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RESPONSE AND LINEARITY OF DIFFERENT LIPID COMPOUNDS WHEN ANALYZED BY THIN-LAYER CHROMATOGRAPHY WITH FLAME IONIZATION DETECTION

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SUMMARY

The response and linearity were studied for about twenty different types of lipids in a quantitative thin-layer chromatographic system equipped with flame ionization detection (TLC–FID). For most compounds the response showed good linearity with loads from 0.4 to 15 μg. The relative response of cholesterol, sterol ester and wax ester in ratio to triglyceride was, however, found to depend on the concentration, and this should be taken into account in quantitative determinations. The relative response of different lipids varied from 0.6 for hydrocarbon to about 1.4 for cholesterol. The application of the TLC–FID technique for skin lipid separations is illustrated.

INTRODUCTION

After Cotgreave and Lynes[1] successfully combined a universal flame ionization detector (FID) with thin-layer chromatography (TLC), fast development of quantitative TLC of lipids resulted. Padley[2] introduced silica gel-coated quartz rods which were passed through the flame of an FID. This technique was developed further by Okumura and Kadano[3] to give rise to an analytical instrument, the Iatroscan analyzer. In this commercially available system the adsorbent layer is sintered on to a quartz rod to form a reusable rod (Chromarod) which, after development by normal chromatographic procedures, is passed through an FID.

The response of organic compounds in gas chromatographic systems with FID depends on the number of C atoms containing oxygen in ratio to those containing none ("active carbon content")[4]. Thus the response of different lipids can be expected to vary also in Iatroscan analysis. Actually the need for appropriate response factors

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has been reported, and the response of some compounds has already been determined[5–8].

In this investigation we determined the relative response of different types of lipids to either triglyceride or lecithin. We also studied the linearity of the response within the concentration range applicable for TLC–FID analysis on the Iatroscan analyzer.

EXPERIMENTAL

Reagents and standards

The specific lipid compounds analyzed in this study are listed in Table I. To minimize the effect of different carbon chain lengths on the response, reference compounds with C_{16} fatty acid or alcohol moieties were used, when available. When compounds with C_{16} moieties were unavailable, natural mixtures of highest purity were purchased.

TABLE I

LIPID CLASSES AND REFERENCE COMPOUNDS

Lipid class	*Designation*	*Reference compound*	*Source*	*Supplier*
Triglyceride	TG	Tripalmitin	Synthetic	Analabs
1,3-Diglyceride	1,3-DG	1,3-Dipalmitin	Synthetic	Appl. Sci.
1,2-Diglyceride	1,2-DG	1,2-Dipalmitin	Synthetic	Appl. Sci.
2-Monoglyceride	2-MG	2-Monopalmitin	Synthetic	Appl. Sci.
Fatty acid	FA	Palmitic acid		Appl. Sci.
Glyceryl ether	GE	1-Hexadecylglyceryl ether	Synthetic	Appl. Sci.
Sterol ester	SE	Cholesteryl palmitate	Synthetic	Appl. Sci.
Sterol	CHOL	Cholesterol		Calbiochem
Wax ester	WE	Hexadecyl palmitate		Analabs
Fatty alcohol	FAlc	1-Hexadecanol		Appl. Sci.
Hydrocarbon	HC	Hexadecane		Appl. Sci.
Lecithin	PC	L-α-Dipalmitoyllecithin	Synthetic	Appl. Sci.
Lysolecithin	LPC	L-α-Monopalmitoyllecithin	Synthetic	Appl. Sci.
Cephalin	PE	L-α-Dipalmitoylcephalin	Synthetic	Appl. Sci.
Lysocephalin	LPE	L-α-Monopalmitoylcephalin	Synthetic	Avanti
Sphingomyelin	SPH	Natural mixture	Bovine	Avanti
Ceramide	Cera	Natural mixture	Bovine	Appl. Sci.
Cerebroside	Cere	Natural mixture	Bovine	Appl. Sci.

