A COMPLEMENTARY THIN LAYER AND GAS–LIQUID CHROMATOGRAPHIC PROCEDURE FOR FATTY ACID ANALYSIS

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Methoxymercuration has proved useful in magnifying differences in properties of fatty unsaturates before attempting to fractionate mixtures according to the number of double bonds. Derivative preparation is usually accomplished through reaction with mercuric acetate in methanol. If need be, the original unsaturates can easily be gained by acid treatment of the derivatized compounds subsequent to the fractionation[1,2].

Chromatographic applications with the methoxy, acetoxymercuri-adducts have included the separation of fatty acids as their methyl or allyl esters[3,4], triglycerides[5], and phospholipids[6] on paper. Column chromatography has been used to separate the saturated and unsaturated esters into groups[7] and to further isolate the individual unsaturated classes[8–11]. A thin layer chromatographic method is available for the isolation of saturates, monoenes, dienes, and trienes individually and more unsaturated members collectively[12]. Resolution of the latter group also by thin layer chromatography was achieved in a report which appeared during the course of this investigation[13].

The present work describes the complementary use of TLC and GLC to define fatty ester unsaturation content and chain length. Unsaturates were converted to their methoxy, bromomercuri-adducts and separated by TLC on the basis of number of double bonds. Following decomposition of the derivatives, members of each unsaturation class were separated by GLC according to chain length.

EXPERIMENTAL

Reagents and apparatus

After various purification measures all solvents were distilled before use. Methanol was refluxed over zinc dust and potassium hydroxide for 3 h. *n*-Heptane and *n*-hexane were passed twice through columns of silica gel, washed with sulfuric acid until no color was present, washed with water and dried over anhydrous sodium sulfate. Dioxane was refluxed with sodium for 5 h.

Thin layer chromatography (TLC) was effected on layers of silica gel G approximately 250 μ thick coated on 20 × 20 cm plates. Chromatograms were run by ascending technique. To remove contaminating matter the coated layer was washed with

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methanol–ethyl ether (80:20, v/v)[14]. Plates following activation at 110° for 1 h were stored in a dry atmosphere.

Gas chromatographic analyses were done on a 4 ft. × 3 mm I.D. Pyrex glass tube packed with 5 % polydiethyleneglycolsuccinate (LAC-728) coated on Diatoport S 80-100 mesh. On column injections of 5–20 μg of mixture were made. Operating conditions were helium flow rate of 75 ml/min, temperature programming from 100° to 210° at 3°/min, and 400 to 800 attenuation of signal. A flame ionization detector was used.

Highly purified fatty acid methyl ester standards were obtained from the National Heart Institute and the Hormel Institute. A check by gas chromatography showed all samples to be within the limits of purity stated.

Adduct preparation

The reference mercury addition compounds for TLC were made by refluxing the fatty ester standard (20 mg) for 1 h at 80° in 1–2 ml methanol containing mercuric acetate in 20 % excess of the mequiv. of unsaturated compound present. Mercuric acetate was added as a 0.25 *M* methanolic solution stabilized with a trace of acetic acid. Solutions were not kept longer than 3 days. No difference in degree of adduct formation or performance in the TLC systems was noted when the mercuric acetate was added in solid form.

Following the addition of NaBr–methanol (5:95, w/v) in 10 % excess to the addition reaction mixture dissolved in 10 ml chloroform, the solution was stirred on a Vortex mixer for 30 sec. Water in an amount equal to twice the total volume of methanol present was added and the solution stirred again. The white turbid mixture resulting on addition of water usually separated into two clear phases within 5 min. In the more unsaturated samples, however, additional chloroform and, with docosahexaenoate adduct, centrifugation for a brief period was required for clearing. The chloroform layer was extracted with water repeatedly until completely clear. If not used immediately the mercury derivatives were stored in chloroform at 5° in the dark.

