Measurement of *trans* and Other Isomeric Unsaturated Fatty Acids in Butter and Margarine

L.M. SMITH, W.L. DUNKLEY, A. FRANKE, and **T. DAIRIKI,**
Department of Food Science and Technology,
University of California, Davis, California 95616

ABSTRACT

A procedure is described for gas liquid chromatographic determination of *cis* and *trans* isomers of unsaturated fatty acids after fractionation of the saturated, monenoic, dienoic, and polyenoic fatty acid methyl esters by argentation thin layer chromatography. To test its reliability, the procedure was used for quantitative measurement of *trans*-isomers of unsaturated fatty acids in a known mixture of simple triglycerides containing saturated fatty acids from 4:0 to 24:0 and *cis* and *trans* isomers of 14:1, 16:1, 18:1, and 18:2. Results of the analyses of five margarine and five butter samples are presented, together with results of infrared spectrophotometric analyses for *trans* fatty acid concentrations, ultraviolet spectrophotometric analyses for conjugated fatty acid concentrations, and enzymatic analyses for *cis-cis*-methylene interrupted fatty acid concentrations. The combined argentation thin layer and gas chromatographic procedure is suitable for determination of the principal fatty acids in complex food lipids such as milk fat.

INTRODUCTION

There is increasing interest in the biological utilization and effects of *trans* and other isomeric unsaturated fatty acids on human health (1). Fats from ruminants usually have small amounts of *trans* and conjugated acids (2). Many foods contain vegetable oils that have been partially hydrogenated, resulting in the formation of variable amounts of *trans* and conjugated isomers. Oxidation during processing or storage of foods may cause increases in the content of these constituents. The information presently required for nutritional labeling of foods does not include *trans*-, conjugated, or mono-unsaturated fatty acid contents, but the regulations may be amended in the future to provide this knowledge for the consumer.

A need exists for simple improved methods to determine isomeric unsaturated fatty acids in foods on a routine basis. Total isolated *trans* unsaturation can be estimated by infrared spectroscopy, but this method gives no information concerning specific structures of individual fatty acids. Ultraviolet spectroscopy can be used to determine conjugated dienoic and trienoic double bonds but not double bond positions or carbon chain length. Gas liquid chromatography (GC) of fatty acid methyl esters is widely used to determine the major fatty acids in biological materials. However, most GC systems using packed columns provide little or no separation of *cis* and *trans* isomers of the same carbon chain length and of isomers that differ only in the position of double bonds. An automated glass capillary GC system has recently been developed by Jaeger et al. (3) which gives a greatly improved separation of complex mixtures of fatty acid methyl esters including *cis-trans* isomers. Unfortunately the method requires expensive, complicated equipment and a high degree of technical experience and skill.

The recent development of improved stationary phases, such as Apolar-10C (4), SP-2340 (5), and OV-275 (6), has facilitated the separation on packed GC columns of *cis* and *trans* isomers of the same carbon chain length and number of double bonds. However, lipids of biological and nutritional interest may contain complex mixtures of saturated and unsaturated fatty acids. At present, to resolve such mixtures it is necessary to combine GC with other techniques including thin layer chromatography (TLC). Conacher (7) has reviewed the methodology for the chromatographic determination of geometric and positional isomers in fats and oils.

In the present paper, we describe a packed column GC procedure to determine the fatty acid composition of butter and margarine, including *cis* and *trans* unsaturated isomers, after preseparation into saturated, monoenoic, dienoic, and polyenoic methyl esters by argentation TLC. The procedure was devised primarily for the quantitative analysis of dairy and other foods whose lipids contain a wide variety of fatty acids including those of short chain lengths. Results obtained for the analysis of several margarine and butter samples are compared with data obtained by infrared, ultraviolet, and enzymic analyses.

EXPERIMENTAL PROCEDURES

Materials

Purified fatty acid methyl esters and simple triglycerides (Nu-Chek Prep, Elysian, MN) were used to prepare standard mixtures to assist in the identification and quantification of components found by the various analytical procedures. Solvents and other materials used were of reagent grade.

