T. P. HILDITCH SYMPOSIUM ON ANALYSIS OF NATURAL FAT TRIGLYCERIDES. II

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CARTER LITCHFIELD, Chairman

Quantitative Gas-Liquid Chromatography of Triglycerides[1]

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Abstract

To determine optimum operating conditions, an extensive study was made of the variables affecting quantitative recovery and resolution of model triglyceride mixtures. Parameters investigated included: flash heater temperature, carrier gas flow rate, type of carrier gas, column length, glass and metal columns, temperature program rate, linearity of detector response, physical design of gas chromatograph, and molecular species of triglyceride.

Results indicate that with optimum operating conditions, triglyceride molecular weights through trierucin can be quantitatively analyzed. Accurate calibration is essential, since quantitative response factors vary somewhat with operating conditions, triglyceride carbon number, and the chromatograph used. Cocoa butter and rat adipose tissue triglycerides have been quantitatively analyzed by this technique.

Introduction

GAS-LIQUID CHROMATOGRAPHY (GLC) has established itself over the past decade as an indispensable tool for fats and oils research. Its widespread adoption for fatty acid analysis has been due to its high resolving power, speed of analysis, automated quantitative chart readout, and extreme sensitivity. Quantitative analysis of the triglyceride composition of natural fats has always been a tedious and lengthy process. The discovery by Fryer et al. (1), Huebner (2,3), Kuksis and McCarthy (4), Martin et al. (5), and Pelick et al. (6) that triglycerides could be separated according to molecular weight using GLC promised to bring the advantages of GLC to the field of triglyceride analysis.

The majority of natural fats contain mostly C_{16} and C_{18} fatty acids; hence their triglycerides fall in the molecular weight range of tripalmitin through tristearin. The application of triglyceride GLC to natural fats containing only such high molecular weight triglycerides has been slow in realization. Although Kuksis and McCarthy (4,7) reported quantitative results through tristearin, their subsequent quantitative work has been limited to butter and coconut oils in which the majority of triglycerides have molecular weights below tripalmitin (7–10). In a recent paper, Kuksis (11) has claimed that complete "thermal stripping" of newly packed SE-30 columns is essential for quantitative recoveries of tripalmitin and tristearin. Youngs and Subbaram (12) have reported appreciable losses with the GLC of tristearin, and have turned to GLC analysis of the lower molec-

[1] Presented at the AOCS meeting in Houston, Texas, 1965.

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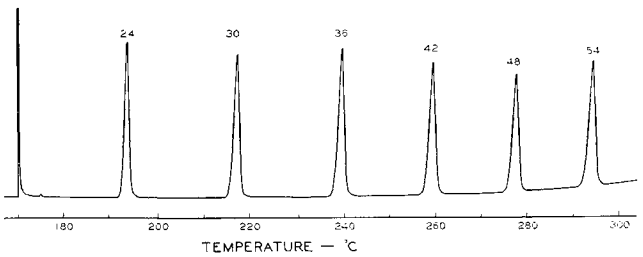

FIG. 1. Typical calibration chromatogram for a mixture of trioctanoin, tridecanoin, trilaurin, trimyristin, tripalmitin, and tristearin run under optimum operating conditions. Operating conditions: chromatograph B; 24 in. glass column programmed at 3C/min.; other conditions as listed under Optimum Operating Conditions in Experimental section.

ular weight permanganate/periodate oxidation products of natural fats containing mostly unsaturated triglycerides. Recently, Jurriens and Kroesen (13) have published quantitative analyses for cocoa butter, palm oil and lard; but they also found significant component loss in the tripalmitin through tristearin molecular weight range. Accurate calibration of these losses was necessary for their quantitative GLC analysis of natural fat triglycerides.

In our early work on GLC of triglycerides, we also encountered high losses and poor resolution with molecules having molecular weights above trimyristin. In an effort to obtain accurate quantitation and resolution with high molecular weight triglycerides, we undertook a detailed study of the various operating parameters. This paper describes our investigation of the effects of flash heater temperature, carrier gas flow rate, type of carrier gas, glass vs. metal columns, column length, temperature program rate, linearity of detector response, and type of gas chromatograph on the quantitation and resolution of high molecular weight triglycerides. Several applications to the quantitative analysis of natural fats will also be considered.

Procedures

Materials

Ninety-nine-plus percent pure monoacid triglycerides were purchased commercially (Applied Science Laboratories, State College, Pa., and Hormel Institute, Austin, Minn.) and used without further purification. GLC of the individual triglycerides revealed traces of diglycerides and/or mixed-acid triglycerides in the trielaidin, triarachidin, tribehenin, and trierucin. The purity of these triglycerides was estimated from their chromatograms, and appropriate purity corrections were applied when they were used as calibration standards.

Cocoa butter was purchased commercially (Hershey Chocolate Corp., Hershey, Pa.) and analyzed as received. Rat adipose tissue triglycerides were obtained from a male albino rat maintained for 55 days after weaning on a standard fat-free diet supplemented with 15 mg of ethyl arachidonate per day (14). Total lipids were extracted from the epididymal fat pads using 2:1 $CHCl_3/CH_3OH$, and the triglycerides were isolated by preparative thin-layer chromatography on silicic acid.

A mixture of C_{45}, C_{46}, C_{47}, and C_{48} triglycerides was synthesized from a 1.00/1.26 molar mixture of pentadecanoic and palmitic acids (99+% pure, Lachat Chemicals, Inc., Chicago, Ill.) by the method of Bourne, et al. (15) using trifluoroacetic anhydride. The final product was not recrystallized, since the presence of monotrifluoroaceto-triglycerides did not interfere with GLC analysis of C_{45}-C_{48} triglycerides.

Methods

Operating Conditions. GLC of triglycerides was carried out on two instruments: (A) an Aerograph Hy-Fi A-600-B gas chromatograph equipped with a hydrogen flame detector and suitable for manual temperature programing; and (B) an F&M 400 gas chromatograph equipped with a hydrogen flame detector and an automatic temperature programer. Instrument A was modified by adding a constant mass flow controller for the carrier gas and a 250C auxiliary oven for preheating air, hydrogen and carrier gas. The latter prevented any possible sample condensation by cold gases coming in contact with the sample. Instrument B was modified by installing an automatic temperature control on the detector block heater.

