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RAPID ANALYSIS OF *trans* FATTY ACIDS ON SP-2340 GLASS CAPILLARY COLUMNS

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SUMMARY

The application of 15-m glass capillary columns coated with SP-2340 in the analysis of *trans* fatty acids is demonstrated. Although resolution of *cis* and *trans* isomers is incomplete on short columns, appropriate correction factors can be applied which permit the rapid quantitative analysis of fatty acid mixtures containing *trans* isomers. The conditions employed permit a fairly detailed analysis of fatty acids of tissue samples in less than 30 min and the analysis of fatty acids derived from partially hydrogenated food fats in under 15 min after purification and preparation of the methyl esters.

INTRODUCTION

We have been interested in dietary sources, nutritional adequacy and metabolic fate of *trans* fatty acids. Our research required a procedure which was rapid, reproducible and sensitive and one which would provide quantitative information on *trans* fatty acids as well as the typical fatty acids present in lipids derived from foods and tissues.

Classical procedures for the analysis of *trans* fatty acids were of limited utility. Although useful in certain situations, infrared spectrophotometry (IR) or combinations of IR or gas–liquid chromatography (GLC) and argentation thin-layer chromatography (TLC) were undesirable for the rapid routine analysis of fatty acid mixtures containing *trans* isomers. As has been reported by others[1,2] packed column GLC procedures specifically designed for the resolution of geometric isomers of fatty acids were also inadequate. They provided no time advantage over some of the classical methods and in our hands failed to provide reproducible data on polyunsaturated fatty acids derived from tissue lipids.

We now employ short glass capillary columns coated with SP-2340 in the routine analysis of fatty acid mixtures containing *trans* isomers. Heckers *et al.*[3] demonstrated the potential of glass capillary GLC columns coated with SP-2340 for the analysis of *cis*/*trans* isomeric fatty acid pairs and Lanza and Slover[2] have reported on the suitability of glass capillary columns coated with SP-2340 for the analysis of food fatty acids. Although others[4,5] have shown that analysis time can be significantly re-

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duced when short glass capillary columns are used and the overall advantages and disadvantages of short glass capillary columns coated with SP-2340 have been described[5], more attention has been devoted to the longer glass capillary GLC columns partly because *cis* and *trans* isomers are less adequately resolved on the shorter columns.

In our experience, the significant reduction in analysis time provided by the shorter columns more than compensates for the incomplete resolution of isomeric fatty acids. We find that 15-m glass capillary GLC columns coated with SP-2340 are more than adequate for the analysis of *trans* fatty acids, provided that appropriate correction factors are utilized and herein present data which demonstrate the efficacy of the short column procedure for the rapid routine quantitative determination of fatty acid mixture containing *trans* isomers.

EXPERIMENTAL

Materials

Unless otherwise noted, all solvents employed in this study were reagent grade or better. Isooctane used in glass capillary GLC was high purity reagent purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Fatty acid methyl ester standards (99% purity) were purchased from Nu Chek Prep. (Elysian, MN, U.S.A.) and aldehyde standards were purchased from Supelco (Bellefonte, PA, U.S.A.). Silica gel G-60 used in the preparation of thin-layer chromatographic plates was a product of E. Merck (Elmsford, NY, U.S.A.) and Biosil A used in column chromatography was purchased from Calbiochem (La Jolla, CA, U.S.A.). Margarines and shortenings were purchased locally.

Lipid extraction and preparation of methyl esters

Margarines and shortenings were melted at 40°C and appropriate aliquots were dissolved in methylene chloride containing 1% (w/v) butylated hydroxytoluene (BHT). After filtering through anhydrous sodium sulfate, the extracts were concentrated to near dryness under nitrogen and transesterified in the dark at room temperature for 72 h in 0.6 *N* sulfuric acid in methanol (2 ml/100 mg lipid) or by refluxing for 4 h in 1.5 *N* anhydrous methanolic HCl (2 ml/50 mg lipid). Some samples were transesterified in the presence of known amounts of *cis*- and *trans*-heptadecenoate (17:1) to aid in quantitation after argentation TLC.