Reference compounds were divided into groups according to their chromatographic behavior, and standard solutions with 0.4, 1.0 and 5.0 mg/ml of each component were prepared in chloroform–methanol (2:1). Each solution had either tripalmitin or dipalmitoyllecithin as a common link. Additionally, both these components were present in one solution.

All the solvents were reagent grade and distilled in glass before use.

Natural lipid samples

The chloroform–methanol extraction[9] was used to obtain mouse skin lipids, human meibomian gland lipids and a sample of human eyelid skin. These demonstrate

the separation of neutral lipids, wax esters and sterol esters, and polar lipids, respectively.

Thin-layer chromatography

The Chromarod S or S-II quartz rods with a coating of silica gel (Iatron Laboratories, Tokyo, Japan) were stored in 4.5 *M* H_2SO_4. Immediately before use the rods were rinsed with distilled water and activated by passing them through the flame of the detector of the Iatroscan analyzer. The particle sizes of the adsorbent layers of Chromarod S and S-II are 10 μm and 5 μm, respectively.

Disposable micro pipettes (1 μl, Drummond Microcap; Drummond, Broomall, PA, U.S.A.) were used for spotting. The sample varied from 1 to 3 μl and ranged from 0.4 to 15 μg of individual lipid components.

The glass frames containing the spotted rods were placed into paper-lined glass tanks and developed with appropriate solvents. After development, the solvent was removed by flushing the rods with a stream of nitrogen and drying in air for about 5 min, or placing the frame with the rods into an oven (60°C) for 5 min. The rods were then transferred to the instrument and scanned immediately.

Light petroleum (b.p. 30–40°C)–diethyl ether–99% formic acid (65:3:0.7) was used to separate most neutral lipids. The development was performed twice for 25 min in the same solvent with air-drying between developments. Development time is usually given because solvent fronts are not always visible. Di- and monoglycerides were developed for 25 min in benzene–chloroform–99% formic acid (30:45:1.5). More polar lipids were chromatographed twice for 25 min in chloroform–methanol–water (40:15:2.2). Separation of wax esters and sterol esters was achieved on Chromarod S-II rods by developing with hexane–benzene (27:33) for 25 min.

Instrumentation

An Iatroscan Model TH-10 analyzer (Iatron) in combination with a Spectra-Physics Model 4100 computing integrator was used for quantitative lipid analyses. The FID was operated under the following conditions: hydrogen pressure 0.7 kg/cm^2; air flow 2000 ml/min and the scanning speed 40 sec per scan (Gear No. 30 of the instrument). The integrator was used also to plot the chromatograms with the chart speed 16.0 cm/min, peak window 2 and threshold 18. Attenuation was adjusted between 8 and 512 according to the sample concentration.

RESULTS AND DISCUSSION

The response of the reference compounds was determined as the average value of five to eight determinations for each of the seven concentrations from 0.4 to 15 μg. These average values were used to plot the linearity curves (Figs. 1 and 2) as well as to calculate the relative response of the reference compounds in ratio to TG or PC (Tables II and III).

All the compounds showed a fairly good linearity although the response at the lowest loads was often higher than anticipated. This may be due to background noise which became significant near the lower detection limit. At the highest loads the slope of the response curves decreased, especially for di- and monoglycerides, possibly because of saturation of the detector. Percentage standard deviation of the