GLC of triglycerides on dimethylsiloxane polymers such as SE-30 (General Electric Co., Waterford, N.Y.) and JXR (Applied Science Laboratories, State College, Pa.) separated the molecules by molecular weight only. Each peak is usually referred to by its carbon number (the number of carbon atoms in the fatty acid moiety of the triglyceride). Thus, tripalmitin, myristo-palmito-olein, and arachido-dimyristin would all appear in the same peak of carbon number 48. The exact columns and operating conditions used in various experiments are described in the figures and tables listing the results of each separate experiment. Optimum operating conditions are listed in the Experimental section and a typical calibration run using these conditions is shown in Figure 1. SE-30 coatings were applied to the solid support using the solvent evaporation technique (24). The 3.0% JXR on 100/120 mesh Gas Chrom Q was purchased already coated (Applied Science Laboratories, State College, Pa.). All columns were conditioned for 2 hr at 350C with 150 ml/min gas flow prior to use. The first 2–6 runs on a newly conditioned column were discarded as nonquantitative. Peaks were identified by comparison with the elution times and temperatures of known compounds.

Normal samples were injected in CS_2, $CHCl_3$, or xylene solution using 1, 10, and 50 μl microsyringes (Hamilton Co., Whittier, Calif.). Solid triglycerides were injected using a Hamilton SS-60 solid sampler.

Problems were encountered in making a leak-free glass-to-metal seal at the ends of a glass column at temperatures as high as 350C. Silicone rubber O-rings (F & M Scientific Corp., Avondale, Pa.) were used for this purpose, but they turned brittle and cracked after 1–2 days of use. The used O-rings stuck tightly to the glass and metal surfaces, making it difficult to remove the column to replace the leaking O-rings without breaking the fragile glass column. No satisfactory solution to this problem has yet been found.

Since chromatograph B was equipped with 0.25 in. tubing fittings, 0.125 in. O.D. stainless steel columns were modified for the use of 0.25 in. fittings by welding 0.25 in. O.D., 0.125 in. I.D. sleeves on each end. This conversion eliminated the use of reducing unions, minimized the amount of connective tubing before and after the column, and maintained on-column injection.

During our early work with triglyceride GLC, we often obtained a group of extraneous minor peaks resembling a homologous series of compounds between 200 and 260C. These peaks interfered with proper sample quantitation, especially at high signal amplification. They appeared even when no sample was injected and were largest immediately after the installation of a new septum. The extraneous peaks

were finally shown to originate in the septum. Solvent extraction of septums did not diminish the effect. The problem was finally eliminated by heating the septums overnight in a 250C oven prior to use.

Quantitation. A model mixture containing approximately equal weights of trioctanoin, tridecanoin, trilaurin, trimyristin, tripalmitin, and tristearin was used to evaluate the influence of various parameters on triglyceride GLC. A typical chromatogram is shown in Figure 1. Peak areas were measured by the standard triangulation method (16). Quantitative weight response factors (f_w) and molar response factors (f_m) for individual triglycerides were calculated by the internal normalization method (17,19):

$$f_w = \frac{\text{Weight \%}}{\text{Area \%}} \qquad f_m = \frac{\text{Mole \%}}{\text{Area \%}}$$

A value of 1.00 was assigned to f_w and f_m for trilaurin, so that the correction factors from all experiments would be comparable. Comparison of f_w values under different operating conditions gave an indication of sample recovery. The correction factors were also directly usable for the quantitative analysis of unknown triglyceride mixtures. If the area of each peak was multiplied by its respective f_w or f_m correction factor, then the relative weight or molar amounts represented by each peak were obtained.

Resolution. The resolution of triglyceride peaks by GLC was measured in terms of a value ΔC, defined as the minimum carbon number difference between two triglycerides which could be separated with baseline resolution in the C_{42} to C_{48} region of the chromatogram. This value ΔC gave a clear, easily understood picture of the separating capabilities of a given column under specific operating conditions. ΔC values were considered more useful for triglyceride GLC than the usual expressions for peak resolution (25) because: (a) triglyceride GLC peaks deviate widely from the pure Gaussian form (see below) upon which standard formulas are based; and (b) it is of more practical interest to define peak resolution in terms of "baseline resolution" rather than "band intercept resolution" (25).

The value of ΔC was calculated from trimyristin and tripalmitin peaks of approximately equal height using the measurements shown in Figure 2. The formula for ΔC was derived as follows:

$$w_{B42/48} = \frac{w_{B42} + w_{B48}}{2} \cong 1.6\,(w_{h42} + w_{h48})$$

$$M = \frac{\Delta t}{w_{B42/48}} = \frac{\Delta t}{1.6\,(w_{h42} + w_{h48})}$$

$$\Delta C = \frac{6}{M} = \frac{9.6\,(w_{h42} + w_{h48})}{\Delta t}$$

where

$w_{B42/48}$ = average baseline width of triglyceride peaks in the C_{42} to C_{48} region of the chromatogram.
w_{B42} = baseline peak width for trimyristin peak.
w_{B48} = baseline peak width for tripalmitin peak.
w_{h42} = peak width at half peak height for trimyristin.
w_{h48} = peak width at half peak height for tripalmitin.
M = maximum number of peaks which could be separated with baseline resolution in the C_{42} to C_{48} region of the chromatogram.
Δt = distance between the apexes of the trimyristin and tripalmitin peaks.
ΔC = minimum carbon number difference between two triglycerides which could be separated with baseline resolution in the C_{42} to C_{48} region of the chromatogram.

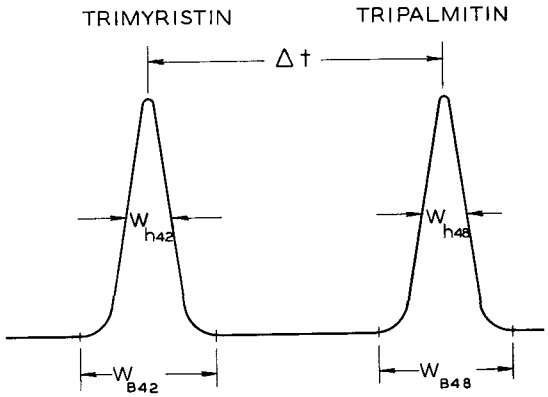

FIG. 2. Diagram of peak dimensions used to calculate peak resolution (ΔC). w_h = peak width at half peak height. w_B = baseline peak width.

To determine the number of peaks which could be resolved in the distance Δt, the average baseline peak width $w_{B42/48}$ in this region was calculated by averaging the baseline peak widths for trimyristin and tripalmitin. Since the peak width at half peak height was more reproducible and could be measured more accurately than baseline peak width, $w_{B42/48}$ was expressed in terms of w_{h42} and w_{h48}. Examination of over 50 different chromatograms run under widely varying conditions showed that this relationship was approximately $w_{B42/48} \cong 1.6\,(w_{h42} + w_{h48})$. This deviated substantially from the relationship one would have predicted if ideal Gaussian-shaped peaks were obtained (18); but it represented the actual relationship found with triglyceride peaks under typical experimental conditions. Once the average baseline peak width was known, the maximum number of peaks M which could be separated in distance Δt was obtained by dividing Δt by $1.6(w_{h42} + w_{h48})$. Since there was a difference of six carbon numbers between trimyristin and tripalmitin, it followed that the minimum carbon number difference between two triglycerides which could be separated with baseline resolution was 6/M. This value, ΔC, was then expressed in terms of w_{h42}, w_{h48}, and Δt so that it could be calculated directly from these distances on the chromatogram.