Five different brands of margarine, including both hard and soft types, were purchased locally. The five brands of butter purchased were representative of butter made in California and in the midwestern U.S.

Extraction of Lipids

An extraction method was employed which would be applicable to most foods. A 2-g sample of margarine or butter was weighed into a 100-ml Virtis Homogenizer jar, and 60 ml of chloroform-methanol (2:1, v:v) was blended with the sample at high speed for 2 min. The mixture was decanted through a filter (Whatman 2V) into a 250-ml separatory funnel. Another 60 ml of chloroform-methanol was added to the jar, blended for 2 min, and transferred to the filter. Then 30 ml of deionized water was added to the combined extracts in the separatory funnel. The mixture was shaken 60 sec and allowed to stand overnight in a refrigerator for phase separation. The lower phase was run into a 100-ml volumetric clask and made up to the mark with methanol. A 5-ml aliquot of the solution was evaporated, and the residue was weighed. A volume of the solution calculated to contain 600 mg of lipid was measured into a 50-ml centrifuge tube and evaporated to dryness at 60 C with nitrogen. The lipid was dissolved in 15 ml of iso-octane, and the solution was stored under nitrogen at -20 C until analyzed.

GC of Butyl Esters

The major fatty acids of the extracted lipids were determined by a procedure based on that of Parodi (8). Butyl

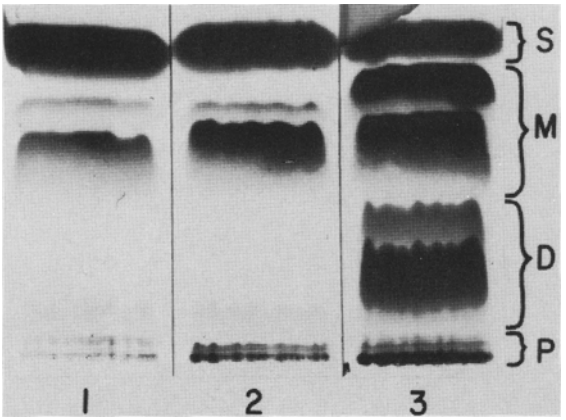

FIG. 1. Separation by argentation thin layer chromatography of methyl esters from (1) butter, (2) mixture of simple triglycerides and (3) margarine. Fractions: S, saturated; M, monoenoic; D, dienoic and P, polyenoic. Conditions are described in the text.

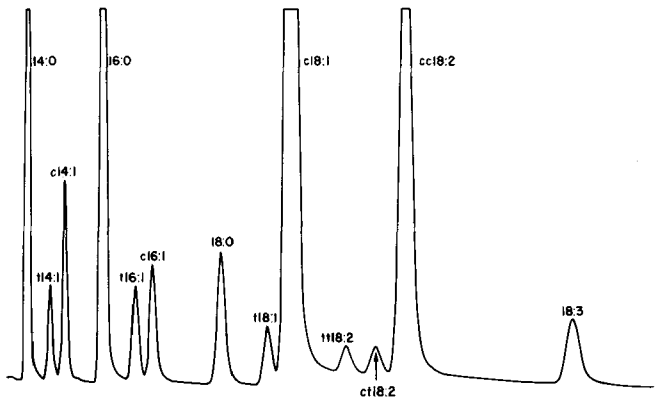

FIG. 2. Separation of a mixture of fatty acid methyl ester standards containing *cis* and *trans* isomers. Gas liquid chromatography conditions: 730 cm x 0.27 cm SS column packed with 10% Apolar-10C on 100/120 Gas-Chrom Q; column temperature, 190 C; injector and detector, 250 C; carrier gas N_2, flow rate, 15 ml/min.

