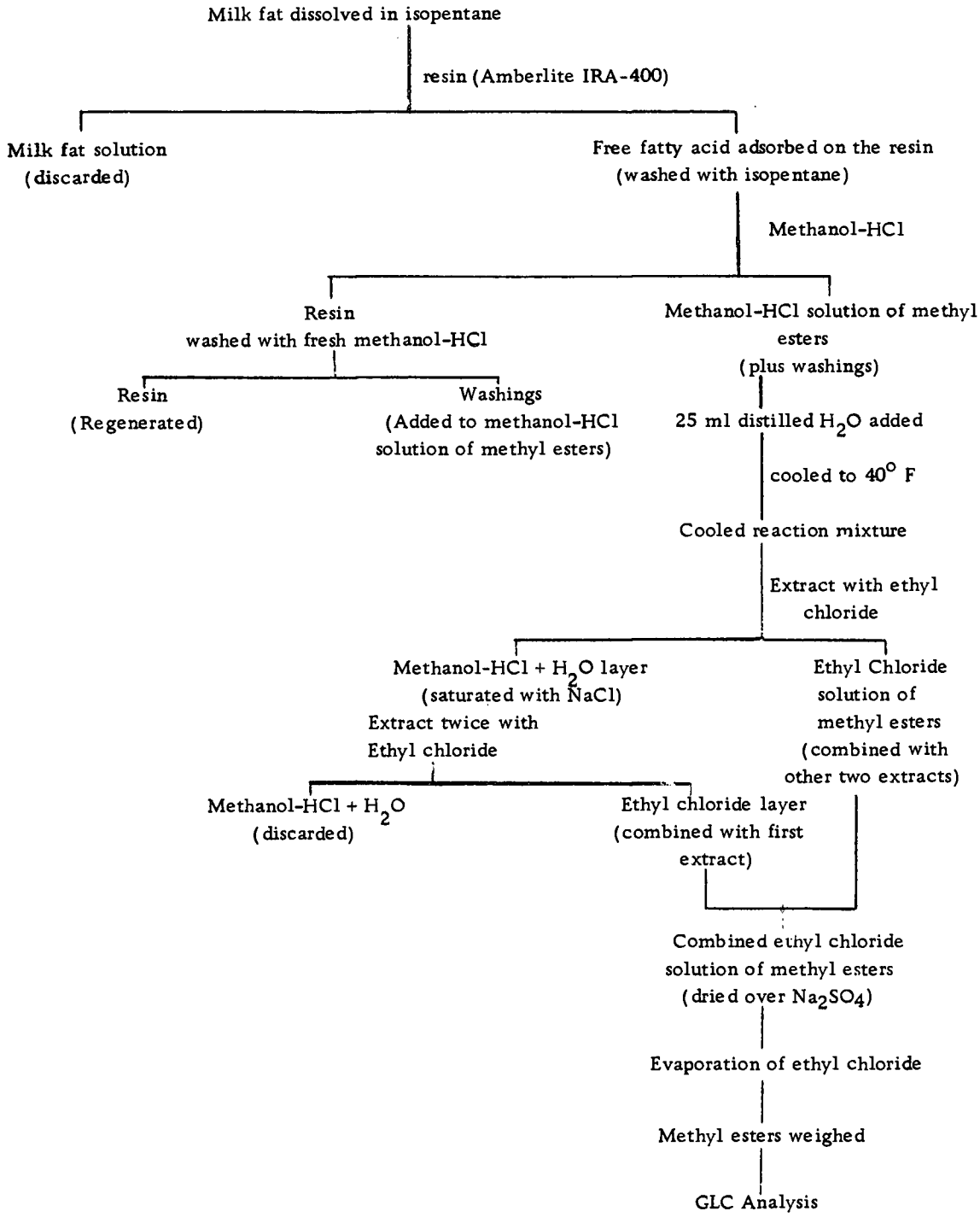

Figure 1. An outline of free fatty acid analysis procedure for milk fat.

QUALITATIVE AND QUANTITATIVE ANALYSIS OF THE METHYL ESTERS OF FREE FATTY ACIDS

Preparation of Column for GLC: Celite 545 was size graded to 80-100 mesh and was washed with acid and alkali as described by Farquhar et al. (22, p. 9-10). The Celite was then coated with stationary phase in the following manner: Ten grams of DEGS (LAC-3R-728 from Cambridge Industries, Cambridge, Massachusetts) was dissolved in chloroform and mixed with 30 g of 80-100 mesh Celite 545. The resulting slurry was heated on a steam bath and the mixture was stirred during the evaporation of the chloroform to assure even distribution of the stationary phase. The column packing thus obtained was dried in an oven at 110° C for 12 - 14 hours.

The column was prepared by adding the prepared packing to a 9 feet x 1/4 inch O. D. aluminum tube and vibrating the tube to assure uniform packing. The packed column was wound into a spiral shape to fit the gas chromatograph oven. The column was conditioned for 24 -48 hours in an oven at 180 ± 5° C under 20 psi of nitrogen. The column was conditioned for an additional 12 hours at 200° C under five psi of helium. Every new column was conditioned by the above procedure.

Analysis of Methyl Ester Mixture by GLC: The Aerograph model A-100 with a four filament thermal conductivity detector was the chromatograph employed. Operating parameters were: temperature, 200° C; helium flow rate, 85 ml per minute; recorder, 1 millivolt full scale; and filament current, 200 milliamps.