Adduct decomposition

Silica gel bands containing the ester components and diphenylcarbazone used for detection of organomercurials[15] were scraped off the thin layer plates into tubes containing 5 ml HCl–methanol (2:4, v/v). The mixture was swirled with a Vortex mixer and allowed to stand for 5 min. Addition of pentane (5 ml) and water (10 ml) was followed by thorough mixing and removal of the hypophase. One other water wash preceded the three-fold extraction of the hydrocarbon epiphase with 1 % aqueous potassium hydroxide solution. The latter treatment was found necessary for complete removal of indicator. Two more washes with water were followed by drying the pentane layer with anhydrous sodium sulfate and solvent removal at 0° under a gentle stream of nitrogen.

RESULTS

Oleate, elaidate adduct study

The particular alcohol used as reaction solvent influenced the completeness of addition compound formation. Methanol was found best in a series of C_1 through C_4

straight and branched chain monohydric alcohols for promoting adduct synthesis from methyl oleate and elaidate (Table I). Exemplary of the speed of reaction in this solvent was the near completion of double bond saturation achieved in 3 min at 80°. Increased length and branching of the alcohol chain slowed the reaction.

TABLE I

DERIVATIZATION OF OLEATE AND ELAIDATE

Reaction conditions			*Reaction completion**	
Time (*h*)	*Temp.* (°C)	*Solvent*	*Oleate* (%)	*Elaidate* (%)
0.05	80	Methanol	97	96
0.5	80	Methanol	100	100
0.5	80	Ethanol	67	63
0.5	80	*n*-Propanol	54	36
0.5	80	Isopropanol	33	22
0.5	80	*n*-Butanol	44	28
0.5	80	*tert.*-Butanol	23	9
0.5	30	Methanol	98	77
24	30	Methanol	100	96

* To 60 mg sample was added 1 ml 0.25 *M* mercuric acetate in the solvent shown. Following reaction 0.2 g sodium bromide was added and the mixture titrated with standard base[16].

A comparison of the effect of geometrical isomerism on mercuration rate showed that under the different reaction conditions studied the *cis* form methoxymercurated faster than the *trans*. In the one exception to this conclusion seen in Table I analyses were made too late to detect any difference. Experimental variables that tended to slow the reaction accentuated the difference in performance of the *cis*, *trans* isomers.

Methoxymercuration

Methyl esters of fatty acids containing 1 to 6 double bonds reacted readily with mercuric acetate in methanol to form the addition compounds in high yield (Table II).

TABLE II

EXTENT OF MERCURATION

*Fatty acid**	% *Reaction*
18:0	0.0
11:1[10]	97.6
cis-18:1[9]	100.0
trans-18:1[9]	99.6
22:1[13]	97.8
18:2[9,12]	99.0
18:3[9,12,15]	97.0
20:4[5,8,11,14]	94.7
20:5[5,8,11,14,17]	95.8
22:6[4,7,10,13,16,19]	95.1

* Fatty acids are expressed as numbers describing in order the chain length, number of double bonds, and position of unsaturation, the latter as superscripts[17].

The possibility of reactions other than the saturation of double bonds resulting in the production of titratable acidity was dispelled by the negative result with methyl stearate. High mercuration yields obtained with oleate, linoleate, and linolenate, all greater than 99 % pure, showed a slightly downward trend with increasing unsaturation. The reaction with arachidonate, eicosapentaenoate, and docosahexaenoate, all greater than 90 % pure, averaged 95 % completion. Double bond position in the monounsaturates, whether terminal or internal, as illustrated by 10-undecenoate and 13-docosaenoate, respectively, did not affect yield.

Thin layer chromatography

A clear separation by TLC of the methoxy, acetoxymercuri-derivatives of methyl oleate (R_F 0.80), linoleate (R_F 0.54), and linolenate (R_F 0.30) was obtained using the developing system hexane–dioxane–acetic acid (60:40:5, v/v/v). The solvent traveled 14 cm from the origin in 1 h at room temperature. Linolenate and arachidonate adducts did not completely separate under these conditions.