The value of ΔC was a more useful guide than the number of theoretical plates in determining whether a specific GLC column would yield a desired triglyceride separation under a given set of operating conditions. If we desired to separate triglycerides of carbon numbers 42, 44, 46, and 48, then a column with a ΔC value of 2.0 was needed. If triglycerides of carbon numbers 42, 43, 44, and 45 were to be separated, then a column with a ΔC value of 1.0 was needed. By comparing ΔC values under different operating conditions, optimum conditions for best resolution were determined.

For the present paper, all ΔC values were calculated for the C_{42} to C_{48} region of the chromatogram. In actual practice, ΔC values for the C_{48} to C_{54} region were of greater interest for the GLC of natural fats containing common C_{16} and C_{18} fatty acids. It was not possible in the current study, however, to obtain accurate w_{h54} and w_{B54} measurements on chromatograms where adverse experimental conditions caused

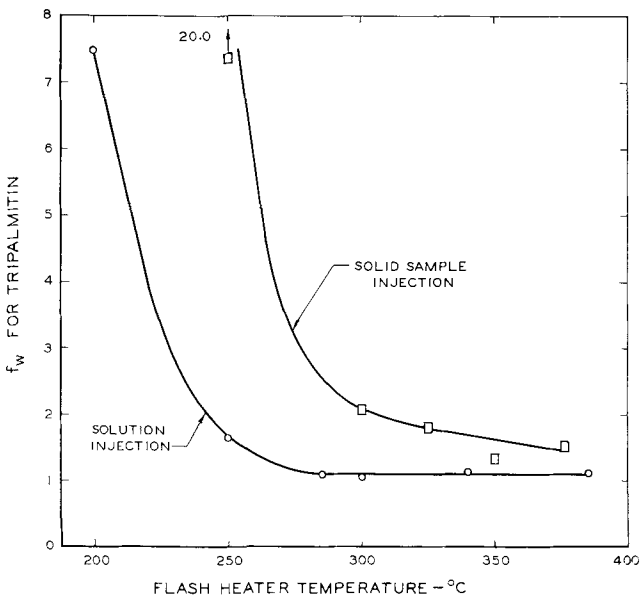

FIG. 3. Effect of flash heater temperature and type of injection on the quantitation of tripalmitin. Operating conditions: chromatograph A; 24 in. stainless steel column containing 2.7% SE-30 on 60/80 mesh acid washed Chromosorb W; 150 ml/min. N_2 carrier gas; oven temperature programmed at 2.5C/min.; other conditions as listed under Optimum Operating Conditions in Experimental section.

large losses of tristearin. Therefore, a ΔC in the C_{42}–C_{48} region was adopted as the uniform measurement of resolution.

Peaks for pure monoacid triglycerides were always slightly better resolved than the equivalent peaks for natural fat triglycerides. This peak broadening was undoubtedly due to the multiplicity of compounds eluting in each carbon number peak with natural fats.

Experimental

In our early work on GLC of triglycerides, we encountered high losses and poor resolution with molecules having carbon numbers above 42. In an effort to overcome these problems, we made a detailed study of the influence of various operating parameters on triglyceride quantitation and resolution as measured by f_w and ΔC values.

Flash Heater Temperature

The effect of varying the flash heater temperature from 200 to 385C on the f_w value for tripalmitin was determined (Fig. 3). When the sample was injected in CS_2 solution, the f_w for tripalmitin was constant (ca. 1.10) from 285 to 385C, but increased rapidly at flash heater temperatures below 285C. Injecting the triglyceride sample in solid form gave higher f_w values than solution injection at all flash heater temperatures, indicating substantial losses with solid sample injection.

TABLE I

Effect of Carrier Gas Flow Rate on f_w Values for Tripalmitin and Tristearin in Glass and Steel Columns[a]

Carrier gas flow rate (ml/min)	f_w			
	Tripalmitin		Tristearin	
	Glass column	Steel column	Glass column	Steel column
50	1.00	1.01	1.14	1.12
100	0.98	0.96	1.08	1.08
150	0.97	0.95	1.01	1.02
200	0.98	0.99	1.07	1.06

[a] Operating conditions: 24 in. column programmed at 3.0C/min.; other conditions as listed under Optimum Operating Conditions in Experimental section.

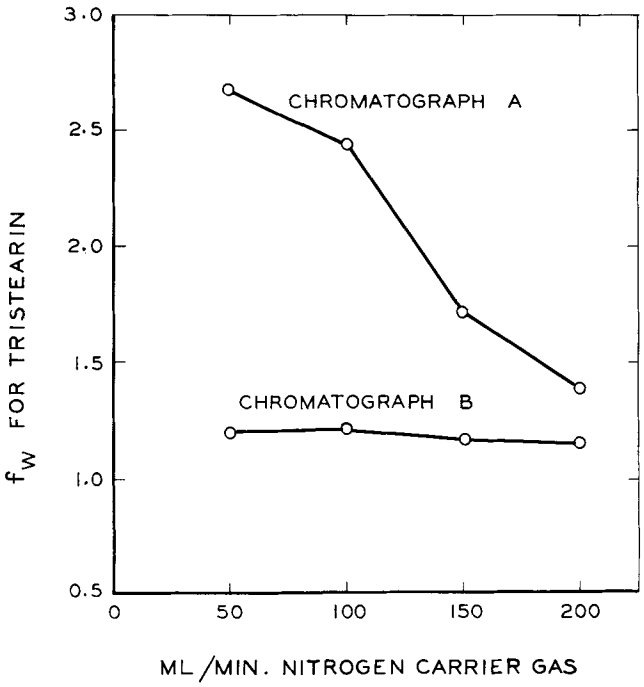

FIG. 4. Effect of carrier gas flow rate on the quantitation of tristearin. Operating conditions for chromatograph A: 24 in. stainless steel column containing 2.7% SE-30 on 60/80 mesh acid washed Chromosorb W; N_2 carrier gas; oven temperature programmed at 2.5C/min; other conditions listed under Optimum Operating Conditions in Experimental section. Operating conditions for chromatograph B: 24 in. stainless steel column programmed at 3.0C/min; N_2 carrier gas; other conditions as listed under Optimum Operating Conditions in Experimental section.

Carrier Gas Flow Rate

To determine the effect of carrier gas flow rate on the quantitation of high molecular weight triglycerides, the f_w values for tristearin were determined at 50, 100, 150, and 200 ml/min nitrogen flow on two different gas chromatographs (Fig. 4). On chromatograph A, the f_w for tristearin decreased sharply with increasing carrier gas flow. On chromatograph B, the f_w for tristearin was approximately constant at all four flow rates tested. When glass and steel columns were compared on chromatograph B, both showed equivalent f_w values that were almost independent of carrier gas flow rate (Table I).

