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Precision and Accuracy in Gas Liquid Chromatography of C_{14}-C_{18} Fatty Methyl Esters[1]

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Abstract

Repetitive analyses of four primary standards for fatty methyl esters by gas liquid chromatography (GLC) with polyester columns and thermal conductivity detection established standard deviations ranging from ±0.3–0.5% corresponding to coefficients of variation of 1.0–2.0%. These data, representing a relative error of measurement of 1.5–3.0% at a 99% confidence level, suggest a precision approaching that of conventional spectrophotometric measurements.

Proportionality factors, calculated from known mass or molar concentration divided by area % from GLC analysis, were found to be reproducible correction factors which may be generally applicable to GLC analysis of fatty methyl esters with polyester columns and thermal conductivity detectors.

Mass response to a thermal conductivity detector was found to decrease with either increasing molecular weight for saturated C_{14}–C_{18} fatty methyl esters or with unsaturation among the C_{18} unsaturated esters, while molar response increases with molecular weight and decreases with degree of unsaturation.

The use of uncorrected area % data can introduce significant absolute mass errors ranging from about +11% for myristic acid to −17% for linolenic acid.

THE EXPANDING USE of gas liquid chromatography (GLC) in routine fatty acid analysis lends importance to the precision and accuracy of this technique. Very little information is available on the precision of GLC analysis of fatty methyl esters, particularly the commonly encounted C_{14}–C_{18} acids. More attention has been given to the relative thermal conductivity detector response of the individual methyl esters. From the work of several investigators (1–3) it is apparent that the response of a thermal conductivity detector is not a simple function of either weight or molar concentration. The need for suitable correction factors has prompted study of the response of individual fatty methyl esters, relative to the response of various arbitary internal standards. These relative response factors have been proposed as suitable constants of general applicability to GLC analysis with thermal conductivity detectors (2,3), although the factors vary with the internal standard chosen.

The current work was undertaken to obtain information both on the precision of GLC analysis for the C_{14}–C_{18} fatty methyl esters, and to determine the proportionality factors for correction of GLC data.

Materials and Methods

Four primary standards (Table I) were used. Standards A, B, and C were prepared, with a weight accuracy of at least one part per thousand, from highest purity methyl esters (ca. 99% +), each of which was independently analyzed by GLC to insure the absence of impurities. Standard D was a calibration standard furnished by the Hormel Foundation. All standards were stored at 0F prior to analysis. A Beckman GC-2A chromatograph equipped with a thermal conductivity detector was used for all analyses. The flash chamber was modified for controlled operation at 325C, and equipped with a syringe guide to insure reproducible sample injection.

Two 6 ft copper columns 0.25 in. O.D., each packed with Gas Chrom A, 80–100 mesh, and coated with 20% diethylene glycol succinate polyester were used for the analyses reported here. Both column and detector were operated at 190C, with 250 ma detector current, and helium at 25 lb pressure as carrier gas. Samples of approximately 0.10 μl were injected by use of a 1 μl syringe.

Relative concentrations of components in each standard were estimated from peak areas, by triangulation, and are reported as area percent (Table I).

Relative mass response factors for the individual fatty methyl esters were calculated by dividing the average area response per unit weight, from repetitive analysis of each standard, by the corresponding response of methyl palmitate as an internal standard. Average values for the four standards are reported in Table II.

Absolute proportionality factors (PF) were calculated from the average data from replicate analysis of each standard from the relationships:

PF (Mass) = Known, wt. %/Found, area %

PF (Molar) = Known, Mole %/Found, area %

These proportionality factors obtained over a period of several months were found to be reproducible, and the factors reported (Table III) are average data from the same number of replicates as reported for the relative response factors (Table II).