From the standpoint of fractionating fatty acid methyl esters containing 3 to 6 double bonds the methoxy, bromomercuri-addition compounds proved a better choice than the more polar methoxy, acetoxymercuri-derivatives discussed above. In Fig. 1

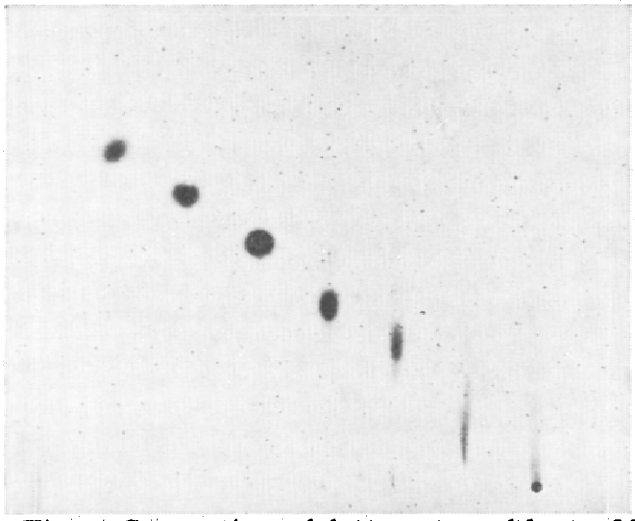

Fig. 1. Separation of fatty ester adducts. Methyl stearate (upper left) and, in progressive order, oleate, linoleate, linolenate, arachidonate, eicosapentaenoate, and docosahexaenoate (lower right). Amount of each ester: approximately 5 μg exclusive of addition components.

can be seen the results obtained for methyl stearate (R_F 0.73), oleate (R_F 0.63), linoleate (R_F 0.47), linolenate (R_F 0.30), arachidonate (R_F 0.19), eicosapentanenoate (R_F 0.07), and docosahexaenoate (R_F 0.01) in a chromatogram run 15 cm from the origin in 1 h 15 min at room temperature using the developing system heptane–dioxane (60:40, v/v). Although the sequence of components on different chromatograms never changed, variation in their R_F values was noted. Single spots were seen for methyl stearate, oleate, and linoleate. Small spots representing either a more saturated impurity or incompletely derivatized sample were found immediately above the much larger spots for methyl linolenate and arachidonate. The pentaene sample streaked more than the other standards. Docosahexaenoate containing 94.0 % hexaene, 5.7 % pentaene, and 0.3 % tetraene showed the pentaene contaminant clearly.

Complete resolution of a mixture containing approximately equal amounts (5 μg) of the standard esters with 0 through 6 double bonds was difficult to obtain when the sample was spotted with a micropipette or microsyringe. The triene, tetraene adduct separation, in particular, was sensitive to the degree of activation and uniform thickness of the thin layer. Pentaenoic and hexaenoic samples were not always separable.

Consistently superior results were obtained by placing the sample on the chromatoplate as a continuous thin line without cutting through the adsorbent with an applicator[18] designed for this purpose rather than as discrete spots with a microsyringe. A typical example of the separation achieved with a synthetic mixture containing 18 standard fatty acid methyl esters is seen in Fig. 2.