Comparison of ΔC values for the different carrier gas flow rates showed that peak resolution improved when the carrier flow rate decreased (Fig. 5). This improvement was most pronounced on a steel column with a nitrogen carrier and rather small on a glass column with a helium carrier.

Type of Carrier Gas

The influence of the type of carrier gas on the quantitation of tripalmitin and tristearin was determined using nitrogen and helium on two different chromatographs (Fig. 6). With chromatograph A, f_w values with nitrogen carrier gas were considerably lower than those using helium. With chromatograph B, helium and nitrogen both gave equivalent f_w values for tripalmitin and tristearin.

Comparison of ΔC values for helium and nitrogen carrier gases (Fig. 5) showed that helium gave significantly better peak resolution than nitrogen at all flow rates tested.

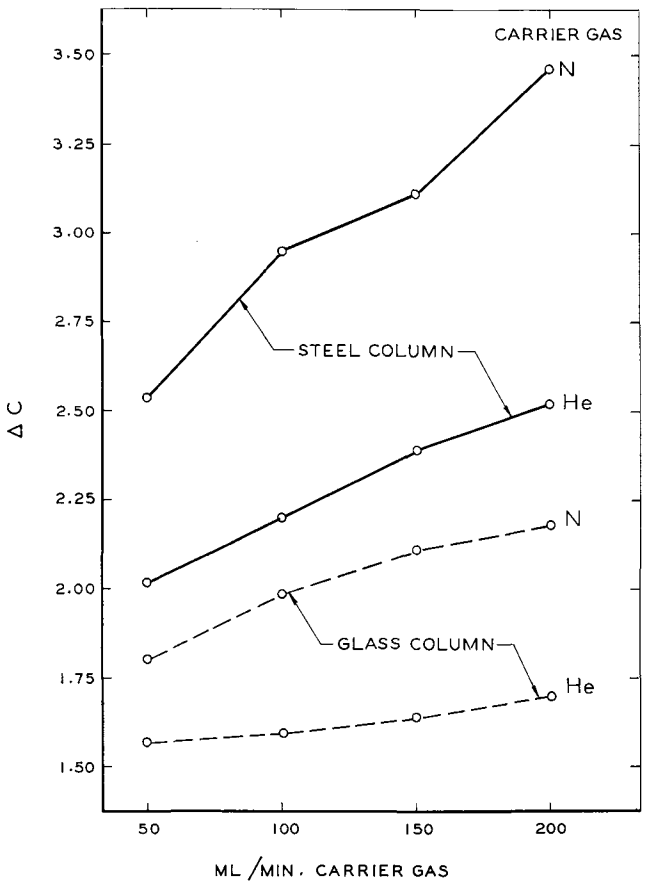

FIG. 5. Effect of carrier gas flow rate on peak resolution in glass and metal columns. Operating conditions: chromatograph B; 24 in. column programmed at 3.0C/min; other conditions as listed under Optimum Operating Conditions in Experimental section.

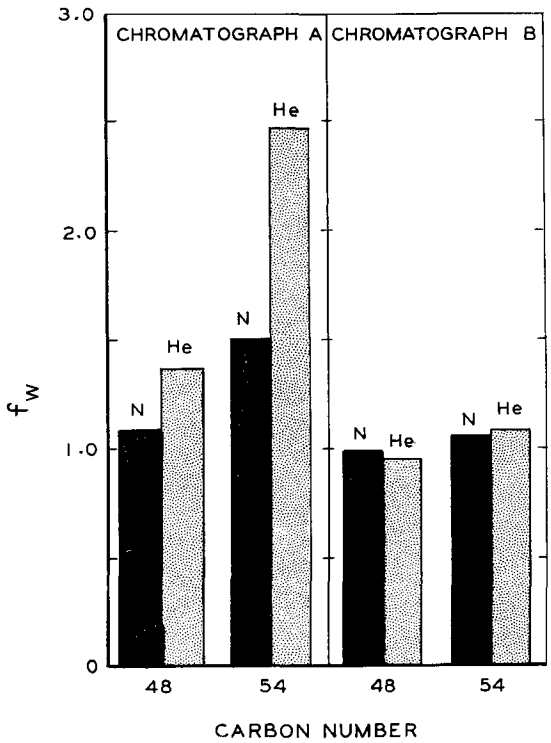

FIG. 6. Effect of type of carrier gas on the quantitation of tripalmitin and tristearin. Operating conditions for chromatograph A: 24 in. stainless steel column containing 2.7% SE-30 on 60/80 mesh acid washed Chromosorb W; oven temperature programmed 2.3C/min.; other conditions as listed under Optimum Operating Conditions in Experimental section. Operating conditions for chromatograph B: 24 in. stainless steel column programmed at 3.0C/min; other conditions listed under Optimum Operating Conditions in Experimental section.

Glass vs. Steel Columns

To determine if glass and stainless steel columns were equivalent for the GLC of high molecular weight triglycerides, they were compared on chromatograph B using both helium and nitrogen carrier gases at several flow rates (Table I). The f_w values for tripalmitin and tristearin were almost identical on both glass and steel columns. Comparison of the respective ΔC values, however, revealed that glass columns gave superior peak resolution under identical operating conditions (Fig. 5).

Column Length

The effect of column length on the quantitation and resolution of high molecular weight triglycerides was studied on chromatograph A using 6, 18, and 60 in. long columns (Fig. 7). The f_w values for tripalmitin and tristearin were both close to 1.00 on a short 6 in. column. Some loss was encountered on the 18 in. column, and large losses occurred on the 60 in. column. Tristearin losses were always greater than tripalmitin. As expected, ΔC values decreased significantly as the columns became longer.

Similar results were also obtained using chromatograph B with helium as the carrier gas.

Temperature Program Rate

The effect of temperature program rate on the quantitation and resolution of high molecular weight triglycerides was investigated on chromatograph B. With a 24 in. steel column under optimum operating conditions (see below), the f_w values for tristearin were 1.02 at 1.0C/min, 1.03 at 2.0C/min, and 1.01 at 4.0C/min. Varying the program rate had no effect on the quantitative recovery of tristearin.

Comparison of the ΔC values at different program rates on glass and steel columns revealed that slower program rates gave significantly better peak resolutions with steel columns (Fig. 8). With glass columns, however, peak resolution was independent of program rate over the range of conditions studied.