Results and Discussion

Precision

Standard deviations, coefficients of variation and the 99% confidence limits of the means, from repetitive analysis of four standards are listed in Table I. These analyses were conducted over a period of several days and reflect variations associated with day-to-day changes in operating parameters. Average values for the standard deviations and coefficient of variation indicate no significant differences in the pre-

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TABLE I

Precision of GLC Analysis for C_{14}-C_{18} Fatty Acid Methyl Esters

Standard No.	Property	Fatty acid methyl ester				
		Myristic	Palmitic	Stearic	Oleic	Linoleic
A (N = 5)[a]	Known-mole %	5.82	18.89		25.07	50.22
	Known-wt %	4.91	17.77		25.86	51.45
	Found-area %	5.68 ± 0.31[e]	19.15 ± 0.53		26.90 ± 0.72	48.26 ± 0.45
	σ[c]	± 0.18	±0.31		±0.43	±0.27
	CV[d]	3.24%	1.64%		1.59%	0.56%
B (N = 5)[a]	Known-mole %	22.61	22.37		28.80	26.22
	Known-wt %	19.72	21.77		30.73	27.79
	Found-area %	22.04 ± 0.94[e]	23.26 ± 0.77		29.60 ± 0.47	25.11 ± 0.48
	σ[c]	±0.56	±0.46		±0.28	±0.28
	CV[d]	2.55%	1.98%		0.96%	1.13%
C (N = 5)[a]	Known-mole %	25.24	23.99	22.90		27.86
	Known-wt %	22.13	23.46	24.73		29.67
	Found-area %	24.38 ± 0.61[e]	24.59 ± 0.39	24.50 ± 0.36		26.66 ± 0.44
	σ[c]	±0.36	±0.23	±0.21		±0.26
	CV[d]	1.48%	0.95%	0.86%		0.98%
D[b] (N = 7)	Known-mole %		21.46	19.43	19.57	19.70
	Known-wt %		20.01	19.99	20.00	20.00
	Found-area %		22.10 ± 0.33	20.32 ± 0.30	21.06 ± 0.45	19.46 ± 0.32
	σ[c]		±0.28	±0.24	±0.38	±0.27
	CV[d]		1.28%	1.19%	1.78%	1.39%
	Average σ[c]	±0.46	±0.32	±0.23	±0.36	±0.27
	Data CV[d]	2.02%	1.46%	1.03%	1.44%	1.02%
	Error[f]	3.3%	2.3%	1.5%	2.1%	1.5%

[a] Number of replicate analyses, each standard.
[b] Standard also contains linolenic acid. Known-mole %, 19.89; Known-wt %, 20.00; Found area %, 17.06 ± 0.38; σ, ±0.35; CV, 2.05%.
[c] Standard deviation.
[d] Coefficient of variation, standard deviation as % of mean.
[e] 99% confidence level of mean, $CL = 6t/\sqrt{N}$.
[f] Error of mean at 99% CL.

cision of measurement for methyl palmitate through linolenate. Difficulties in the triangulation of the sharp methyl myristate peaks are probably responsible for the slightly lower precision of measurement for this ester. Coefficients of variation ranging from 1.0–2.0% at a 99% confidence level suggests that the precision of these GLC analyses approaches that of conventional colorimetric or spectrophotometric methods, generally considered to be from 0.5 to 2.7 relative error (4).

Detector Response

In GLC analysis of fatty acids as methyl esters it is frequently assumed that the response of a thermal conductivity detector is a linear function of weight concentration (5,6). Average recovery values from replicate analysis of four standards (Table I) indicates that peak area is not a simple function of concentration expressed on either a weight or molar basis. For the saturated esters, fortuitous molecular weight relationships results in area percent values for methyl myristate close to mole concentration, while those for methyl palmitate and stearate are closer to weight concentration. Among the C_{18} unsaturated esters, peak area is lower than weight or molar concentration. These results substantiate the observations of Messner et al. (1), Horrocks (3), and Killheffer and Jungermann (2) who demonstrated that peak area is proportional to mole percent, or weight percent, only when the detector response of individual esters is adjusted by means of empirical relative response constants which are approximately linear for a structurally similar homologous series. These relative response factors are calculated in terms of percentage of the response of some internal standard which is arbitrarily assigned a value of unity. Relative response factors may be calculated on a weight or molar basis, and although useful, the corrected data are always relative to some arbitrary standard.

When the mass response factors of the individual esters in Table I are calculated relative to methyl palmitate as 100, relative mass response factors (Table II), obtained here with a polyester column, are in essential agreement with comparable data obtained by Horrocks et al. (3) with a silicone resin column. Relative mass response of saturated esters decreases with increasing molecular weight. Among the C_{18} unsaturated esters, the response of methyl oleate is about the same as methyl stearate, and there is a further decrease in response with increasing unsaturation.