About 0.5 μl of the weighed methyl ester mixture, representing the free fatty acids from a known weight of milk fat, was injected into the gas chromatograph and the separated fractions were represented as peaks on the chromatogram. The separation was complete within 20 minutes. The peaks obtained were identified by comparing their retention times with those of known methyl esters obtained under exactly the same conditions. The area under each peak was measured by the method of triangulation and the percent composition of each ester in the mixture was determined by dividing the area under the peak by the total area under all of the peaks of the chromatogram. The weight of each ester present in the mixture was calculated by taking the percentage of the total weight of ester mixture represented by each peak and these data were multiplied by the recovery factors. The recovery factor for each ester was obtained by determining the percent recovery of the esters from a known mixture. The weights of the methyl esters were converted to the weights of the corresponding fatty acids and each fatty acid was expressed as grams of free fatty acid per kilogram of milk fat.

Determination of Percent Recovery of the Fatty Acids: Paraffin oil was used rather than milk fat for recovery studies in order to eliminate possible errors due to triglyceride hydrolysis by the resin. Twenty grams of "Shell" paraffin oil was twice treated with the Amberlite IRA-400 resin to remove any substances which might have affected the recovery results. A known mixture of even numbered saturated fatty acids from C_4 to C_{18} was prepared. The fatty acids were weighed in the sequence C_{18} first and C_4 last, to minimize the loss by volatilization of short chain fatty acids during weighing. The mixture was added to paraffin oil and the free fatty acids were isolated by the resin procedure, converted to their methyl esters and the esters analyzed by GLC as previously described. The percent recovery of each fatty acid in the mixture was determined by comparing the weight of acid added to the oil with the weight of acid obtained by GLC. A duplicate run was made with a fresh mixture of fatty acids and their percent recoveries were determined. The average of two recovery analysis was used as the percent recovery for each fatty acid.

A blank run was made in the above experiment with 20 g of paraffin oil containing no fatty acids. The weight of the residue, obtained after evaporation of all the ethyl chloride, was subtracted from the weight of the fatty acid esters obtained. When the residue

was analyzed on the gas chromatograph, it did not contain fatty acid esters.

Hydrolytic Capacity of the Ion Exchange Resin: This experiment was performed to study the possibility of fat hydrolysis by the strongly basic Amberlite IRA-400 resin. Twenty-five grams of milk fat were weighed and the free fatty acids were isolated and converted to their methyl esters. The esters were isolated and weighed in the usual manner. The isopentane solution of milk fat, after isolating the free fatty acids with the resin, was saved along with the washings. The isopentane was partly evaporated at room temperature, and the remaining solution was again analyzed for free fatty acids. The fat solution was once again saved and free fatty acid analysis carried out for the third time. The weight of the residue obtained was noted each time. A second fresh milk fat sample was analyzed in the same manner as described above and the weights of the isolated esters noted.

Evaluation of Silver Salt-Alkyl Iodide Method: Before adapting the modified Hornstein et al. method (50), the free fatty acids were analyzed by the method of Gaedke (26). A known mixture of fatty acids was prepared and added to 10 g of milk fat dissolved in ethyl ether. The fatty acids were adsorbed on pretreated Amberlite IRA-400 resin. After the fat was washed from the resin, the adsorbed acids were

eluted with 10 percent nitric acid in ethyl ether. The fatty acids were then converted to their potassium salts by neutralizing the reaction mixture containing eluted fatty acids and some added water, with 1N 80 percent ethanolic potassium hydroxide and then with 0.3 N ethanolic (80 percent) potassium hydroxide to the phenolphthalein end point. The silver salts of the fatty acids were formed by adding 1N silver nitrate to the potassium salts solution. The ethyl ether was evaporated and the silver salts centrifuged out. The silver salts were dried by washing with methanol and ethyl ether. These were then covered with methyl iodide and allowed to react overnight in a tightly covered vial. The methyl esters that formed were extracted with ethyl ether and the extract was evaporated until exactly one ml was left. This solution was then analyzed by GLC. The areas under the peaks were measured and compared to those obtained with known mixtures of methyl esters. A blank run was made with 10 g of milk fat that did not contain added fatty acids. The correction for the areas under the peaks obtained with the blank was made.

In order to calculate the amount of fatty acids recovered, a known mixture of fatty acid methyl esters was made and diluted to one ml with ethyl ether. An aliquot of this was analyzed by GLC and the area obtained under each peak was compared to the area obtained under the corresponding peak of the chromatogram representing the free fatty acids of milk fat.

RESULTS AND DISCUSSION

EVALUATION OF THE ION EXCHANGE RESIN

The Problem of Excessive Leaching of a Low Molecular Weight Polymer: It was observed in the course of isolation and esterification of free fatty acids that the resulting methyl esters contained a pale yellow color. This color was later found to be due to a brown residue that leached from the resin. Fresh resin gave more residue than one that had been used repeatedly. Since the quantitative studies were based on weighing the ester mixture, excessive leaching of polymers from the resin interfered with the analysis. Therefore, the amount of residue given by the Amberlite IRA-400 resin was determined. This was accomplished by treating the fresh resin, after regeneration to the hydroxide form with anhydrous methanol-HCl; extracting the resulting reaction mixture with ethyl chloride, and weighing the residue obtained after evaporating the ethyl chloride. The entire procedure for esterification of fatty acids and extraction of the esters was followed but without any acids adsorbed on the resin. Table 2 shows the amount of residue leached from two 25 g aliquots of fresh Amberlite IRA-400 resin during repeated treatments. It can be seen from this table that the amount of residue given by fresh resin is quite high in both cases. The amount of residue leached from the

TABLE 2

Residue Leached From Fresh Amberlite IRA-400 Resin
And From Subsequent Treatments

Treatment Number	Weight of the Residue (mg) Sample 1 *	Sample 2 *
1. Stearic Acid	35.0	--
2. Methanol-HCl	13.7	19.5
3. Methanol-HCl	5.0	5.4
4. Methanol-HCl	5.3	4.2

* The weight of the fresh resin taken in each case was 25 g.

resin decreased with repeated treatments and it reached a fairly constant value after the third treatment. Average values for the residue leached from used resin was found to be 5.0 mg per 25 g of resin. Hence, all resin batches were initially prepared by one stearic acid treatment followed by three successive treatments with methanol-HCl and 5 mg was taken as the average residue to be subtracted from the weights of methyl esters isolated from fat. The stearic acid pretreatment as described by Benedict (7), and the subsequent methanol-HCl treatment outlined on page 25 of this manuscript, effected a reduction of resin leachings and improved the gravimetric recovery of acids from the resin. This agrees with the findings of McCarthy and Duthie (73), who reported that the quantitative recovery of free fatty acids became possible after the same resin was used four or five times.