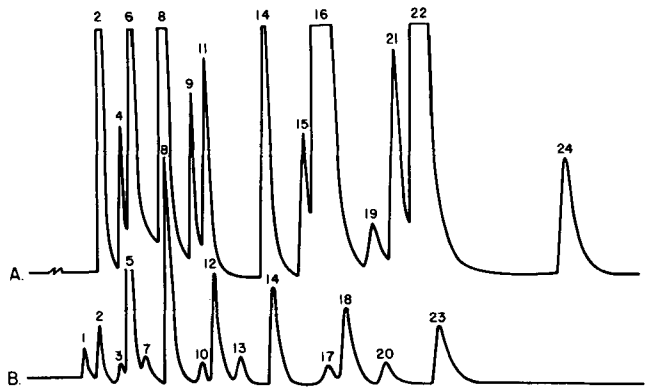

FIG. 3. Chromatograms illustrating the possible peak overlap between (A) *cis* and *trans* isomers of unsaturated fatty acids and (B) saturated branched and straight chain fatty acids. Gas liquid chromatography conditions same as for Figure 2. Identity of methyl ester standards: 1, 14 br; 2, 14:0; 3, 15 br; 4, *t*-14:1; 5, 15:0; 6, *c*-14:1; 7, 16 br; 8, 16:0; 9, *t*-16:1; 10, 17 br; 11, *c*-16:1; 12, 17:0; 13, 18 br; 14, 18:0; 15, *t*-18:1; 16, *c*-18:1; 17, 19 br; 18, 19:0; 19, *t,t*-18:2; 20, 20 br; 21, *c,t*-18:2; 22, *cc*-18:2; 23, 20:0; 24, *ccc*-18:3.

esters were prepared by alcoholysis of a 4% solution (w/v) of the lipids in iso-octane with a 3 N solution of butanolic KOH. The esters were washed with dilute aqueous NaCl and dried with Na_2SO_4 before being separated by GC. The chromatograph (Hewlett-Packard 5700A) was equipped with a carbon dioxide system for rapid cooling between runs, and a data analyzer (Hewlett-Packard 3352B) for operation of the automated sample injector and for calculations. The butyl esters were separated with a 244 x 0.27 cm ID stainless-steel column packed with 15% EGSS-X on 100/120 mesh Gas-Chrom P (Applied Science Laboratories, State College, PA). Column temperature was held 2 min at 70 C, then was programmed at the rate of 4 C/min to 180 C and held for 32 min. Injection and detector temperature was 250 C. Nitrogen carrier gas flow rate was 20 ml/min in the column. At the flame ionization detector, gas flow rates (ml/min) for nitrogen, hydrogen, and air were 51, 39, and 240, respectively.

Standard mixtures of simple triglycerides were butylated and chromatographed to obtain relative retention times and response factors.

TLC of Methyl Esters

Methyl esters of the extracted lipids were prepared by the method of Christopherson and Glass (9). TLC glass plates (20 x 20 x 0.3 cm) were coated with a 0.5 mm layer of Silica Gel G containing 10% $AgNO_3$. Plates were predeveloped with diethyl ether and then activated at 110 C for 10 min. One ml of a 4% (w/v) solution of methyl esters in iso-octane was applied to the plate as a 5-mm band, leaving a 1-cm space at both edges. Each plate was developed twice, to minimize tailing, with petroleum ether-diethyl ether (92:8). Four fractions—saturates, monenes, dienes, polyenes—were visualized by spraying the TLC plate edges with 0.1% dichlorofluorescein in methanol and marking the bands under UV light. The separation is illustrated in Figure 1. Each fraction was scraped off into a screw-cap centrifuge tube. Sufficient 1% NaCl (w/v in methanol-water, 9:1) was added to wet the gel, the esters were eluted three times with 10-ml volumes of diethyl ether, and the combined elutes were stored in screw-cap centrifuge tubes at -20 C until analyzed.