Proportionality Factors

Absolute response factors for individual esters may be calculated from the recovery data listed in Table I. These PF's, obtained by dividing the known weight or molar concentrations in a standardization mixture by the area percent as determined by GLC, serve as simple multiplication factors to correct peak area data to true weight or molar concentration. Similar factors have been proposed by Kaufmann et al. (7).

Mass PF values for the saturated esters, shown in Table III, exhibit a gradual increase from C_{14}–C_{18} corresponding to decreasing mass response with an increase in molecular weight, with the value for methyl stearate being close to unity. For esters of unsaturated C_{18} acids, the trend is reversed. PF values increase, denoting decrease in absolute mass response with degree of unsaturation. These trends are illustrated by the good linearity of the data plotted in Figure 1.

Mass PF values were calculated from recovery data reported by several invesitgators with polyester col-

TABLE II

Relative Mass Response of Fatty Acid Methyl Esters

Methyl ester	N[a]	Relative mass response[b]	
		Present work %	From literature[c] %
Myristic	46	106.0	102.6
Palmitic	46	......[d]	......[d]
Stearic	25	93.6	93.7
Oleic	34	94.9	88.2
Linoleic	46	87.9	91.0
Linolenic	13	78.2	84.8

[a] Total No. GLC analyses from replicate analysis of 4 standards.
[b] Area per unit weight of each ester relative to value for methyl palmitate = 100.
[c] From data reported in reference (3). 25% Silicone resin (SF-96), 5′ column at 208–228C, detector current 200–250 ma, He as carrier gas.
[d] Internal standard—response taken as 100.

TABLE III

Relation Between Molar Volume of Fatty Acid Methyl Esters and Proportionality Factors

Methyl ester	Molar volume $V_m^{25°a}$	Proportionality factor [b]		
		Mass		Molar
		Present work	From literature [3]	Present work
Myristic	281.36	0.894	0.925	1.034
Palmitic	314.32	0.945	0.925	0.978
Stearic	345.43	1.001	0.974	0.949
Oleic	340.66	0.985	0.980	0.947
Linoleic	333.70	1.078	1.071	1.035
Linolenic	326.36	1.169	1.182	1.166

[a] Calculated from data reported in references 13, 14 from relationship

$$V_m^{25°} = \frac{\text{Mol wt}}{d^{25°}\ \text{g/cc}}$$

[b] Values reported for each acid are mean values from replicate analysis of 4 standards ($N = 10–46$)
From relationship:

PF (mass) = Known-Wt %/Found-Area %
PF (molar) = Known-Mole %/Found-Area %

[c] Average data calculated from recovery values reported in references 8–11.

umns (8–11) and the average of these (Table III), are in remarkably good agreement with data developed in the present study. This suggests that PF values may be of general applicability to polyester columns and thermal conductivity detectors.

Calculation of PF factors on a molar basis (Table III) shows that the absolute mass and molar response of saturated fatty acid esters are reversed, i.e., mass response decreases and molar response increases with increasing molecular weight. For the C_{18} unsaturated esters, both mass and molar response decrease (PF values increase) with increasing unsaturation. Assuming that differences in thermal conductivity response of individual esters is a function of the kinetic energy of solute particles, observed differences in the rate of heat transfer from detector filaments to the walls of the cell should be related to the collision frequency of solute molecules (3,12). Interrelated factors such as molecular weight, geometry of the molecule and perhaps association all influence net collision frequency, and should be related to molar volume in the vapor state. Lacking such data, molar volume in the liquid state may be used to approximate these effects, assuming that the factors influencing molar volume in the liquid state are operative in the vapor state. Accordingly, density data reported by Bonhorst et al. (13) and Gros and Feuge (14) for pure methyl esters were extrapolated to 25C and used to calculate molar volume from the relationship:

$$VM^{25°} = \frac{\text{Mol wt}}{\text{Density}^{25°}}$$

These calculated values (Table III) when plotted against mass proportionality factors show good linear relation for saturated esters (Figure 2). Although the C_{18} unsaturated esters depart from the relationship, when the absolute difference between the molar volume of each unsaturated ester and methyl stearate is plotted against PF values, an equally good correlation is obtained. This suggests that either association or molecular geometry is primarily responsible for the behavior of C_{18} unsaturated esters.