Livers obtained from mice fed a diet containing margarine fat (as 10% of the diet) were used to obtain a polar lipid fraction. Total lipids were extracted in methylene chloride–methanol (2:1) (19 volumes/gram of tissue) essentially as described by Folch *et al.*[6] except that the tissue was homogenized in methanol containing BHT (25 μg/mg expected lipid) prior to extraction and methylene chloride was used in place of chloroform throughout the procedure. The crude lipid extract was separated into upper and lower phases by the addition of 0.2 volumes of 0.58% NaCl solution and the lower phase was carefully concentrated at less than 30°C to remove traces of water and methanol prior to chromatography on silicic acid columns[7,8] to obtain a polar lipid fraction. Transesterification of the polar lipid fraction was conducted at 80°C for 16 h in 1.5 *N* anhydrous methanolic HCl (2 ml/50 mg lipid) in tubes sealed with PTFE-lined caps.

Purification of methyl esters

Hexane and water were added to the transesterification mixtures and the esters were extracted into the hexane phase, concentrated under nitrogen at less than 35°C and purified by preparative TLC on 20 × 20 cm glass plates coated to a thickness of 0.5 mm with the adsorbent. All samples were applied to plates that had been activated at 100°C for 1 h and prerun in diethyl ether. The developing solvent employed depended upon the nature of the transesterified sample. When the transesterification mixture contained dimethylacetals (derived from plasmalogens present in tissue samples), 100% xylene(s) was used to permit isolation of both methyl esters and dimethylacetals[9]. Other transesterification mixtures were developed in light petroleum (b.p. 30–60°C)–diethyl ether (95:5, v/v).

Developed plates were visualized under UV light or by spraying a portion of the plate with bromthymol blue reagent[10]. Appropriate areas of the silica gel were scraped from the plates with the aid of a PTFE "policeman,, (scraper), transferred to a glass column fitted with a plug of glass wool and extracted with 4 column volumes of methylene chloride to elute the purified fatty acid methyl esters (PFAME). The PFAME were concentrated to dryness under nitrogen and diluted with isooctane to approximately 25 mg/ml for analysis by glass capillary GLC.

Argentation TLC

Preparative TLC for the isolation of *cis*- and *trans*-monoene fractions as methyl esters was conducted on 25 × 20 cm glass plates coated to a thickness of 0.5 mm with silica gel G containing 8.5% silver nitrate (w/w). The plates were activated and prerun as described above and developed in the 25-cm direction using chloroform–ethanol (99.25:0.75, v/v). Bands were visualized under UV light after spraying the plates with 0.2% 2,7-dichlorofluorescein in ethanol (w/v) and the appropriate areas were treated as described above to isolate the *trans*- and *cis*-monoene fractions, except that methylene chloride–1 *N* anhydrous methanolic HCl (99:1) was used to elute the esters from the silica gel.

Ozonolysis of monounsaturated PFAME

Microozonolysis was conducted essentially as described by Beroza and Bierl[11]. Approximately 100 μg of the monoene fraction was dissolved in 100 μl of carbon disulfide and flushed with nitrogen for 15 sec. Ozone was bubbled through the sample at −70°C for 1 min at a flow-rate of 10 ml/min. Excess ozone was removed from the sample by flushing with nitrogen for 30 sec, *ca.* 1 mg of triphenylphosphine was added and aliquots were injected into a Perkin-Elmer Model 900 GLC (Norwalk, CT, U.S.A.) equipped with a flame ionization detector (FID) and fitted with a 1.8 m × 0.32 mm I.D. stainless-steel column packed with 15% DEGS on Chromosorb Z (100–200 mesh). The injector port was maintained at 225°C and the column temperature was programmed from 60 to 205°C at 3°C/min with a nitrogen carrier gas flow of 25 ml/min. Areas corresponding to the aldehyde ester peaks were used to quantify the positional isomers present in the monoene fractions subjected to ozonolysis. To minimize interference from other components, the injector end of the column contained a 7-cm portion packed with 2% zinc oxide (w/w) coated on prewashed sand. Identification of aldehyde and aldehyde ester peaks was accomplished with the aid of known aldehydes, standard PFAME subjected to the ozonolysis procedure and from relative retention times of components analyzed at selected constant temperatures.