GC of Methyl Ester Fractions

The ether was evaporated from each eluted fraction (saturates, monoenes, dienes, or polyenes) below 30 C with nitrogen. Each sample was dissolved in 1 ml of iso-octane and transferred to a 2-ml vial, which was sealed and placed in the automatic sample injector. The methyl esters were separated and identified with a 730 cm x 0.27 cm ID stainless-steel column packed with 10% Apolar-10C on 100/120 Gas-Chrom Q (Applied Science Laboratories). The column was operated isothermally at 190 C. Injector and detector temperature was 250 C. Nitrogen carrier gas flow rate was 15 ml/min in the column. At the flame ionization detector, gas flow rates (ml/min) for nitrogen, hydrogen, and air were 51, 39, and 240, respectively.

Standard mixtures of fatty acid methyl esters or of simple triglycerides were used to obtain relative retention times and response factors. These data were used to calibrate the mini-computer of the data system.

GC Calculations

Averages of three separate analyses of the major fatty acids by GC of the butyl esters were used to obtain the amounts of 4:0-12:0, 16:0, 18:1, 18:2, and 18:3 in each butter or margarine sample. To obtain the more complete final analysis for each sample, the corresponding methyl ester fractions separated by argentation TLC were also analyzed, and the results were combined with the butyl ester data.

After computing the fatty acid composition (weight %) of the saturated methyl ester fraction, the concentration of 16:0 found in the butyl ester analysis, was used as an internal standard to normalize all the other saturates from 13:0 through 24:0. For the monoene fraction, the concentration of 18:1 was used as an internal standard. Since the

TABLE I

Comparison of Theoretical and Analytical Results for a Standard Mixture of Simple Triglycerides[a]

Saturated acids (wt %)			Unsaturated acids (wt %)		
Fatty acid	Theoretical[b]	Found[c]	Fatty acid	Theoretical[b]	Found[c]
4	2.7	2.5	*t*14:1	0.5	0.5
6	2.6	2.8	*c*14:1	0.9	1.0
8	1.4	1.5	*t*16:1	0.5	0.5
10	3.2	3.2	*c*16:1	1.5	1.9
12	2.7	2.6	*t*18:1	2.9	2.6
13	0.1	0.1	*c*18:1	28.8	27.8
14	8.4	8.6	*tt*18:2	0.4	0.6
15	0.8	0.8	*cc*18:2	2.4	3.0
16	22.2	23.2	18:3ω6	0.3	0.4
17	0.7	0.7	18:3ω3	0.9	1.1
18	12.2	11.9	*c*20:1	0.6	0.2
19	0.5	0.3	20:3ω6	1.0	1.4
20	0.4	0.1	20:3ω3	0.6	0.9
22	0.3	—	20:4ω6	0.6	0.7
24	0.1	—			

[a]Esterified, fractionated by argentation thin layer chromatography, and fractions analyzed by gas chromatography.
[b]Based on weights of simple triglycerides taken to prepare standard.
[c]Average of three analyses.

cis- and *trans*-isomers of 18:1 were separated on the Apolar-10C column, their total was equated to the 18:1 found in the butyl ester determination. For the diene fraction, the total of *trans,trans*-, *cis,trans*-, and *cis,cis*-18:2 was accepted as equal to the 18:2 measured as butyl ester. For the polyene fraction, the *cis,cis,cis*-18:3 was equated to the difference between the 18:3 found in the butyl ester determination and the 20:0 in the saturated methyl ester fraction, because in the GC analysis of the butyl esters, the 18:3 and 20:0 peaks overlapped.

Infrared Spectroscopy

Isolated *trans*-unsaturation was determined by the AOCS Tentative Method Cd 14-61 (10) using methyl elaidate for standard.

Ultraviolet Spectroscopy

The percentage of conjugated double bonds was determined by AOCS Official Method Cd 7-58 (11).

Enzymatic Determination of Polyunsaturated Fatty Acids

The content of *cis,cis*-methylene interrupted (CCMI) polyunsaturated fatty acids was determined using the lipoxidase procedure of Sheppard et al. (12).