TABLE IV

Effect of Column Temperature on Relative Mass Response and Proportionality Factors

Methyl ester	Relative response [a]			Proportionality factor [b]		
	170C[c]	190C[d]	220C[e]	170C	190C	220C
Myristic	110.2	105.2	120.3	0.880	0.908	0.834
Palmitic	......	......	....	0.969	0.954	0.954
Stearic	93.6	94.5	101.2	1.039	1.010	1.000
Oleic	96.2	95.4	96.1	0.982	0.980	0.957
Linoleic	88.0	85.3	83.5	1.064	1.071	1.122
Linolenic	81.6	77.1	68.5	1.121	1.172	1.297

[a] Mass response relative to methyl palmitate = 100.

[b] Proportionality factor $= \frac{\text{Known–Wt. \%}}{\text{Found–Area \%}}$

[c] GLC conditions: column and detector 170C, 35 lb helium, 250 ma, 0.15 µl sample.

[d] GLC conditions: column and detector 190C, 25 lb helium, 250 ma, 0.10 µl sample.

[e] GLC conditions: column and detector 220C, 15 lb helium, 250 ma, 0.04 µl sample.

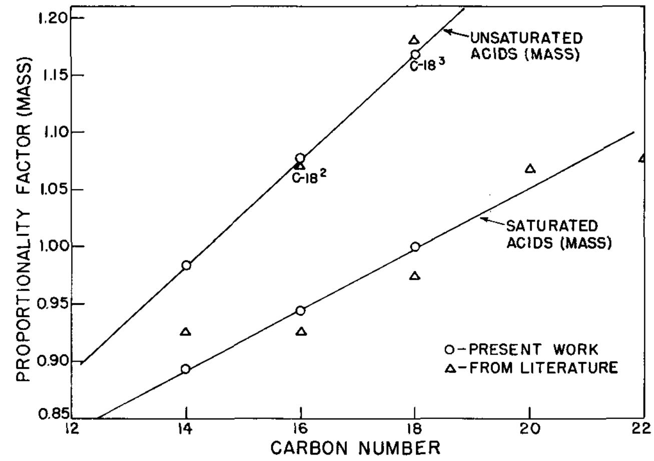

FIG. 1. Mass proportionality factors vs. carbon number or unsaturation.

Temperature Effects

There are little available data on the effect of column temperature on the response of a thermal conductivity detector to fatty acid methyl esters. The temperature dependence was evaluated by replicate analysis of two primary standards ($N = 6$, each standard) at column temperatures of 170, 190 and 220C. Relative response factors (Methyl palmitate = 100) and absolute PF values as a function of temperature are reported in Table IV. These data suggest that PF values for saturated C_{14}–C_{18} fatty acid methyl esters, and for methyl

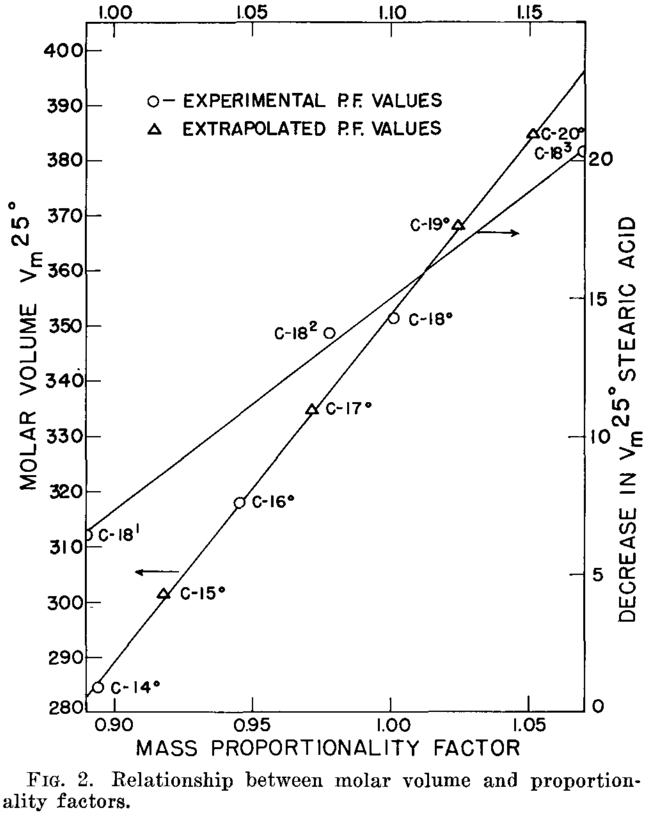

FIG. 2. Relationship between molar volume and proportionality factors.

oleate, are reasonably constant over a temperature range of 50C. Although other parameters were changed, column temperature seems to be the most critical parameter for both linoleic and linolenic acids, and the temperature dependence would probably restrict the use of the PF values reported here to a narrow range, probably 190–200C.