TABLE I

OPERATING PARAMETERS FOR SP-2340 GLASS CAPILLARY GLC COLUMNS

Parameters	*15 meter*	*75 meter*
Gas held-up time (t_m) for methane	1.05 min	7.36 min
Retention time for isooctane	1.05 min	7.42 min
Average linear gas velocity ($\bar{u}$)	24 cm/sec	17 cm/sec
Partition ratio (k, 18:0)	3.34 at 180°C	5.59 at 160°C
Split ratio at room temperature	70:1	100:1
Split ratio (operating temperature)	160:1 (180°C)	200:1 (160°C)
Sample size (µg/injection)	*ca.* 7 µg	*ca.* 10 µg

Infrared analysis

Aliquots of PFAME were analysed for *trans* unsaturation essentially as described by Allen[12] using a Perkin-Elmer 1320 IR spectrophotometer and methyl elaidate and methyl oleate as standards.

Glass capillary gas liquid chromatography

Glass capillary columns (15 m or 75 m × 0.25 mm I.D.) coated with SP-2340 were purchased from Quadrex (New Haven, CT, U.S.A.) and installed in the Hewlett-Packard (Avondale, PA, U.S.A.) Model 5830 or 5840 gas–liquid chromatographs equipped with FID detectors. The instruments were modified to accept glass capillary columns essentially as described by Slover and Lanza[13] except that the glass splitter tube was packed with silanized glass beads and silanized glass wool and the regulator on the gas cylinder was used to maintain the helium carrier gas inlet pressure. Make up gas (nitrogen) was maintained at a flow of approximately 50 ml/min and the temperatures of the injection port and the detector were maintained at 250 and 275°C, respectively. Samples were analyzed on the 15-m column with the initial temperature at 180°C. After 9 min, the column temperature was programmed from 180 to 195°C at a rate of 1°C/min. The final temperature was maintained until the end of the run (30 min or less).

The initial temperature for the 75-m column was maintained at 160°C for 28 min. At 28 min, the temperature was raised to 190°C at a rate of 2°C/min. At 80 min after injection of the sample, the temperature was again programmed at a rate of 2°C/min to a final temperature of 205°C, which was maintained until all components had emerged (120 min or less). Sample injection volumes were 0.5 µl or less of isooctane solutions. Other conditions pertinent to the operation and performance of the capillary columns are listed in Table I. Identification of fatty acid methyl esters was based on available standards and comparisons of retention times to those reported in the literature[13,14].

RESULTS AND DISCUSSION

Fig. 1 is a typical chromatogram obtained for PFAME of partially hydrogenated fats analyzed on a 15-m SP-2340 glass capillary GLC columns. Peaks of interest are identified according to the chain length and number of double bonds

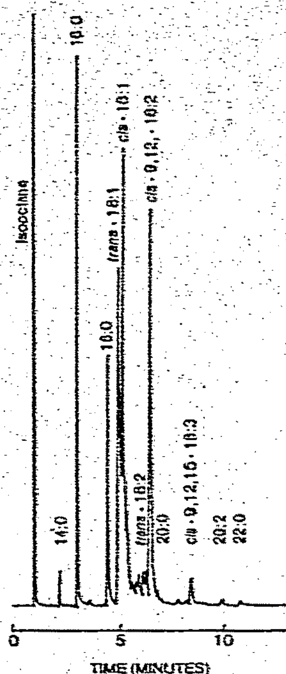


Fig. 1. Chromatographic trace of fatty acid methyl esters of a margarine sample (sample E) analyzed on a 15-m glass capillary GLC column coated with SP-2340.

present in the molecule (*e.g.*, *trans*-octadecenoates = *trans*-18:1). Although the *cis*- and *trans*-18:1 in this sample were not completely resolved, baseline resolution on such a column was routinely achieved for mixtures of methyl elaidate and methyl oleate as well as for other pairs of geometric isomers.