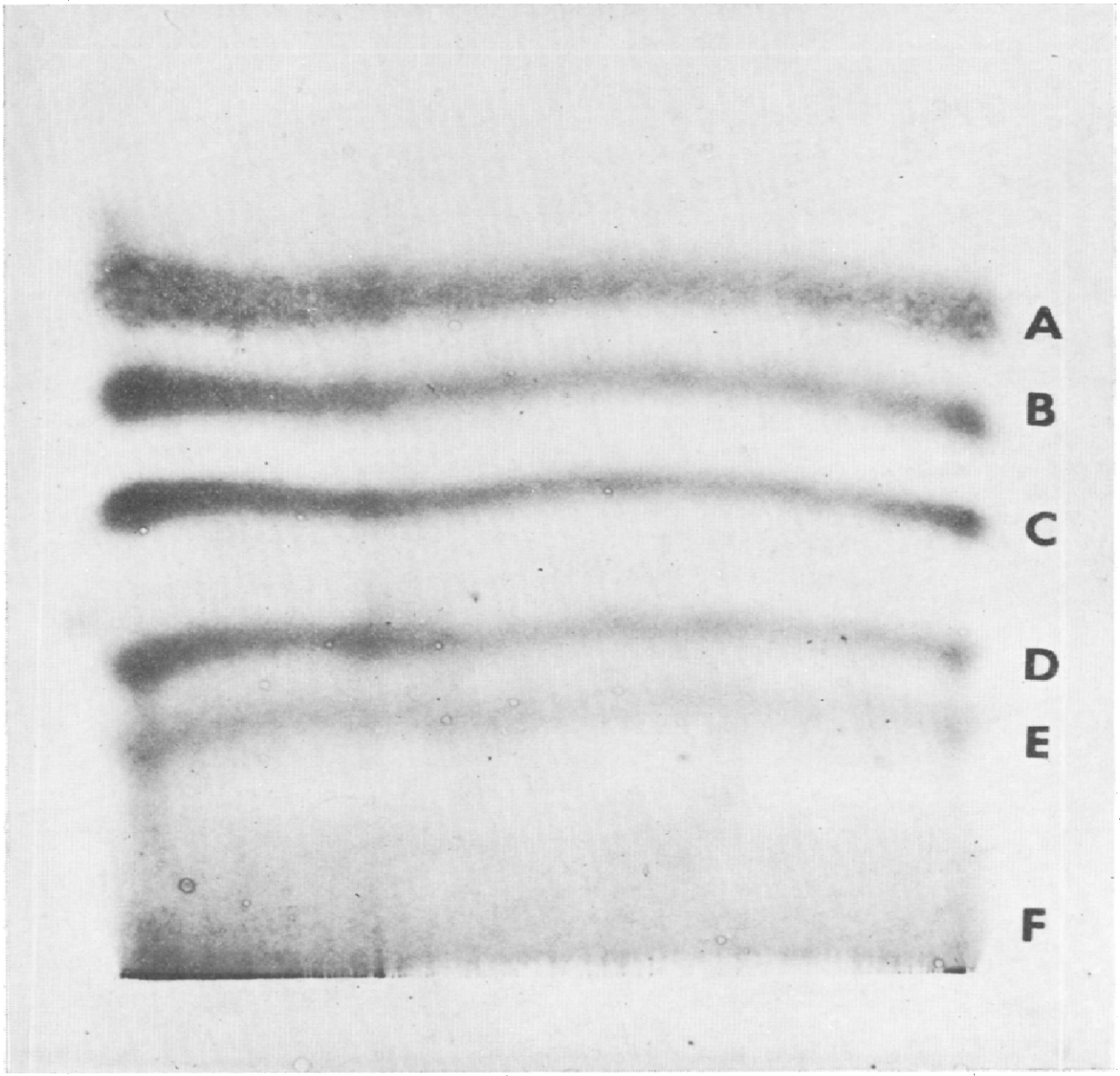

Fig. 2. Fractionation of fatty esters according to number of double bonds. A = saturates; B = monoenes; C = linoleate; D = linolenate; E = arachidonate; F = combined eicosapentaenoate and docosahexaenoate. Amounts not including derivative elements: 200 μg saturates, 130 μg monounsaturates, and 420 μg of polyunsaturates distributed equally among the component groups.

Influence of the remainder of the molecule on the migration rate of mercurated monoenes in the heptane, dioxane developing system was investigated. With the straight chain C_{18} members tested movement was in the decreasing order hydrocarbon, ester, aldehyde, and alcohol (Table III). Within the fatty acid methyl ester group longer chain length increased movement. In the glycerides free hydroxyl groups reduced movement. Double spots obtained for the diolein standard probably reflect fractionation of the two isomers differentiated on the basis of the free hydroxyl group position. Triolein, methyl oleate and elaidate had the same R_F value.