Linearity of Detector Response

The linearity of the hydrogen flame detector response for trilaurin and tristearin was evaluated using a standard calibration mixture (Fig. 1) containing 18.0 wt % trilaurin and 17.9 wt % tristearin. The resulting plot of peak area vs. amount of trilaurin injected (Fig. 9, top) was linear from 0–45 μg of triglyceride. Even when the 0–5 μg region was expanded to a larger scale, the plot was linear and passed through the origin. A similar plot of peak area vs. amount of tristearin injected (Fig. 9, bottom) was equally linear in the 0–20 μg range, but showed a different slope in the 23–45 μg range. Since under normal operating conditions a full scale tristearin peak represented only 10–20 μg of triglyceride, this nonlinearity could only occur on overloaded columns and represented no problems in normal analyses.

Optimum Operating Conditions

Optimum conditions for the GLC of triglycerides were chosen based on the experimental results reported above. For best results, a gas chromatograph designed for steroid analysis and equipped with a hydrogen flame detector and temperature programming was used. Triglyceride peaks of even carbon number were easily resolved using a 24 in. long, 2.5–3.0 mm. I.D.

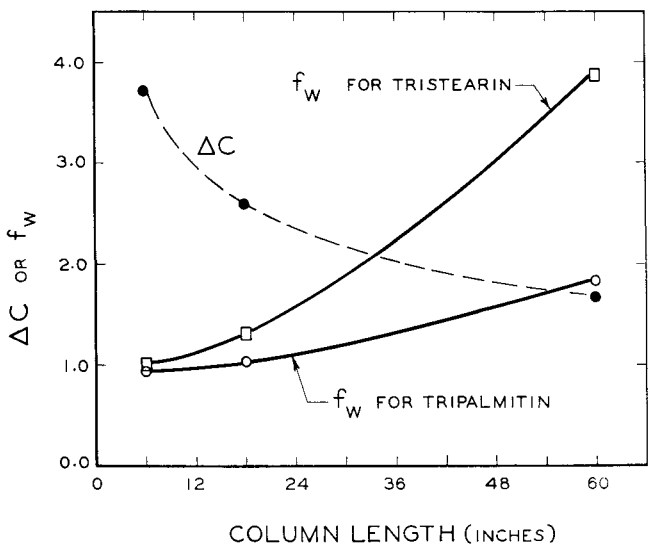

FIG. 7. Effect of column length on peak resolution and quantitation. Operating conditions: Chromatograph A; 6, 18, and 60 in. stainless steel columns programmed at 1.7C/min; 125 ml/min. N_2 carrier gas; other conditions as listed under Optimum Operating Conditions in Experimental section.

glass or stainless steel column packed with 3.0% JXR on 100/120 mesh Gas Chrom Q. The column was programmed from 170 to 325C at 2–4C/min with 100 ml/min helium carrier gas flow. The flash heater was maintained at 320–350C and the detector base at 300–340C. Under these conditions, 10–20 μg of triglyceride produced a full scale peak on a 1 mv recorder. The reasons for choosing these optimum conditions are outlined in the Discussion section.

Applications

The separation of a model mixture of monoacid saturated triglycerides under optimum operating conditions (Fig. 1) showed excellent resolution in the C_{42} to C_{54} region ($\Delta C = 1.6$). The f_w and f_m calibration factors for 16 different monoacid triglycerides were determined using optimum conditions (Table II).

Cocoa butter and rat adipose tissue fat, two natural fats containing only high molecular weight triglycerides, were analyzed by GLC. The resultant chromatograms showed excellent peak resolution allowing accurate quantitation (Fig. 10 and Table III). Calculation of the average fatty acid chain length from both the triglyceride and fatty acid compositions (Table III) using the method of Kuksis et al. (10) showed close agreement between the two types of analyses.

A mixture of C_{45}, C_{46}, C_{47}, and C_{48} triglycerides was separated with good resolution on a 60 in. column

TABLE II

Typical Calibration Factors for Monoacid Triglycerides Using Optimum Operating Conditions [a]

Triglyceride	Carbon number	Double bonds	f_w	f_m
Trioctanoin	24	0	1.12	1.52
Tridecanoin	30	0	1.04	1.20
Trilaurin	36	0	1.00	1.00
Trimyristin	42	0	0.96	0.85
Tripalmitin	48	0	0.98	0.78
Tripalmitolein	48	3	1.01	0.81
Trimargarin	51	0	1.05	0.79
Tristearin	54	0	1.09	0.78
Triolein	54	3	1.03	0.74
Trielaidin	54	3	1.06	0.76
Trilinolein	54	6	1.10	0.80
Trilinolenin	54	9	1.12	0.82
Triarachidin	60	0	1.21	0.79
Tri-11-eicosenoin	60	3	1.10	0.73
Tribehenin	66	0	1.43	0.86
Trierucin	66	3	1.34	0.81

[a] Chromatograph B with stainless steel column programed at 3C/min. Other conditions as listed under Optimum Operating Conditions in Experimental section.

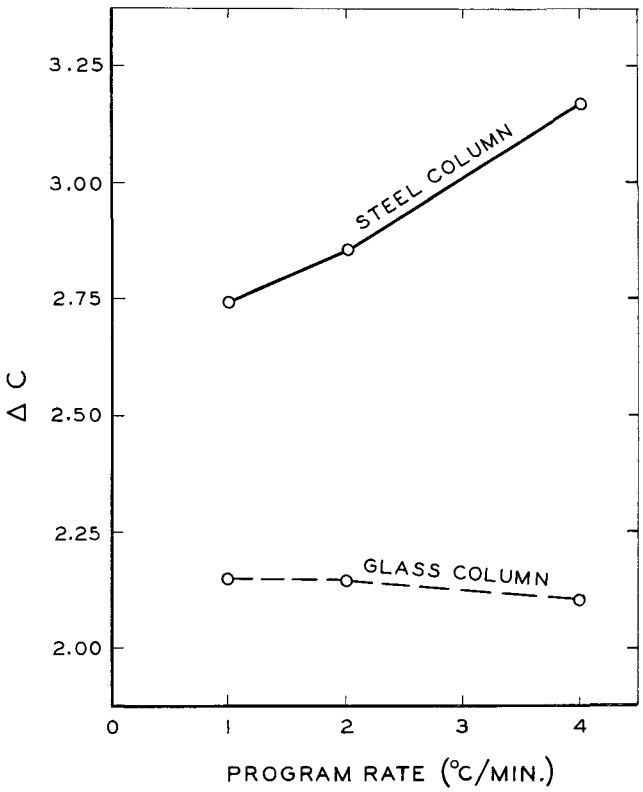

FIG. 8. Effect of program rate on peak resolution. Operating conditions: chromatograph B; 24 in. glass column; N_2 carrier gas; other conditions as listed under Optimum Operating Conditions in Experimental section.

(Fig. 11). However, this high resolution on a long column was gained only at the expense of significant component loss (Fig. 7).

Discussion

Optimum Operating Conditions

Optimum operating conditions for accurate quantitation and resolution of high molecular weight triglycerides were selected based on the experimental data described above. These conditions are given in the Experimental section above and were chosen as outlined below.