Retention by the Resin of the Fatty Acids from Previous Analyses:

McCarthy and Duthie (73) found that the fatty acids from previous analysis were retained on the ion exchange resin and that they were exchanging with the free fatty acids of the sample. To investigate this point, the fresh resin after pretreatment as previously described, was used to methylate a known fatty acid. The resin was then regenerated to the hydroxide form and another fatty acid, with a widely different carbon number from the preceding one, was methylated. The methyl ester obtained in the latter case was analyzed by GLC to

check whether the peak due to the methyl ester of the preceding fatty acid could be seen. Various combinations of known fatty acids were tested; for example, butyric acid was methylated after myristic acid; palmitic acid after butyric acid; hexanoic acid after palmitic acid; stearic acid after hexanoic acid; etc. In no case did the chromatogram of one ester show a peak due to the preceding ester. In this study it was necessary to select fatty acids with widely different carbon chain lengths, because the authentic fatty acids had traces of fatty acids differing by one or two carbons as impurities. My results are in contrast to those obtained by McCarthy and Duthie (73) who found that fatty acids retained on the resin from previous use were exchanging with the free fatty acids of the sample.

Hydrolytic Capacity of the Ion Exchange Resin: Amberlite IRA-400 is a quaternary ammonium resin which exhibits strong basic properties. The possibility that the resin, when in contact with triglycerides, could effect hydrolysis was investigated as previously described on page 35 of this manuscript. The weights of the residues obtained after each successive treatment of the fat samples are given in Table 3. Two different milk fat samples were used in this study. The weights of the residues given in this table are corrected for the polymeric residue leached from the resin itself. These results show that the

TABLE 3

Methyl Esters Obtained by Successive Analysis of the Same Milk Fat

Analysis Number	Weight of Methyl Esters* (mg)	
	Sample 1	Sample 2
1	115.5	73.1
2	15.6	5.6
3	6.0	1.3

* Obtained from 25 g of milk fat.

weight of the esters obtained from each successive treatment of the fat continues to decrease, which indicated that the hydrolyzing capacity of the resin had no pronounced affect upon measurement of free acids.

IDENTIFICATION OF METHYL ESTER PEAKS REPRESENTING THE FREE FATTY ACIDS FROM A MILK FAT SAMPLE

Gas chromatograms of the methyl esters of the fatty acids of milk fat are well represented in the literature (79). Also Figure 2