TABLE III

MOVEMENT OF MERCURATED MONOENES

Sample	*R_F value*
1-Octadecene	0.80
Oleate	0.67
Oleylaldehyde	0.61
Oleylalcohol	0.47
15-Tetracosaenoate	0.75
13-Docosaenoate	0.74
Elaidate	0.67
Palmitoleate	0.66
10-Undecenoate	0.58
Ricinoleate	0.58
Triolein	0.67
Diolein	0.58
	0.54
Monoolein	0.25

Gas–liquid chromatography

Gas chromatographic analyses revealed that the fractionation by TLC of fatty acid methyl esters according to degree of unsaturation was successful. Each group of esters from the chromatogram shown in Fig. 2 was recovered and analysed by gas chromatography, according to chain length. The saturated and monoenoic bands with 8 and 5 members, respectively, showed each component clearly (Fig. 3). Moreover, overlapping of the longer homologues in the monoenes and the shorter homologues in the saturates did not occur. The polyunsaturated esters showed sharp, distinct peaks within their expected unsaturation classes.

Trace amounts of saturated and monoenoic members in the polyunsaturated classes probably represented material strongly bound to the silica gel which was released in the acid treatment for derivative decomposition. It was subsequently found that removal of fine particles in the silica gel powder by flotation in ethyl ether decreased but did not eliminate entirely this problem. Control experiments showed that a peak appearing in all chromatograms at approximately 29 min following sample injection was not a fatty acid ester.