It is evident from Figures 4 and 6 that the f_w values for high molecular weight triglycerides were not always the same on different gas chromatographs, even under equivalent operating conditions. Therefore, choice of the proper instrument is important for best results. Chromatograph B consistently gave lower f_w values than chromatograph A, and the calibration factors on instrument B were not sensitive to changes in the type and flow rate of the carrier gas (Fig. 4 and 6). Chromatograph B was specifically designed for the high temperature GLC of C_{18}–C_{35} steroids, and included such design features as on-column injection, an absolute minimum of connective tubing between the column and detector, and minimal "dead spaces" in the injection port to minimize component loss, thermal decomposition, and peak tailing. Since both steroids and triglycerides are high molecular weight compounds, it appears likely that those chromatograph design parameters which favor steroid GLC would also be desirable for triglyceride GLC. For this reason, a gas chromatograph designed for steroid analysis is recommended for triglycerides, even though thorough comparative studies of the various design features have not been carried out.

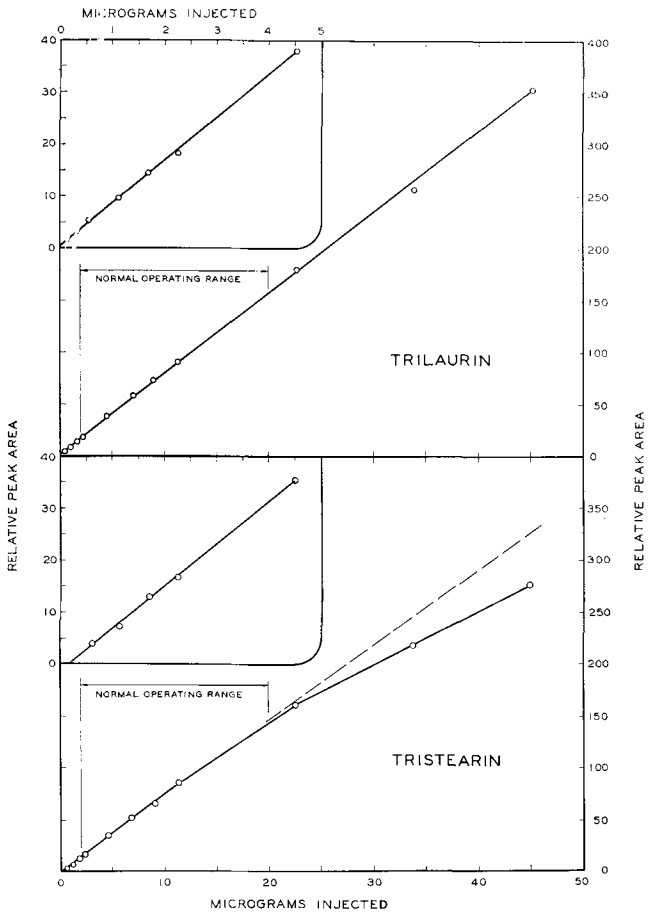

FIG. 9. Linearity of detector response for trilaurin and tristearin. Operating conditions: chromatograph B; 24 in. stainless steel column programmed at 3.0C/min; N_2 carrier gas; other conditions as listed under Optimum Operating Conditions in Experimental section.

A hydrogen flame detector was used for triglyceride GLC because it is more sensitive to carbon compounds than a thermal conductivity detector and less sensitive to silicone bleed than an argon ionization detector.

Columns packed with 3.0% JXR or SE-30 on 100/120 mesh Gas Chrom Q were quite effective for triglyceride work. Undoubtedly other similar supports could prove equally effective. In our experience, unsilanized and silanized supports gave equivalent calibration factors under equivalent operating conditions. A 24 in. long by 2.5–3.0 mm I.D. column of glass or stainless steel proved successful for separating natural fat triglycerides having carbon numbers 48, 50, 52, and 54 (Fig. 10). Shorter columns gave insufficient resolution while longer columns resulted in greater component loss for high molecular weights (Fig. 7). Kuksis (11) has reported similar large losses with 72 in. columns.

Glass columns were best for maximum resolution (Fig. 5), but their frequent breakage when changing O-ring seals (see Methods) made steel columns more practical for routine use. Apparently stainless steel exhibits a marked adsorption effect with triglycerides but glass does not. This adsorption effect decreases peak resolution, but appears to be reversible since f_w values for tristearin are the same on both glass and steel columns (Table II).

Helium carrier gas gave significantly better peak resolution than nitrogen (Fig. 5) with equivalent calibration factors on chromatograph B (Fig. 6). A standard carrier flow rate of 100 ml/min of helium

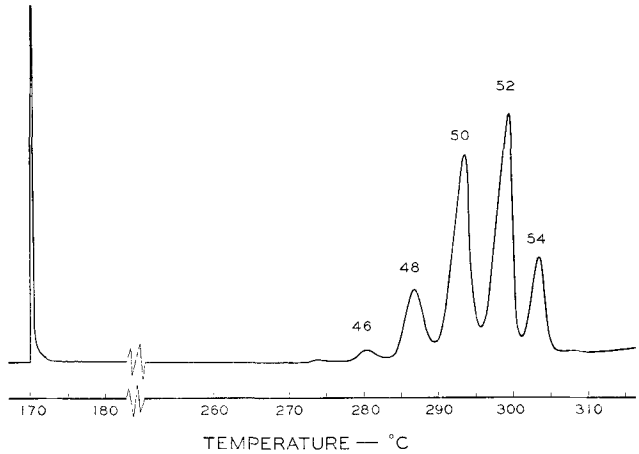

FIG. 10. Chromatogram of rat adipose tissue triglycerides. Operating conditions: 24 in. glass column programmed at 4.0C/min; N_2 carrier gas; other conditions as listed under Optimum Operating Conditions in Experimental section.

was selected as low enough for best peak resolution and high enough for good peak height with minimum sample size. The reason for superior peak resolution with helium is not completely understood. Barr and Sawyer (20) have reported that helium also gave better peak resolution than nitrogen in the GLC of 3-pentanone.

A flash heater temperature between 320 and 350C assured efficient vaporization of triglycerides injected in solvent solution (Fig. 3) without subjecting the sample to higher temperatures than necessary. Column temperature was programmed from 170 to 325C for the standard calibration mixture, but the starting temperature could be increased to 200C when no triglycerides below C_{36} were present. A slow programming rate (2C/min) was used with steel columns for best resolution (Fig. 8), while glass columns could be programed at 4C/min without loss of resolution. Tristearin usually eluted between 290 and 310C, the exact temperature depending on the age of the column and the specific operating conditions. The detector base block was always maintained at 300 to 340C to minimize any effect of large temperature variations on detector sensitivity.