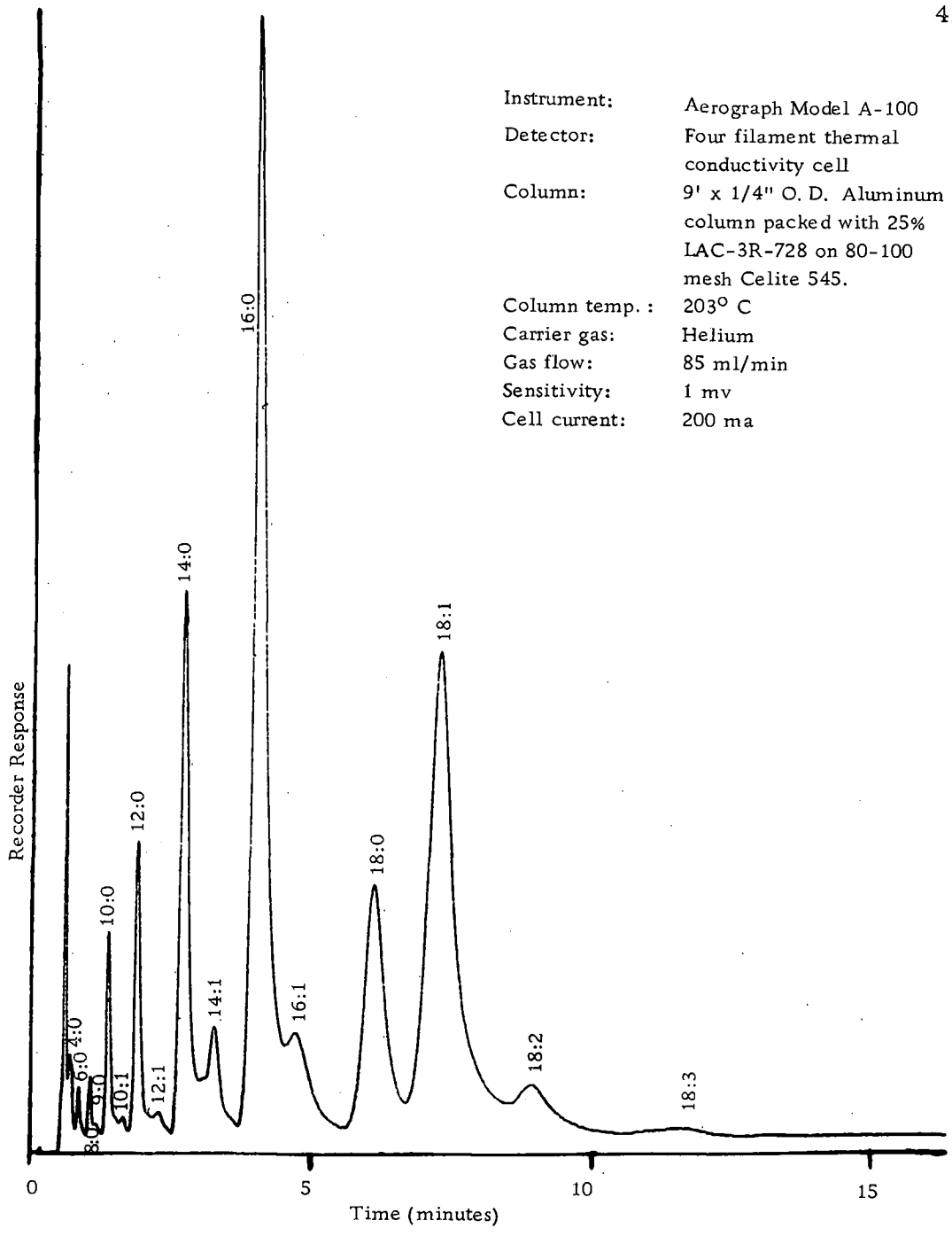

Figure 2. Gas chromatogram of methyl esters of free fatty acids from sweet cream fat.

shows a typical chromatogram obtained from a sweet cream fat sample using the modified method. The patterns are very characteristic and one can not easily mistake the major esters in the mixture. Nevertheless, it was necessary to confirm the identity of major peaks by comparing relative retention times (Tr/Tr) of known peaks with those of unknowns. Table 4 compares the Tr/Tr for methyl esters of authentic acids with the values for esters of the free fatty acids from a fresh milk fat sample. With the exception of 16:0, values for the authentic and unknown esters are in good agreement. Palmitic acid (16:0) is the most abundant saturated acid in milk fat. Therefore, even though the Tr/Tr of the authentic and unknown (Table 4) do not agree as well as the remaining data, the chromatographic peak can not be mistaken for others because of its relative size.

The enrichment technique was employed in the case of the unsaturated esters. By adding a small amount of an authentic ester to the unknown mixture and observing the resulting chromatogram for increases in relative peak size, peak locations for the 18:1, 18:2 and 18:3 were confirmed.

TABLE 4

Relative Retention Times of Methyl Esters of Fatty Acids

Fatty Acid Methyl Ester	Relative Retention Time	
	Authentic	Unknown
4:0	0.354	0.353
6:0	0.453	0.456
8:0	0.569	0.569
10:0	0.733	0.734
12:0	1.000	1.000
14:0	1.435	1.432
16:0	2.112	2.157
18:0	3.233	3.228

PERCENT RECOVERY OF THE FATTY ACIDS

The results obtained for the percent recovery of the free fatty acids are given in Table 5. The value for each acid represents the average of two separate determinations. Moreover, the values for each recovery study were the average of three values from three different chromatograms of the same sample. The recoveries of 9:0, 10:1, 12:1, 14:1 and 16:1 acids were not determined experimentally. These values were estimated from the values of adjacent saturated fatty acids. In the case of 18:1, 18:2, 18:3 and 20:4 acids, the percent recoveries were estimated on the basis of the curve and on recovery data obtained by Smith (93, p. 613).

Before using ethyl chloride as a solvent for extracting the methyl esters from the reaction mixture, petroleum ether and isopentane also were tried. It was found, however, that during the evaporation process there was appreciable loss of the 6:0 and 8:0 methyl esters and 4:0 was completely lost. It will be noted that the percent recoveries of 8:0 and 10:0 are less than that of 6:0. The reason for this has not been determined. For comparison purposes, recoveries by the silver salt procedure of Gaedke (26) are included in Table 5.

TABLE 5

Percent Recovery of Free Fatty Acids

Fatty Acid	Percent Recovery(a)	Percent Recovery(b) by Gaedke's Method
4:0	71.4	0.0
6:0	86.5	16.0
8:0	66.6	43.1
9:0	71.0	--
10:0	75.1	37.9
10:1	85.5	--
12:0	94.3	120.4
12:1	97.0	--
14:0	100.2	53.8
14:1	100.0	--
16:0	99.5	87.5
16:1	96.0	--
18:0	92.5	72.6
18:1	92.5	--
18:2	90.0	--
18:3	85.0	--
20:4	80.0	--

(a) Obtained from paraffin oil
(b) Obtained from milk fat by method of Gaedke (26)

FREE FATTY ACIDS IN MILK FATS

Sweet Cream: The chromatogram shown in Figure 2 is typical of the methyl esters of the free fatty acids obtained from milk fat. It will be noted that the peaks are well separated and they give some indication of the approximate proportion in which the various free acids are present in the fat. There was generally one minor peak between each even numbered saturated fatty acid ester from 8:0 to 18:0. The peak between 8:0 and 10:0 was identified by Tr/Tr to be that of the 9:0 methyl ester. The other minor peaks had retention times comparable to the odd numbered saturated fatty acid methyl esters from 11:0 to 17:0 and the even numbered monounsaturated fatty acid methyl esters from 10:1 to 16:1. Since the monounsaturated fatty acids are in larger concentrations than the odd numbered fatty acids (91, p. 174), the minor peaks were designated and calculated as monounsaturated fatty acids.