The reason for the poor resolution in Fig. 1 is the heterogeneity of the *trans* and *cis* components in partially hydrogenated fats. As can be seen in Fig. 2, the monooctadecenoates obtained from partially hydrogenated fats by argentation TLC consist of various positional isomers. The results obtained after ozonolysis of such monoene fractions (Fig. 2) indicate that *trans*-monoenes with double bonds ranging from Δ-6 to Δ-16 were present in all the samples and most of these positional isomers were also present in the *cis* fraction.

As can be seen in Fig. 3, the longer column provided better resolution of the *trans* and *cis* components, however resolution of all *trans* isomers from all *cis* isomers was still incomplete. Moreover the analysis time required for the 75-m column was much longer.

Because of the time advantage associated with using the 15-m column, we investigated the utility of employing correction factors to aid in quantifying *trans* and *cis* components in PFAME. Four samples were examined which differed in the proportion of positional isomers (samples A–D, Fig. 2) as well as in the percentage of total *trans* fatty acids. After transesterification in the presence of known amounts of *trans*- and *cis*-17:1, the *trans*- and *cis*-monoene fractions in each sample were isolated

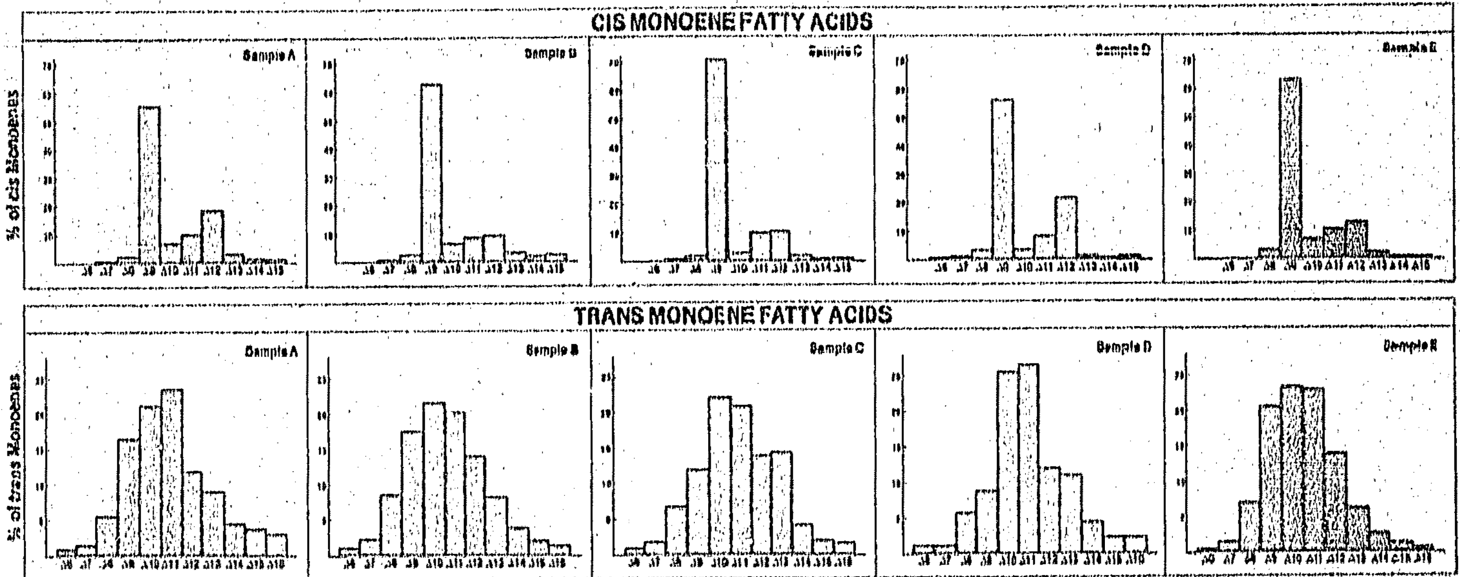


Fig. 2. Distribution of positional isomers of *cis* and *trans* monoenes estimated by ozonolysis of fractions isolated by argentation TLC of methyl esters derived from selected margarines and shortenings.