To obtain maximum peak resolution, electrometer attenuation was turned up as far as practical (until baseline noise or baseline rise at higher temperatures became objectionable), and sample size was kept as small as possible. With chromatograph B at optimum

TABLE III

Triglyceride and Fatty Acid Compositions of Cocoa Butter and Rat Adipose Tissue Fat

Triglycerides	Cocoa Butter	Rat adipose tissue fat
	mole %	mole %
46	—	1.8
48	—	12.3
50	16.0	34.3
52	45.6	37.6
54	37.4	14.0
56	1.0	—
Fatty acids		
12:0	—	0.1
14:0	0.2	2.7
14:1	—	0.4
16:0	28.7	31.9
16:1	tr	18.3
18:0	35.0	1.8
18:1	32.0	44.5
18:2	3.3	0.3
20:0	0.8	—
Average fatty acid chain length		
From triglyceride data	17.49	17.00
From fatty acid data	17.43	16.86

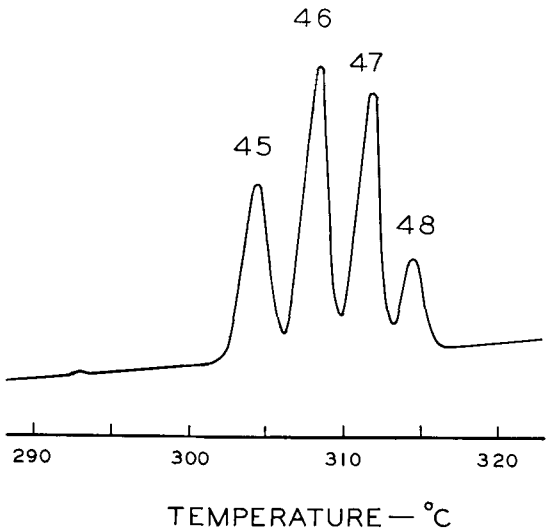

FIG. 11. Chromatogram of a mixture of C_{45}, C_{46}, C_{47}, and C_{48} triglycerides. Operating conditions: chromatograph A; 60 in. stainless steel column programmed at 1.7C/min; 125 ml/min. N_2 carrier gas; other conditions as listed under Optimum Operating Conditions in Experimental section.

operating conditions, about 10–20 μg of triglyceride was required to produce a full scale peak on a 1 mv recorder. Detector response was shown to be directly proportional to sample size in this range (Fig. 9).

We have not found it necessary to perform a lengthy "thermal stripping" of new columns in order to obtain accurate quantitative results as Kuksis (11) has reported. After 2 hr of conditioning at 350C and an initial 2–6 trial analyses, new columns gave typical low calibration factors for tripalmitin and tristearin. In one instance, however, a very tightly packed new column loaded by the suction technique did show high initial f_w values for tristearin, and they decreased significantly with further conditioning. This may indicate that longer conditioning is required for more tightly packed columns to avoid loss of high molecular weight triglycerides.

Calibration Factors

Theoretically, the flame detector calibration factors for triglycerides should be approximately equivalent to the calibration factors for the corresponding methyl esters. A triglyceride molecule can be thought of as three methyl ester molecules bound together. For example, one molecule of tristearin has the same atomic composition and the same number of C–C, C–H, and C–O bonds as one molecule of methyl stearate plus two molecules of methyl oleate; therefore, they should both have approximately equivalent detector responses.

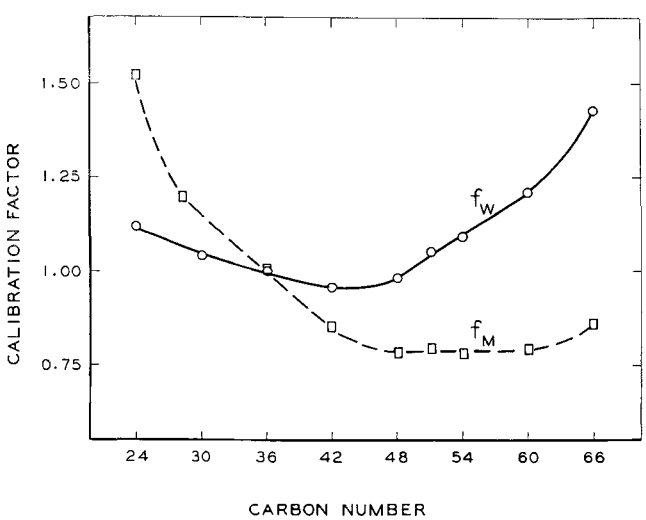

FIG. 12. Variation of calibration factors with carbon number for saturated, monoacid triglycerides. Operating conditions: chromatograph B with stainless steel column programmed at 3.0C/min. Other conditions as listed under Optimum Operating Conditions in Experimental section.

For a hydrogen flame detector, the plot of f_w vs. carbon number for long chain fatty acid methyl esters approximates a horizontal line (21,22) which has a slight negative slope (23). This negative slope is attributed to the decreasing weight percent of oxygen as the carbon number increases (23). A plot of f_w vs. carbon number for the triglycerides of long chain fatty acids should theoretically have the same shape, assuming that all of the injected sample reaches the flame detector. On the other hand, as the molecular weight of compounds in a homologous series increases, volatility decreases. At some point the molecules become so large and so nonvolatile that they may enter the GLC column as a mist rather than a vapor, and thus be unable to participate in the gas-liquid partitioning effect necessary for GLC. One would expect this problem with high molecular weight triglycerides.

The f_w calibration factors for saturated monoacid triglycerides chromatographed under optimum conditions have been plotted vs. carbon number in Figure 12. It is evident that the f_w plot approximates a horizontal line with a slight negative slope only from C_{24} through C_{42}. Above C_{42}, the plot has a positive slope which apparently increases with increasing carbon number. We conclude that there are no losses of injected triglycerides up through C_{42}, since the actual plot resembles the theoretical in this region. Above C_{42}, the positive slope indicates that some sample loss does occur; and the higher the molecular weight the greater the loss. The linear detector response obtained for 0–20 μg of tristearin in Figure 9, even at sample sizes below 2 μg, indicates that this loss is constant and is approximately proportional to the amount of sample injected. Thus, the loss can be accurately compensated for with calibration factors, as Jurriens and Kroesen (13) have reported.

Comparison of the f_w values for saturated and unsaturated triglycerides of equal carbon number (Table II) indicates that unsaturated triglycerides can also be quantitatively analyzed by GLC. We have not encountered the severe degradation of unsaturated molecules reported by Jurriens and Kroesen (13). However, not all triglycerides of the same carbon number have the same f_w value. Tripalmitin and tripalmitolein have approximately equal calibration factors. So do tristearin, trielaidin, trilinolein, and trilinolenin; but triolein may have a slightly lower f_w value. Tri-11-eicosenoin has a lower calibration value than triarachidin; and trierucin shows a lower loss than tribehenin. This may indicate a tendency for unsaturated triglycerides of carbon numbers 60 and 66 to show lower losses than their corresponding saturated compounds.