Ten samples of milk fat prepared from fresh sweet cream were analyzed for free fatty acid content. The quantity of each free acid was calculated and expressed as milligrams of free fatty acids per kilogram of fat. The results of the analyses are compiled in Table 6. Included in the table are; the mean values of the fatty acids for ten samples, the standard deviation, and the mean weight percent of each

TABLE 6

Free Fatty Acids in Milk Fat Obtained From Sweet Cream (mg/kg of fat)

Sample No. / Fatty Acid	1	2	3	4	5	6	7	8	9	10	Mean	S. D.	Wt. % of Total Free Fatty Acids
4:0	295.1	247.7	234.6	128.9	10.4	21.4	4.7	8.5	14.2	17.1	98.26	353.12	2.80
6:0	13.3	2.4	0.00	0.00	1.5	4.4	0.00	3.1	4.1	5.8	3.46	12.03	0.10
8:0	20.8	49.2	20.0	26.6	8.4	24.5	2.1	27.6	11.4	27.0	21.76	39.01	0.62
9:0	27.4	5.2	6.9	13.3	0.00	8.6	1.7	0.00	9.8	9.2	8.21	24.20	0.23
10:0	89.0	223.9	111.4	121.2	85.1	104.7	52.3	169.3	96.3	124.3	117.75	144.32	3.36
10:1	35.2	27.0	11.5	11.9	9.4	19.6	5.5	23.8	13.1	15.7	17.27	27.32	0.49
12:0	152.9	241.5	161.7	141.5	133.4	120.9	104.8	208.2	136.5	147.3	154.87	122.86	4.42
12:1	22.5	18.9	10.2	9.8	11.7	19.6	7.6	20.0	22.4	30.3	17.30	21.66	0.59
14:0	316.0	451.7	299.8	290.4	295.0	276.9	232.2	472.1	292.9	312.8	323.98	241.12	9.25
14:1	96.3	130.5	70.5	84.5	78.5	72.2	61.0	126.8	77.6	83.8	88.17	69.95	2.52
16:0	867.4	1188.1	716.4	749.6	732.1	685.2	598.1	1138.9	732.4	790.5	819.87	582.08	23.40
16:1	127.8	196.6	118.1	105.7	105.4	86.3	96.1	181.2	110.5	131.8	125.95	108.06	3.59
18:0	296.5	366.5	196.2	368.5	370.5	369.3	286.7	534.6	329.2	357.6	347.56	257.76	9.92
18:1	1126.8	1500.0	753.2	1179.8	1129.4	926.0	852.4	1633.8	1003.9	1077.2	1118.25	819.25	31.91
18:2	123.9	197.7	123.8	161.7	134.9	108.9	116.9	271.3	124.3	150.7	151.41	148.82	4.32
18:3	26.6	87.1	81.8	56.4	55.1	48.0	44.2	91.6	61.1	49.8	60.17	62.12	1.72
20:4	0.00	0.00	42.6	60.6	0.00	58.3	0.00	60.6	63.3	20.6	30.60	87.33	0.87
Total	3637.5	4934.0	2958.7	3510.4	3159.1	2954.8	2466.3	4971.4	3103.0	3351.5	3504.84		100.11

free fatty acid in the total free fatty acid mixture. The results in Table 6 show considerable variation in the amount of free fatty acids between samples. This is evident from the standard deviation values. The standard deviations are high for all of the fatty acids. The reason for this could be that the amount of free fatty acids in different samples of milk fat is not constant (3). Samples 1 to 4 in Table 6 have very high values for 4:0 as compared to the remaining six samples. This may be due to the fact that the 4:0 methyl ester is the most volatile of all the methyl esters in the mixture and it is possible that some of it was lost in the case of other samples (from 5 to 10) when ethyl chloride was evaporated at room temperature. The results probably will be more consistant, if the evaporation step is standardized.

Cultured Butter: Nine samples of cultured butter and one sample of sweet cream butter, made from the sweet creams used for the above fatty acid analysis, were analyzed. The results of these analyses are presented in Table 7. Here also the amounts represented by the small peaks were calculated as monounsaturated fatty acids. As in the case of the fat samples from sweet creams, the free fatty acid content of milk fat from the cultured butter samples varied considerably from sample to sample. The standard deviation values are also high, probably because of the same reason. The sample numbers of the butters given in Table 7 correspond to those of the cream samples in Table 6

TABLE 7

Free Fatty Acids in Milk Fat Obtained From Cultured Butter (mg/kg of fat)

Sample No. Fatty Acid	1	2	3	4	5	6	7	8	9*	10	Mean	S. D.	Wt. % of Total Free Fatty Acids
4:0	236.6	162.4	162.6	176.4	7.1	6.8	9.2	19.8	18.4	24.0	82.33	271.32	2.27
6:0	0.00	24.6	0.00	16.9	0.00	0.00	1.0	2.2	7.4	3.0	3.46	25.63	0.15
8:0	36.4	95.1	13.6	67.2	14.6	10.9	24.5	28.4	27.8	33.4	35.19	79.46	0.97
9:0	5.1	6.2	2.1	15.8	0.00	0.00	0.00	0.00	0.00	0.00	2.92	15.27	0.08
10:0	121.2	234.4	136.9	171.5	101.2	82.6	116.8	211.4	116.5	155.4	144.79	145.82	3.99
10:1	13.6	25.5	13.9	15.6	11.3	11.2	14.5	25.8	12.3	21.2	16.49	11.45	0.45
12:0	157.4	275.7	173.8	176.3	150.3	111.4	155.2	253.7	132.5	173.7	176.00	153.27	4.85
12:1	7.2	14.6	13.6	12.2	11.1	9.8	11.9	20.2	19.3	29.7	14.96	19.60	0.41
14:0	349.7	558.1	367.1	360.3	312.5	272.9	326.4	622.7	275.8	372.4	381.79	349.11	10.53
14:1	98.9	148.4	89.7	87.6	76.6	68.2	82.9	147.0	72.1	92.6	96.40	85.83	2.66
16:0	748.4	1320.4	763.6	790.6	612.9	599.3	653.1	1262.5	687.1	867.4	820.53	710.39	22.62
16:1	131.2	208.2	140.8	123.3	102.8	85.4	114.2	201.9	109.8	156.9	137.45	122.49	3.79
18:0	277.7	362.8	222.1	375.1	289.5	326.6	291.4	566.8	306.1	391.6	340.97	282.61	9.40
18:1	1130.5	1551.7	847.8	1252.7	930.6	807.7	874.1	1778.5	978.9	1167.9	1132.04	910.24	31.21
18:2	135.7	209.3	145.0	167.4	130.2	103.8	124.8	240.7	128.4	178.4	155.37	130.07	4.28
18:3	54.7	91.5	38.5	35.6	44.8	50.7	52.4	92.9	54.1	57.8	57.30	59.18	1.58
20:4	92.1	0.00	0.00	89.8	0.00	52.7	0.00	0.00	39.7	0.00	27.43	115.65	0.76
Total	3596.4	5188.9	3131.1	3931.3	2785.5	2600.0	2852.4	5474.5	2986.2	3725.4	3627.47		100.00