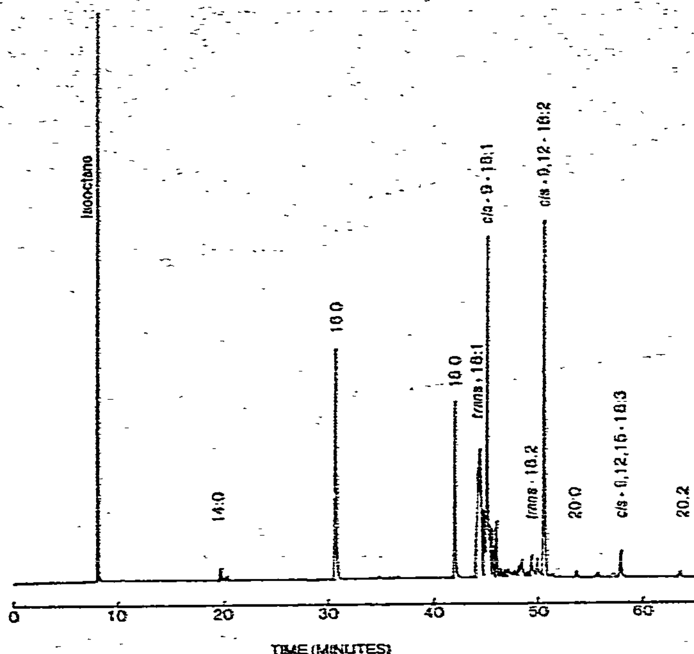


Fig. 3. Chromatographic trace of fatty acid methyl esters of a margarine sample (sample E) analyzed on a 75-m glass capillary GLC column coated with SP-2340.

by $AgNO_3$-TLC and mixtures of the isolated fractions were prepared for analysis by glass capillary GLC on the 15-m SP-2340 column. With the aid of the internal standards, comparisons were made of the observed areas with the expected areas for the *trans*- and *cis*-18:1 peaks and correction factors were calculated for each mixture.

Regardless of the sample used to prepare the mixture, the correction factor obtained for a particular ratio of *trans* to *cis* was quite similar. As can be seen in Fig. 4, over the range examined (*ca.* 4–45% of the 18:1 as *trans*) there was a linear relationship between the correction factors for *trans*- or *cis*-18:1 and the proportion of 18:1 in the samples which were *trans* isomers. The specific correction factor obtained for a mixture reflected the constant amount of *trans*-18:1 which tailed into the *cis*-peak (see Fig. 1) under the conditions employed and the relatively constant proportion of *trans* isomers which emerged in the *cis* area. Thus, as the ratio of *trans* to *cis* decreased, a larger correction was required to increase the observed *trans* area counts to the expected amount and a smaller one was needed to reduce the observed *cis* counts to those expected for the *cis*-18:1 components. Although the correction factors were variable, they were readily computed using the observed areas for the 18:1 peaks in a glass capillary GLC analysis and the equations for the best fitting straight lines given in Fig. 4.