RESULTS AND DISCUSSION

Analysis of Known Samples

The chromatogram shown in Figure 2 demonstrated that a standard methyl ester mixture containing wide ranges of concentration of saturated and unsaturated components can be resolved by packed column GC. Near base line separation was obtained between the *cis* and *trans* isomers of 14:1, 16:1, 18:1, and 18:2. These results are comparable to but extend those reported by Ottenstein et al. (6) who employed 15% OV-275 as stationary phase.

The analysis of fatty acids from foods by GC may present problems because peaks corresponding to some components may be hidden under the larger methyl ester peaks. Figure 3 illustrates the overlap between peaks corresponding to *cis* and *trans* isomers of unsaturated fatty acids and saturated branched chain (odd and even carbon number) and straight chain (odd) fatty acids. It is obvious that complete resolution of complex mixtures of these acids is not possible with the packed column employed in this study.

TABLE II

Fatty Acid Composition of Five Margarines[a]

	Margarine (wt %)				
Fatty acid	F	H	I	M	S
10	—	—	—	—	0.1
12	0.1	0.2	0.1	0.1	0.7
14	0.1	0.1	0.3	0.2	0.7
*t*14:1	0.1	0.1	0.1	0.1	—
15	—	0.1	—	—	—
16	13.5	12.5	19.2	14.9	12.1
*t*16:1	—	TR[b]	—	—	—
*c*16:1	0.1	0.2	0.1	0.2	0.1
17	0.1	0.1	0.2	0.1	0.1
18	6.9	7.5	5.9	7.3	7.8
*t*18:1	16.7	20.9	14.0	16.3	12.9
*c*18:1	26.2	32.2	27.8	27.2	21.5
*tt*18:2	0.1	—	—	—	—
*ct*18:2	0.3	2.3	2.5	1.8	0.8
*cc*18:2	35.4	22.6	26.9	31.3	41.4
18:3ω6	—	0.2	0.3	—	TR
18:3ω3	0.3	1.4	2.6	0.2	1.6
20	0.3	0.3	0.3	0.3	0.3

[a]Average of three analyses.
[b]TR = < 0.1 and > 0.01%.

Therefore, we explored the possibility of preseparating methyl ester mixtures on the basis of degree of unsaturation by argentation TLC before GC analysis. Figure 1 shows separations of esters from butter, margarine, and a standard mixture of simple triglycerides into saturated, monoenoic, dienoic, and polyenoic fractions. GC patterns of the four fractions from the standard triglyceride mixture showed that each contained only the expected peaks.

A known mixture of 29 simple triglycerides was prepared that contained many of the major fatty acids in the approximate concentrations found in bovine milk fat (13) and was analyzed by the combined esterification, TLC and GC procedure. Table I gives a comparison of theoretical and analytical results for the mixture. The agreement of both saturated and unsaturated acids is generally good, except for the 22:0 and 24:0 which were not detected at the low levels expected in milk fat. We concluded that the procedure should be satisfactory to estimate the amounts of the major fatty acids, including *cis* and *trans* isomers of 14:1, 16:1, 18:1, and 18:2 fatty acids in milk fat and less complex food lipids.

TABLE III

Fatty Acid Composition of Five Butters[a]