McCarthy, Kuksis, and Beveridge (4,7) have reported that peak area was proportional to component weight for trilaurin, trimyristin, tripalmitin, and tristearin using a hydrogen flame detector. Their average f_w values were (our calculation): $C_{36} = 1.00$; $C_{42} = 1.14$; $C_{48} = 1.10$; $C_{54} = 1.14$. These calibration factors are in the same range as those reported in Table II. However, the use of actual f_w calibration factors, rather than approximating weight % equal to area %, should yield more accurate analytical results.

In actual practice, triglyceride compositions are usually determined in mole percent values. A plot of f_m vs. carbon number is made for the saturated monoacid triglycerides and the appropriate curve is drawn (Fig. 12). The f_m values for mixed-acid triglycerides (i.e. C_{40}, C_{50}, C_{52}, etc.) are read from the graph. All saturated triglycerides of the same carbon number are assumed to have the same f_m value. Where the f_m values for saturated and unsaturated triglycerides of the same carbon number are different, an average f_m value is assigned to each peak based on its estimated fatty acid composition.

The above considerations emphasize that *accurate calibration is essential for quantitative GLC of triglycerides.* Since calibration factors vary substantially with the operating conditions and the chromatograph used, the f_m and f_w values given in Table II do not necessarily apply to other laboratories. Even with the same instrument, column, and operating conditions, we have found that calibration factors vary slightly over a period of weeks.

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REFERENCES

1. Fryer, F. H., W. L. Ormand and G. B. Crump, JAOCS *37*, 589 (1960).
2. Huebner, V. R., *Ibid. 36*, 262 (1959).
3. Huebner, V. R., *Ibid. 38*, 628 (1961).
4. Kuksis, A., and M. J. McCarthy, Can. J. Biochem. Physiol. *40*, 679 (1962).
5. Martin, A. J., C. E. Bennett and F. W. Martinez, Jr. Paper presented at 3rd Symposium on Gas Chromatography, June 1960, Edinburgh, Scotland.
6. Pelick, N., W. R. Supina and A. Rose, JAOCS *38*, 506 (1961).
7. McCarthy, M. J., A. Kuksis and J. M. R. Beveridge. Can. J. Biochem. Physiol. *40*, 1693 (1962).
8. Kuksis. A., and M. J. McCarthy, JAOCS *41*, 17–21 (1964).
9. Kuksis, A., M. J. McCarthy and J. M. R. Beveridge, *Ibid. 41*, 201 (1964).
10. Kuksis, A., M. J. McCarthy and J. M. R. Beveridge, *Ibid. 40*, 530 (1963).
11. Kuksis, A., *Ibid 42*, 269 (1965).
12. Youngs, C. G., and M. R. Subbaram, *Ibid. 41*, 218 (1964).
13. Jurriens, G., and A. C. J. Kroesen, *Ibid. 42*, 9 (1965).
14. Bottino, N. R., and Raymond Reiser. In preparation.
15. Bourne, E. J., M. Stacey, J. C. Tatlow and J. M. Tedder, J. Chem. Soc. —2976 (1949).
16. Burchfield, H. P., and E. E. Storrs. "Biochemical Applications of Gas Chromatography," Academic Press, New York, 1962, p. 122.
17. *Ibid.* pp. 117–121.
18. Purnell, H., "Gas Chromatography," Wiley, New York, 1962, p. 105.
19. Kaufmann, H. P., A. Seher and G. Mankel, Fette Seifen Anstrichmittel *64*, 501 (1962).
20. Barr, J. K., and D. T. Sawyer, Anal. Chem. *36*, 1753 (1964).
21. Ettre, L. S., and F. J. Kabot, J. Chromatog. *11*, 114 (1963).
22. Moore, J. L., T. Richardson and C. H. Amundson, J. Gas Chromatog. *2*, 318 (1964).
23. Ackman, R. G., and J. C. Sipos, JAOCS *41*, 377 (1964).
24. Burchfield, H. P., and E. E. Storrs, "Biochemical Applications of Gas Chromatography," Academic Press, New York, 1962, p. 45.
25. Ettre, L. S., J. Gas Chromatog. *1* (2), 36 (1963).

Determination of Hydroxy-Acid Triglycerides and Lactones in Butter[1]

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Abstract

The free and esterified ("bound") hydroxy acids from butterfat were isolated by column- and thin-layer chromatography on silica. The 4- and 5-hydroxy acids forming the γ- and δ-lactones were separated from the other hydroxy acids by TLC on silica. The concentrations of a number of free and bound lactones in butterfat were determined by means of a radio-gas chromatograph using the isotope dilution method. In addition the total concentration of the free and bound hydroxy acids which cannot be lactonized, was determined.

Introduction

Lactones are important flavor components in various natural products. Boldingh and Taylor (1) established the presence in butter of various δ-lactones and also, though in smaller amounts, of γ-lactones. By means of the isotope dilution technique they determined the amounts of δ-octalactone, δ-decalactone, δ-dodecalactone and δ-tetradecalactone. The concentrations of these lactones in butter strongly depend on the time of year and range from about 1 to 40 ppm. Their amounts are increased if butter is heated at 140C, which these authors explained by postulating that butterfat contains esterified 4- and 5-hydroxy acids (bound lactones) in the form of monohydroxyacyl triglycerides.

In a short communication (2) the observations which would substantiate the presence of these compounds in butterfat, are described.

In this paper the quantitative determination of the absolute amounts of "free" lactones as well as those of the "bound" lactones will be described. To check the correctness of the method of analysis, the total amounts of lactones have also been determined. For these determinations the isotope dilution technique (3) has been used.

Experimental Procedure for the Isolation of Lactones from Butterfat

Materials and Methods

The solvents diethyl ether, light petroleum (bp 40–60C), iso-octane (ex Shell) and chloroform (ex Merck analytical grade) were all freshly distilled; benzene (ex Merck) was analytical grade and used as such.

The butterfat in a commercial packet of Dutch butter (250 g) was extracted by addition of 250 ml light petroleum and separation of the water and petroleum layers by centrifugation. The petroleum layer was filtered to remove undissolved components and evaporated at low temperature and reduced pressure.

Silica columns (length 30 cm, diameter 2 cm) were used, made from 30 g silicic acid (ex Mallinckrodt, containing 6% physically bound water) and 15 g Hyflo (dried overnight at 120C). Thin-layer plates with a thickness of 0.25 mm were prepared by mixing 60 g silicagel G (ex Macherey and Nagel) with 120 ml water and spreading the slurry on glass plates of

[1] Presented at AOCS meeting in Houston, Texas, 1965.
† Died September 4, 1965.