Data obtained using correction factors and the 15-m glass capillary GLC analyses were comparable to those obtained using more time consuming techniques. Results for total *trans* fatty acid determinations on five food samples using several methods are summarized in Table II. Determinations by glass capillary GLC analyses

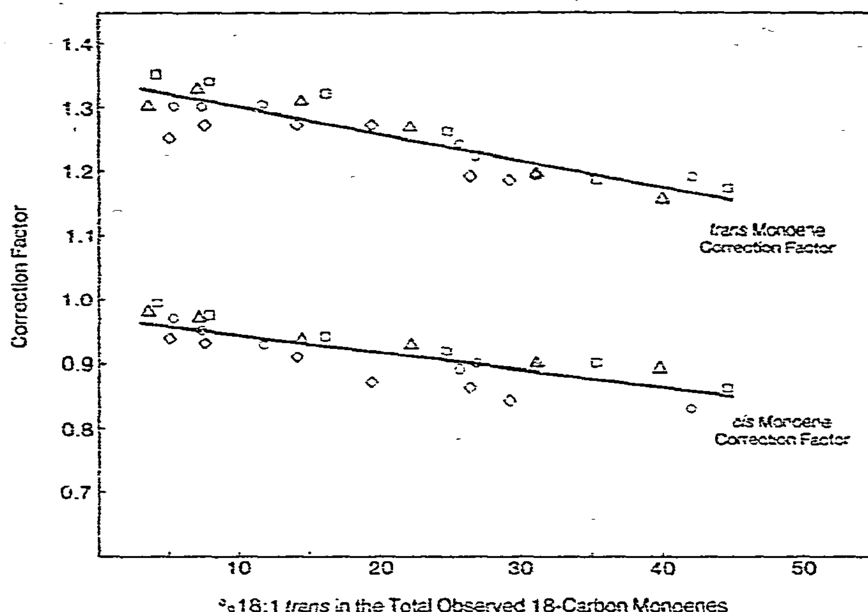


Fig. 4. Relationship between correction factors for *trans*- and *cis*-18:1 and the percentage of total observed 18:1 as *trans* isomer when analyzed as methyl esters on a 15-m SP-2340 glass capillary GLC column. The equations for the best fitting straight lines were $y_t = -4.17 \cdot 10^{-3}\,x + 1.34$ ($r = 0.88$) and $y_c = -2.84 \cdot 10^{-3}\,x + 0.975$ ($r = 0.83$), where y_t and y_c are correction factors for *trans*- and *cis*-18:1, respectively, and x = *trans*-18:1 as a percentage of the total observed 18:1. ○, sample A; △, sample B; □, sample C; ◇, sample D.

were in good agreement with those obtained using the combined argentation TLC + glass capillary GLC method and were generally higher than estimates obtained by the IR method employed. An exception was sample C. In all instances, the glass capillary GLC techniques resulted in lower estimates of *trans* fatty acids for sample C than would be predicted by the combined TLC + glass capillary GLC method. The reason for the discrepancy is not known but it is apparent that it was not a defect restricted to the 15-m glass capillary method.

To test the applicability of using correction factors derived from Fig. 4, data obtained from the 15-m column were corrected in two ways. In Table II, the values shown in column A were obtained after the *trans*-18:1 counts were corrected using factors specifically calculated for each sample whereas those shown in column B were the result of applying a common correction factor based on the equation for the best fitting straight line (Fig. 4). As expected, values derived using the specific correction factors deviated least from the TLC + glass capillary GLC determinations; however, estimates of *trans* fatty acids employing the common correction factor were more than adequate.

The 15-m glass capillary GLC procedure provided relatively rapid and accurate estimates of *trans* fatty acids, although it was more time consuming than IR alone. For some studies, IR may be satisfactory for the estimation of total *trans* fatty acids, but the accuracy of the method we employed was inadequate. Recently[15] an IR procedure has been described which is reported to yield *trans* values that are more

TABLE II

COMPARISONS OF *trans* FATTY ACID ESTIMATES FOR SELECTED SAMPLES USING DIFFERENT METHODS

Average (and standard deviation) for *trans* isomers as weight percent of total fatty acid methyl esters. $n = 5$ for all except samples B, C and D under TLC + glass capillary GLC where $n = 4$.