Analysis by complementary procedure

To test the validity of the complete chromatographic approach for quantitative

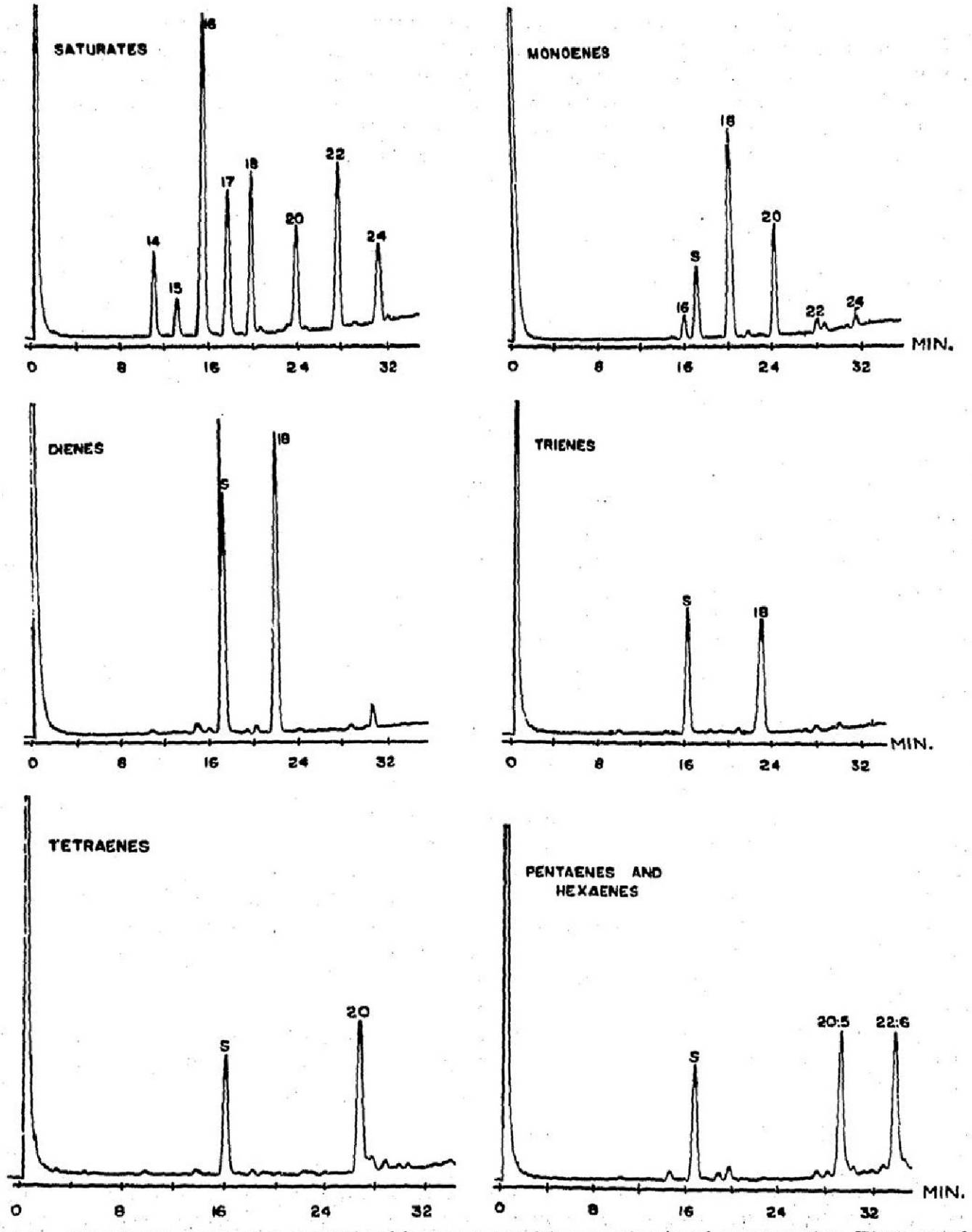

Fig. 3. Gas chromatographic analysis of fatty esters with same degree of unsaturation. Photographs of gas–liquid chromatograms showing components of bands in Fig. 2. Chain length within an unsaturation class denoted by a number appearing at peak apex. S, internal standard (methyl margarate), added as an aid in peak identification.

fatty acid analysis within an unsaturated class another synthetic mixture of saturates and monoenes was prepared. More unsaturated acids were not included because of the inability to obtain commercially members varying widely in chain length. Each of the two groups was analysed by gas chromatography before and after the thin layer fractionation. Results in Table IV show good agreement between the calculated composition and both sets of gas chromatographic data.

TABLE IV

FATTY ACID ANALYSES BY COMPLEMENTARY TLC, GLC PROCEDURE

Acid	*Known (wt. %)*	*Composition of mixture found by GLC analysis, %**					
		Before TLC			*After TLC*		
		Mean	*Range*	*S.D.*	*Mean*	*Range*	*S.D.*
Saturates							
14:0	7.3	7.6	0.5	0.3	7.3	0.2	0.1
15:0	9.5	9.3	0.3	0.1	9.5	0.3	0.1
16:0	11.9	12.4	0.5	0.3	12.8	0.5	0.3
17:0	8.8	9.2	0.1	0.1	9.6	0.2	0.1
18:0	22.5	22.0	0.4	0.2	23.1	0.5	0.3
20:0	19.8	19.2	0.7	0.4	19.8	0.9	0.6
22:0	12.7	12.9	0.4	0.2	11.9	0.5	0.3
24:0	7.5	7.4	0.1	0.1	6.1	0.3	0.2
Monenes							
16:1	13.7	13.6	0.3	0.1	14.1	0.3	0.2
18:1	33.8	33.0	0.5	0.2	34.0	0.9	0.5
20:1	24.0	23.5	0.3	0.2	24.2	0.5	0.3
22:1	12.6	13.7	0.5	0.3	13.5	0.5	0.3
24:1	15.8	16.3	0.3	0.2	14.1	0.3	0.2

* Composition was calculated by comparison of peak heights on gas chromatograms. Each result was based on at least 5 determinations.

Within the saturates there was a trend, particularly noticeable with lignocerate, towards reduced values subsequent to TLC with the longer chain lengths. The C_{24}-monoene percentage also was low. Extraction of a comparable width band of silica gel at a lower level on the chromatoplate confirmed the suspicion that a greater proportion of the long chain saturates and monoenes was bound by the thin layer. In earlier experiments it was found that laurate traveled in the saturate group but was invariably low in percentage composition because of the difficulty in quantitatively retaining this more volatile ester during the experimental manipulations.