Data developed in the present study support the conclusion that the precision of GLC analysis for C_{14}–C_{18} fatty acid methyl esters is comparable to conventional spectrophotometric methods. However the use of uncorrected area percent data can introduce significant absolute errors. It can be calculated from data presented here that the absolute weight error for each methyl ester is as follows: myristic, +10.6%; palmitic, +5.5%; stearic, 0%; oleic, +1.5%; linoleic, −7.8%; and linolenic, −16.9%.

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Quantitative Estimation of Isomeric Monoglycerides by Thin-Layer Chromatography[1]

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Abstract

A method has been developed for the analysis of isomeric monoglycerides by thin-layer adsorption chromatography. Isomeric monoglycerides are separated on silica gel impregnated with boric acid; a mixed solvent system is used. The interaction of hydroxy compounds with boric acid allows the separation of 1- and 2-monoglycerides which cannot be resolved on silica gel alone. In order to make the method quantitative, the resolved components are charred and their spot densities measured. Glycerol, fatty acids, diglycerides, and triglycerides do not interfere. The method does not induce isomerization.

Introduction

ALTHOUGH THE TECHNIQUE of thin-layer chromatography (TLC) has been known for a number of years, it was not until 1956 through the work of Stahl (1,2) that its use in the lipid field was established. Since that time the technique has undergone rapid development for analytical applications, especially for the direct separation of lipids without prior chemical modification.

Lipids are readily resolved into classes (3–7); mono-, di-, and triglycerides can be separated according to the number of hydroxyl groups in the molecule, irrespective of chain length and degree of unsaturation, by adsorption TLC on silica gel (4–8). Subfractionation within classes may be obtained by reversed phase partition TLC, e.g., on siliconized silica gel (5,6,9,10).

Saturated and various unsaturated members of an homologous-vinylogous series are separated according to their degree of unsaturation by TLC on silica gel-silver nitrate (6,11–14) or as their mercuric acetate addition compounds on silica gel (6). In addition, some *cis-trans* and positional isomers are resolved on silica gel-silver nitrate (6,11–13).

Isomeric diglycerides are easily resolved by adsorption TLC on silica gel (8,15).

Although compounds with very small differences in polarity can be separated by TLC, a satisfactory chromatographic method for the analysis of unmodified isomeric monoglycerides was not available until now.

The most successful previous attempt to resolve isomeric monoglycerides is that described by Hofmann (16), who attempted to resolve them by TLC on hydroxyl-apatite. Hofmann shows that at room temp the isomers are resolved; however, considerable isomerization occurs. At 10C the extent of isomerization is less, but resolution of the isomers is drastically reduced.

Boric acid or borate complexing of polyhydroxy compounds has long been employed in carbohydrate chemistry to effect separations (17) and to assign configurations (18). This technique has also been used to remove benzylidene or isopropylidene blocking groups without causing appreciable isomerization of partial glycerides to occur (19–21).

Silica gel-boric acid TLC has been employed to resolve sugars (22), and a similar system was used to resolve *threo* and *erythro* isomers of dihydroxy acids (23).

The present study describes the use of silica gel-boric acid adsorption TLC to separate isomeric monoglycerides. Results are quantified by means of transmittance densitometry.

Experimental

Thin-Layer Chromatography

The analyses were carried out on 8 × 8 in chromatoplates of silica gel impregnated with boric acid. A slurry was prepared from 50 ml of 0.4 M (24.7 g/liter) aqueous H_3BO_3 and 25 g of Silica Gel GF "Merck" from which layers approximately 0.3 mm thick were spread. Chromatoplates were dried at room temp and activated by heating for 2 hr at 110C in a convention-type oven. No special storage conditions were employed. However, the plates were generally used

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