Sample	*Infrared*	*TLC + glas capillary GLC**	*75-m glass capillary GLC***	*15-m glass capillary GLC*	
				*A****	*B[§]*
A	35.6 (0.65)	40.0 (0.72)	38.4 (0.68)	40.2 (0.68)	39.4 (0.60)
B	23.3 (0.16)	24.5 (0.57)	25.3 (0.33)	25.6 (0.47)	26.0 (0.25)
C	18.4 (0.64)	20.2 (0.26)	17.7 (0.32)	18.3 (0.42)	18.1 (0.38)
D	23.8 (0.19)	28.3 (0.42)	27.6 (0.39)	28.7 (0.44)	27.4 (0.26)
E	28.3 (1.16)	30.6 (0.40)	31.5 (0.42)	31.8 (0.28)	31.4 (0.31)

* *Trans* and *cis* monoenes (including 17:1 isomers as internal standards) resolved by argentation TLC and analyzed by 15-m glass capillary GLC. Total ester obtained by 15-m glass capillary GLC before TLC. Values represent total *trans*, including any observed *trans*-polyene.

** Values represent total *trans*, including any observed *trans*-polyene. Monoene values were corrected for *trans* components not resolved from the *cis* components.

*** Values represent total *trans*, including any observed *trans*-polyene, using correction factors for *trans* monoenes specifically calculated for each sample.

[§] Values represent total *trans*, including any observed *trans*-polyene, using a common correction factor based on the regression line for the *trans* correction factor (Fig. 4).

accurate than other existing IR methods and should prove useful to those interested solely in the total level of *trans* fatty acids. Nevertheless, IR alone is unsatisfactory for most studies involving *trans* fatty acids, as it provides less information than glass capillary GLC, which furnishes fairly detailed data on fatty acid composition.

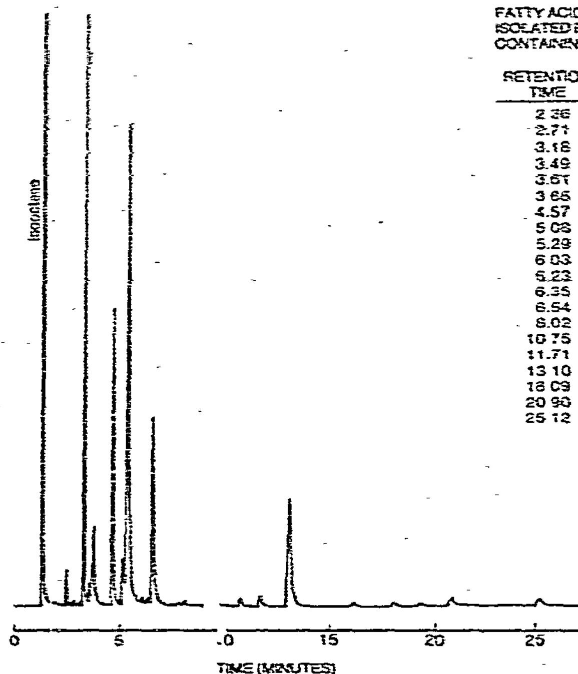
As can be seen in Table III, the 15-m glass capillary GLC method described in this paper provided analyses of fatty acid composition which were reproducible and

TABLE III

FATTY ACID COMPOSITION OF PARTIALLY HYDROGENATED FATS BASED ON 15-m GLASS CAPILLARY GLC

Average (and standard deviation) as weight percent of total fatty acid methyl esters ($n = 5$). tr = trace; less than 0.1%; others include 14:0, 16:1, 20:0 and *trans*-18:3; t = *trans*, c = *cis*.