The percentage recovery of known amounts of methyl stearate, linoleate, and docosahexaenoate carried through the entire chemical and chromatographic procedure was 95.1 %, 99.3 % and 84.9 %, respectively.

DISCUSSION

The ability to resolve fatty acids according to degree of unsaturation by thin layer chromatography of their mercury adducts has been demonstrated in three different systems. The first described was that of MANGOLD AND KAMMERECK[12] using

the methoxy, acetoxymercuri-adducts. In the two developing solvents employed requiring a total of 4.5–6 h, the saturates were isolated from the remainder of the mixture before separation of the unsaturates into classes. Acids more unsaturated than trienoic did not move. Quality of the different fractions as checked by gas chromatography was greater than 98 % pure.

Another system recently described but WAGNER AND POHL[13], in an investigation of brain fatty acids, employed the same addition compounds and extended the fractionation into the highly unsaturated range. Hexaenoic, pentaenoic, and tetraenoic members were clearly resolved, but the tendency also to differentiate on the basis of chain length caused some overlapping of classes to take place. The single developing solvent required approximately 5 h to move 16.5 cm at 20°. Purity of different fractions was not stated.

With the procedure described in the present work using the bromomercuri- rather than the acetoximercuri-compounds overlapping of different unsaturation classes other than pentane and hexaene was minimal. However, it is recognized that only one chain length was represented in each of the polyunsaturated groups. The sharp resolution of classes obtained with the mixture shown in Fig. 2 may be a reflection of the comparatively rapid solvent development time of only 1 h at room temperature. The monoene band containing C_{16} through C_{24} members covered a narrow R_F value spread of 0.60–0.65 (Fig. 2) although individually determined values for the same compounds showed a wider range (Table III). The tendency to separate primarily on the basis of number of additive groups rather than chain length was demonstrated by the similar width of the multicomponent monoenoic band and the single component dienoic band in Fig. 2.

Purity of the saturated and monoenoic groups was 98.6 % and 97.4 %, respectively. Since polyunsaturated classes were contaminated with small amounts of lesser unsaturated esters that travelled ahead of them on the chromatoplate, an effect referred to earlier as the adsorption problem, their purity did not equal that of the saturates and monoenes. However, a significant indicator of the overall fractionation attained was the proportion of more unsaturated material in each fraction. This was 1.3 % in the monoenes, 0.9 % in the dienes, 1.8 % in the trienes, and 6.7 % in the tetraenes.

The fatty ester mercury addition products showed unusual stability for organomercurial compounds. Refrigerated mixtures in chloroform solution kept in the dark for over a month behaved similarly to freshly prepared adducts in the chromatographic systems described here. No attempt was made to exclude light during adduct formation or development of the chromatograms.

A benefit associated with the covering of double bonds through derivatization was the prevention of autoxidation during much of the experimental procedure. Erroneous conclusions that may come about because of air oxidation of polyunsaturated samples before or after development of chromatoplates have been pointed out[19].

Results in Table IV bear out the conclusion of another study[20] concerning the feasibility of using peak height only in gas chromatographic analysis. Percentage composition values determined both from peak height measurements and from peak areas calculated by multiplying the peak height by the width at half height were in close agreement with the true weight %.

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SUMMARY

A procedure is described for the analysis of methyl esters of fatty acids on the basis of number of double bonds and chain length. After conversion of the unsaturates to their methoxy, bromomercuri-adducts separation of the saturated, monoenoic, dienoic, trienoic, tetraenoic, and combined pentaenoic and hexaenoic classes was accomplished by thin layer chromatography on silica gel G in 1 h using the developing solvent heptane–dioxane (60:40, v/v). Following adduct decomposition chain lengths within each unsaturation class were determined by gas chromatography. Analysis of synthetic mixtures revealed close agreement of experimental and known values.

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