* Sweet Cream Butter

from which the butters were made. The butter samples one to four in Table 7 have very high butyric acid content as did the corresponding creams, (Table 6). This indicates that the evaporation technique seems to be satisfactory which is contrary to the assumption made previously. The cause for the abnormally high values for butyric acid (4:0) in the first four samples can not be explained at this time.

By comparing the percent of individual acids of the total free fatty acids given in Tables 6 and 7 with the data of Jack (52) and Herb et al. (47) in Table 1 on pages 5 and 6 of this manuscript, it is evident that the percent composition of free fatty acids in milk fat is similar to that of the esterified fatty acids. The results obtained by the two workers (47 and 52) show considerable variation in some cases. The average values for the percent composition of the free fatty acids lie within the range given by the two sources in Table 1 except for the 4:0 to 8:0 acids. This may be due to the fact that these short chain fatty acids (4:0 to 8:0) are volatile and are lost to some extent during their isolation. A comparison of Tables 6 and 7 also reveals that there is little or no change in the free fatty acid content of milk fat as a result of manufacturing it into cultured cream butter.

Oxidized and Rancid Milk Fats: The free fatty acids of an autoxidized sample and a hydrolytically rancid sample were analyzed to further evaluate the utility of the procedure. The autoxidized sample also was analyzed for peroxides (1, Cd 8-53), 2-thiobarbituric acid number (101) and total carbonyls (8) to ascertain the extent of oxidation. As shown in Table 8, the sample was highly oxidized but the autoxidation does not appear to have had a pronounced effect upon the free fatty acid distribution. This is more evident when the average weight percent of total free fatty acids (Tables 6 and 7) is compared with that of the oxidized fat in Table 8. More samples would be required, however, to draw conclusions relative to this point. Figure 3 shows the distribution of various free fatty acid methyl esters obtained from the autoxidized milk fat.

To study the effect of milk lipase hydrolysis upon the free fatty acid distribution of milk fat, raw sweet cream was divided into two aliquots. One aliquot (the control) was laboratory pasteurized; the other was homogenized and allowed to stand at room temperature for two hours while a distinct rancid flavor developed. The free fatty acids from a portion of each sample were isolated, esterified and analyzed by GLC and the remainder of each sample was titrated with alcoholic KOH as described by Breazeale and Bird (10). The moles of KOH required for titration were converted to weight of free fatty acids

TABLE 8

Free Fatty Acids in Oxidized, Sweet Cream and Rancid Cream Milk Fats

Sample	Oxidized Fat		Sweet Cream Fat		Rancid Fat	
	mg/kg fat	Weight %	mg/kg fat	Weight %	mg/kg fat	Weight %
4:0	47.4	1.29	21.6	0.56	14.2	0.21
6:0	5.1	0.14	12.3	0.32	28.1	0.41
8:0	9.2	0.25	22.6	0.59	92.9	1.34
9:0	13.4	0.37	5.4	0.14	0.00	0.00
10:0	107.4	2.92	85.2	2.21	262.3	3.79
10:1	9.2	0.25	7.2	0.19	32.1	0.46
12:0	141.8	3.86	144.7	3.76	295.7	4.27
12:1	11.4	0.31	14.5	0.38	36.3	0.52
14:0	340.3	9.26	368.2	9.56	672.9	9.71
14:1	79.2	2.15	95.8	2.49	174.0	2.51
16:0	868.9	23.64	1,199.3	31.12	1,966.8	28.39
16:1	118.1	3.21	136.9	3.55	237.2	3.42
18:0	438.6	11.94	480.4	12.47	743.4	10.73
18:1	1,244.9	33.88	1,072.8	27.84	2,006.6	28.96
18:2	132.6	3.61	145.8	3.78	272.8	3.94
18:3	65.2	1.77	40.4	1.05	92.4	1.33
20:4	42.3	1.15	0.00	0.00	0.00	0.00
Total	3,675.0	100.00	3,853.1	100.01	6,927.7	99.99
Free Fatty Acids[a]	--	--	2,468.2	--	5,160.9	--
Peroxide Value	72.64	--	--	--	--	--
TBA No.	34.04[b]	--	--	--	--	--
Carbonyls:Saturated	26.67[c]	--	--	--	--	--
Carbonyls:Unsaturated	10.15[c]	--	--	--	--	--

a. Calculated from acidity of the milk fat determined by Breazeale and Bird method (10), and using a mean molecular weight value of 225.

b. mg of malonaldehyde per kg fat.

c. mmoles per kg fat.