Sample	*16:0*	*18:0*	*18:1-t*	*18:1-c*	*18:2-t,t*	*18:2-c,t*	*18:2-t,c*	*18:2-c,c*	*18:3-c,c,c*	*Others*
A	10.5	6.8	34.1	34.5	2.3	1.4	1.6	7.7	0.2	0.8
	(0.06)	(0.08)	(0.61)	(0.70)	(0.10)	(0.03)	(0.05)	(0.03)	(0.02)	
B	11.1	6.7	24.3	25.5	0.7	0.6	0.4	30.3	0.4	tr
	(0.07)	(0.03)	(0.25)	(0.39)	(0.06)	(0.02)	(0.01)	(0.08)	(0.004)	
C	10.8	6.8	14.7	29.7	1.0	1.2	1.3	31.7	2.2	0.6
	(0.08)	(0.03)	(0.10)	(0.25)	(0.02)	(0.02)	(0.02)	(0.20)	(0.08)	
D	13.9	9.6	22.6	38.5	2.0	1.3	1.6	10.1	tr	0.4
	(0.19)	(0.26)	(0.30)	(0.18)	(0.19)	(0.09)	(0.03)	(0.17)		
E	12.6	8.4	26.9	23.9	1.5	1.6	1.6	18.9	1.5	3.0
	(0.02)	(0.08)	(0.32)	(0.21)	(0.03)	(0.07)	(0.02)	(0.11)	(0.04)	



FATTY ACID COMPOSITION OF POLAR LIPIDS ISOLATED FROM LIVERS OF MICE FED A DIET CONTAINING PARTIALLY HYDROGENATED FAT

RETENTION TIME	TENTATIVE IDENTIFICATION	WEIGHT PERCENT
2.36	14:0	0.8
2.71	15:0	0.2
3.18	16:0	18.9
3.49	16:1-t	1.0
3.61	16:1(n-9)	1.4
3.65	16:1(n-7)	3.6
4.57	18:0	13.0
5.08	18:1-t	4.0
5.29	18:1-c	27.7
6.03	18:2-t,t	0.5
6.23	18:2-c,t	0.2
6.35	18:2-t,c	0.4
6.54	18:2-c,c	12.0
8.02	20:1(n-9)	0.3
10.75	22:0	0.7
11.71	20:3(n-6)	1.1
13.10	20:4(n-6)	11.7
18.09	24:1(n-9)	0.3
20.90	22:5(n-6)	1.0
25.12	22:6(n-3)	1.0

Fig. 5. Chromatographic trace of fatty acid methyl esters of polar lipids isolated from mouse liver and analyzed on a 15-m glass capillary GLC column coated with SP-2340.

sufficiently detailed for many studies involving *trans* fatty acids. In addition to total *trans* values, analyses with the 15-m column can be used to routinely monitor *trans*-polyenoic fatty acid levels and ratios of saturated to polyunsaturated fatty acids in partially hydrogenated fats. Moreover, for studies concerned with dietary *trans* fatty acids, fatty acid compositions of tissue lipids (see Fig. 5) can be obtained in less than 30 min.

A disadvantage of the 15-m glass capillary GLC procedure is that it does not provide the detail on positional isomers of monounsaturated fatty acids that can be obtained with longer glass capillary GLC columns. But, even the longer columns do not resolve all the isomers which are present in most samples encountered in our work. When such detailed information is desirable, we find that combinations of argentation-TLC + glass capillary GLC and ozonolysis are mandatory.

The glass capillary GLC procedure described herein, using the common correction factor was designed to furnish rapid, reproducible and accurate analyses on typical samples encountered in our studies. In our work, the level of *trans*-18:1 as a percentage of the total 18:1 is typically between 4 and 45%. We have not examined values beyond this range, but it is obvious that as the percentage of *trans*-18:1 approaches 0, the correction factors deviate from the best fitting straight line shown in Fig. 4 and approach a value of 1.

The validity of the equations used for computing correction factors can be routinely checked using appropriate samples. We used a well characterized margarine fat as a source of PFAME (sample B, Table III) and mixtures of *trans*- and *cis*-18:1

PFAME isolated from this fat to check the column performance, as factors such as column age may affect the resolution obtained and therefore the degree of correction required. In our experience, with proper care, 15-m glass capillary GLC columns coated with SP-2340 can be used on a routine basis for well over one year for fairly rapid, reproducible and accurate analyses of fatty acid methyl ester mixtures containing *trans* isomers.

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