Fatty acid	Butter (wt %)				
	A	C	D	F	L
4	4.1	4.2	4.2	4.1	4.0
6	2.6	2.7	2.6	2.6	2.5
8	1.5	1.6	1.5	1.5	1.4
10	3.3	3.2	3.2	3.2	3.0
12	3.4	3.4	3.3	3.3	3.2
13	0.1	0.1	0.1	0.1	0.1
14 br	0.1	0.2	0.2	0.2	0.2
14	10.6	10.6	10.3	10.4	10.3
*t*14:1	—	—	0.1	—	TR[b]
*c*14:1	1.3	1.5	1.4	1.3	1.2
15 br	0.6	0.7	0.7	0.7	0.6
15	1.2	1.3	1.3	1.3	1.3
16 br	0.3	0.3	0.3	0.4	0.3
16	28.0	27.2	27.7	27.6	28.1
*c*16:1	2.4	2.6	2.4	2.4	2.4
17 br	0.6	0.6	0.6	0.6	0.6
17	0.8	0.7	0.8	0.8	0.7
18 br	—	0.1	0.1	—	0.1
18	9.7	10.0	9.9	9.9	10.5
*t*18:1	1.8	1.8	1.8	1.8	1.8
*c*18:1	22.0	22.1	22.2	22.2	22.5
*ct*18:2	—	0.2	TR	0.2	0.2
*cc*18:2	4.1	3.7	4.2	4.1	4.1
18:3ω6	0.1	0.1	TR	0.1	0.1
18:3ω3	1.1	1.1	1.0	1.1	1.0
20	0.2	0.1	0.2	0.2	0.1
*c*20:1	—	—	—	—	0.1
20:3ω6	0.2	0.2	0.1	0.2	0.2
20:4ω6	0.1	0.2	0.1	0.2	0.2

[a]Average of three analyses.
[b]TR = < 0.1 and > 0.01%.

Analyses of Margarine Samples

The fatty acid composition of five brands of margarine was determined by the combined argentation TLC and GC procedure (Table II). The margarines contained 34-53% monoenoic, 25-42% dienoic, and 0.2-2.9% trienoic acids. The principal *trans* acid was *t*-18:1 which varied from 12.9 to 20.9%. These values are within the ranges reported by others (6,14,15,16).

Separation by argentation TLC was clean, and GC chromatograms showed only one level of unsaturation in each of the four fractions. Broadening of the *cis* and *trans* 18:1 peaks differed amoung the samples, presumably because of differences in concentrations of positional isomers related to hydrogenation treatments (17).

Analyses of Butter Samples

Table III gives the fatty acid analyses of five brands of butter as determined by the combined argentation TLC and GC procedure. There was little variation among the samples, and the average values for monenoic, dienoic, and trienoic acids were 27.8, 4.1, and 1.3%, respectively. The most important *trans* acid was *t*-18:1 which averaged 1.8%. These values are much lower than for the margarines. The overall composition of the butters was more complex than that of the margarines because of the presence of short-chain, branched-chain, and odd-carbon number saturated acids, and long-chain polyunsaturated fatty acids.

Separation by argentation TLC was not as complete as for the margarines (Fig. 1). The GC chromatograms indicated that small amounts of *trans* monoenes may have been included with the saturates, of *trans,trans* dienes with the monoenes, and of *cis,cis* dienes with the polyenes. These contaminants in the fractions were disregarded in calculating fatty acid composition. Broadening of the 18:1 peaks was less than for the margarines.

Comparison of Analyses by Different Methods

Table IV is a comparison of total *trans*, CCMI polyunsaturated and conjugated fatty acids in the five margarines as determined by GC, spectroscopic, and enzymatic methods. Table V summarizes parallel results for the five butters.

For the margarines, GC gave significantly higher concentrations of *trans* acids than the IR method, but for butters the reverse was found. For both the margarines and butters, the CCMI were higher when determined by GC. The small amounts of total conjugated constituents found in margarine and butter samples were comparable.

The discrepancies between results obtained by different analytical methods are consistent with reports of others. Perkins et al. (18) reported lower *trans* values in margarines by GC than by IR. For CCMI in margarines, Parodi (16) and Nazir et al. (15) found higher values by GC than by the lipoxidase method.