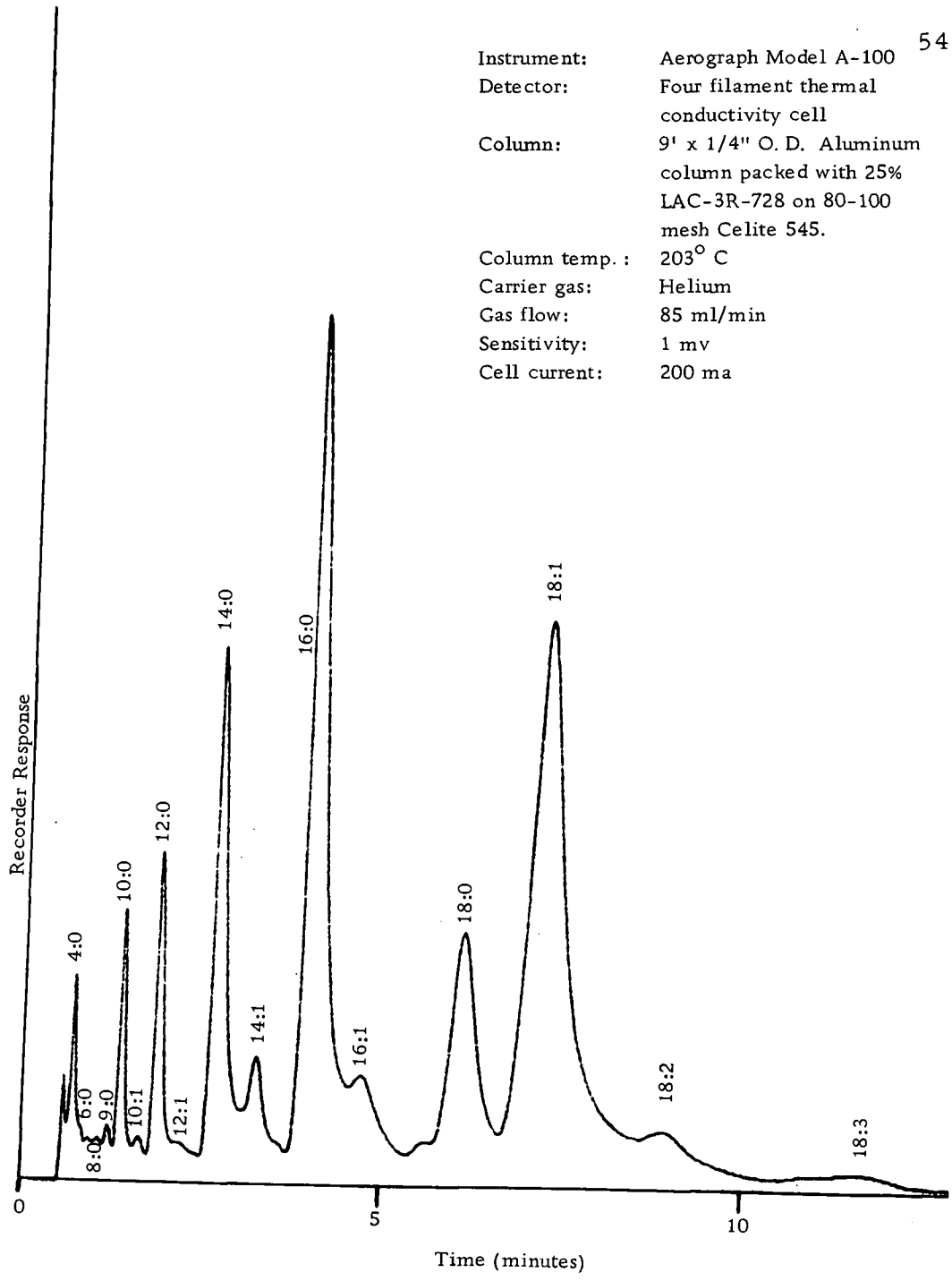

Figure 3. Gas chromatogram of methyl esters of free fatty acids from autoxidized milk fat.

by using a mean molecular weight of 225 for fatty acids of milk fat. The value of 225 is the average saponification value for milk fat. Table 8 gives the findings of the analyses. Some variation in the fatty acid distribution is evident between the sweet cream control and rancidified sample. The differences were most pronounced for the low molecular weight acids. This suggests that the variations in distribution of the short chain acids may be attributed to the uncontrolled evaporation of solvent during analysis which caused variable losses in the more volatile esters. This is especially evident in the case of the ester of butyric acid. The data show a decrease in butyric acid in the rancid sample which is inconsistant with reports in the literature (36). Lipase hydrolysis has been reported as being nonspecific for various fatty acids; however, the enzyme is specific for the 1-position on the triglyceride which has been shown to contain a greater proportion of short chain fatty acids. This is indicated by the increase in the percentage of the 6:0, 8:0 and 10:0 fatty acids in the rancid fat, while the relative proportions of the long chain fatty acids do not change appreciably.

The quantities of total acids determined by the resin procedure are higher than those measured by the direct titration (Table 8). The percent differences between the total free fatty acids by the two techniques are 35. 9 percent for the sweet cream fat and 25. 5 percent for

the rancidified sample. The reasons for this difference is not readily apparent but the values obtained by the resin procedure should approach the real values since each acid was measured individually. In fact, the total acids as measured by the resin procedure would be expected to be low since some of the minor acids were not measured.

The chromatograms of methyl esters of free fatty acids obtained from sweet cream fat and rancidified fat are given in Figures 2 and 4 respectively.

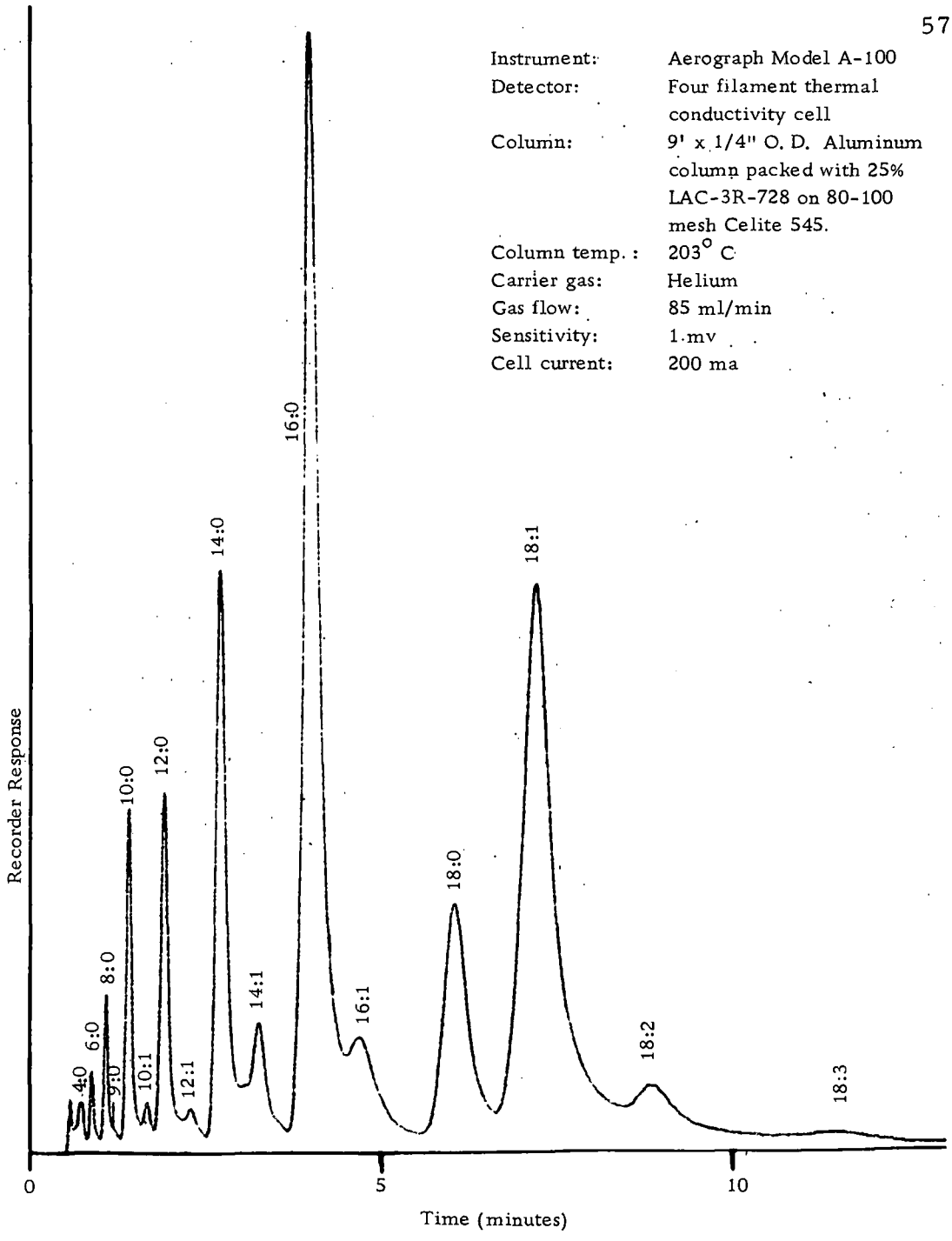

Figure 4. Gas chromatogram of methyl esters of free fatty acids from rancid cream fat.

SUMMARY AND CONCLUSIONS

The anion exchange resin method of Hornstein et al. (50) was adapted with some modifications to determine the free fatty acids in milk fats. The following modifications were made: The ion exchange resin was pretreated with stearic acid (7) and anhydrous methanol-HCl to reduce leachings from the resin. Isopentane was used as the solvent for milk fats to be analyzed and ethyl chloride (b. p. 12.3° C) was used as solvent to extract fatty acid methyl esters from the reaction mixture. The methyl ester mixture representing the free fatty acids was weighed and analyzed by GLC for individual fatty acids.

The modified method was used to determine the free fatty acids in ten sweet cream fat samples, nine cultured butter fat samples, a sweet cream butter sample, a sample of autoxidized fat and a sample of milk fat from each, laboratory pasteurized sweet cream and hydrolytic rancid cream obtained therefrom. The cultured butter was obtained from the sweet cream samples used in the analysis. The composition of free fatty acids obtained in the fat samples was compared to the total esterified fatty acid composition of milk fat and the two were found to be comparable. Various studies were carried out on the ion exchange resin (Amberlite IRA-400) to ascertain its hydrolyzing capacity, retention of fatty acids from previous use and to reduce the leaching of low molecular weight polymers from the resin

during use.

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

1. Fresh ion exchange resin had to be pretreated with stearic acid and anhydrons methanol-HCl to eliminate the leaching of a low molecular weight polymer from resin. Hence, to avoid interference by the polymer with quantitative results, the resin must be pretreated.
2. After pretreatment, the resin continued to give an average of 5.0 mg of residue for every 25 g of resin. The residue did not interfere with the GLC analysis of free fatty acids, because it did not appear as a peak on the chromatograms.
3. Triglyceride hydrolysis by the resin, if it did occur, was undetectable by the procedure used in this investigation.
4. No fatty acids were retained by the resin from its previous use in free fatty acid analysis.
5. The modified method for determining the free fatty acids gave satisfactory results. The recoveries of authentic fatty acid mixtures were good, even for short chain fatty acids.
6. The percent composition of the free fatty acids in the milk fat was similar in many respects to that of the esterified fatty acids of milk fat.

7. There was pronounced variation in the free fatty acid content of the milk fat from sample to sample.
8. The process of manufacturing cultured cream butter had little or no affect upon the free fatty acid content of the butter.
9. The free fatty acid content of milk fat was increased when the fat was hydrolytically rancified; however, contrary to the reports in the literature, the free butyric acid (4:0) content of the rancid fat was decreased. This inconsistancy may be attributed to the variations in the recoveries of short chain fatty acids due to uncontrolled evaporation of ethyl chloride.

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