The choice of analytical procedure is influenced by several factors including the complexity of the fat, equipment and personnel available, and the type of information desired. Infrared and ultraviolet spectrophotometric procedures are simple and rapid but provide limited information. The lipoxidase method requires considerable skill but gives data on a group of polyunsaturated fatty acids important in nutrition and labeling of foods. Many laboratories already employ GC of methyl esters to determine the major

TABLE IV

Comparison of Analyses (Wt %) of Five Margarines by Selected Methods[a]

Fatty acids	Method	Margarine				
		F	H	I	M	S
Totai *trans*						
	GC	17.1	23.3	16.5	18.2	13.6
	IR	14.8	22.1	14.0	15.9	13.8
cis,cis-methylene interrupted						
	GC[b]	35.7	24.2	29.7	31.5	43.0
	Lipoxidase	31.9	15.8	25.2	28.6	41.8
Total conjugated						
	UV	0.40	1.39	0.55	0.73	0.31

[a]Abbreviations: GC, gas chromatography; IR, infrared; UV, ultraviolet.
[b]Summation of *cc*-18:2ω6; *ccc*-18:3ω6; and *ccc*18:3ω3.

TABLE V

Comparison of Analyses (Wt %) of Five Butters by Selected Methods[a]

Fatty acids	Method	Butter A	C	D	F	L
Total *trans*						
	GC	1.8	1.9	1.9	2.0	2.0
	IR	4.0	3.5	4.2	4.2	3.7
cis,cis-methylene interrupted						
	GC[b]	5.4	5.2	5.5	5.6	5.5
	Lipoxidase	3.3	3.3	3.5	3.4	4.0
Total conjugated						
	UV	0.73	0.74	0.69	0.69	0.62

[a]Abbreviations as for Table IV.

[b]Summation of *cc*-18:2ω6; *ccc*-18:3ω6; *ccc*-18:3ω3; *ccc*-20:3ω6; and *cccc*-20:4ω6.

fatty acid composition of lipids. The use of new cyanosilicone stationary phases, such as Apolar-10C, can provide a more detailed analysis that includes *trans* monoenoic and polyenoic fatty acids present in simple fats and vegetable oils.

Complete analyses of complex food fats, such as milk fat, still present problems. Conventional GC of the original fat resolves a limited number of peaks corresponding to the major saturated and unsaturated acids. Although bovine milk fat contains over 400 fatty acids, only about 25 peaks are clearly separated by GC. More detailed analyses require time-consuming preseparation of the acids or esters into a series of simpler fractions by physical methods, such as distillation, crystallization, TLC, or urea adduct formation, prior to their determination by GC and/or spectrophotometry (2,13).

The combined argentation TLC-GC procedure described herein is a practical improvement for routine analyses of milk fat. The preliminary separation of saturated from unsaturated components by argentation TLC avoids overlap of saturated odd-carbon number and branched-chain esters with *cis* and *trans* monoenes of one less carbon. However, the small amounts of conjugated acids, positional isomers, and the many trace (< 0.1%) components known to be present (2,13), are not determined. In the case of simpler fats, such as margarines, there is probably no advantage in using either the butyl ester GC step or preseparation of methyl esters by argentation TLC. This is because such fats usually do not contain appreciable amounts of short-chain fatty acids, and the *trans* isomers are present predominantly in the 18:1 and 18:2 acids. There is a possibility that positional isomers of *cis* and *trans* methyl esters could result in broadened GC peaks and affect quantitation. As discussed by Ottenstein et al. (6), this could present a problem in the analysis of some margarines. However, most nonhydrogenated lipids would be expected to contain relatively minor amounts of *trans* positional isomers.

Compared to packed columns, glass capillary columns provided improved separation of geometric and positional isomers of fatty acid methyl esters from phospholipids of human plasma (3) and red blood cells (19). Nevertheless, there are chromatographic problems that have not yet been solved by capillary GC (3). Separation in a single run of the complex mixtures of positional isomers present in partially hydrogenated edible fats has not been demonstrated. Coating of glass capillaries with highly polar cyanosilicome stationary phases presents technical problems. Moreover, the automated equipment necessary is relatively expensive and demands a high degree of skill to operate and to establish reliable methods.

In laboratories that must work with packed column GC systems, previously unattainable separations can be made routinely by performing a preseparation of fatty acid methyl esters on TLC into saturated, mono-, di-, and polyunsaturated fractions and utilizing improved stationary phases now available.

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