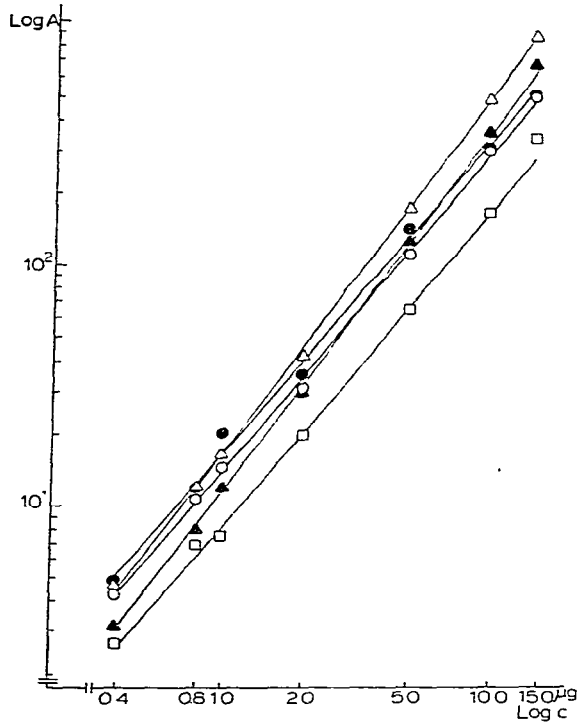

Fig. 1. Linearity of the response of different neutral lipids and lecithin. ○, Tripalmitin; ▲, hexadecyl palmitate; △, cholesterol; □, hexadecane; ●, lecithin.

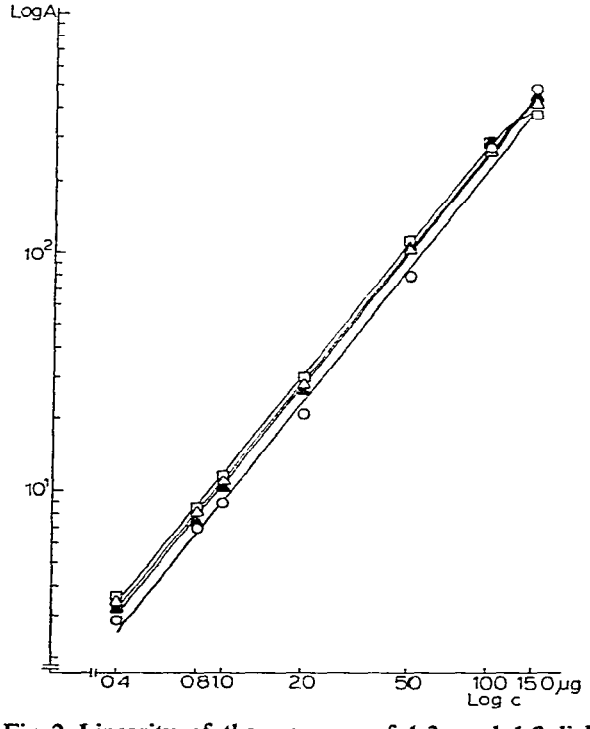

Fig. 2. Linearity of the response of 1,3- and 1,2-diglycerides, 2-monoglyceride and triglyceride. ○, Tripalmitin; ▲, 1,3-dipalmitin; △, 1,2-dipalmitin; □, 2-monopalmitin.

area measurement ranged from 6.0 to 13.5% depending on the reference compound (Tables II and III). These values were obtained at 1-μg load levels. High deviations were measured at the lowest loads (0.4 μg), with the maximum of 25.3% for sterol ester, whereas low deviations (<2%) were obtained for higher loads.

Reproducibility of lipid analysis by Iatroscan was also reported by Sipos and Ackman[5] who used a standard mixture of cholesterol, tristearin, stearic acid and cholesteryl palmitate. They spotted 3.8–7.3 μg of each compound and percentage standard deviations from 5.7 to 12.9% can be calculated from their results. The same reproducibility in the present study was obtained at the 1-μg level. Bradley *et al.*[6] reported reproducibility of 4.4–6.2% for plasma lipid analysis.

The response curve for hydrocarbon in Fig. 1, although parallel to the TG curve, lies at a distinctly lower level. This lower response can be explained by the relatively high volatility of hexadecane which may evaporate slightly before entering the FID during the scanning. Thus a correction factor for hydrocarbon has to be introduced for quantitative determinations. Since all the response curves are not parallel, other factors are also involved. This points up the necessity for determining relative responses.

Relative responses of the reference compounds in ratio to TG or PC were calculated for each concentration. TG was selected as the referent for other neutral lipids because of its widespread occurrence and its migration to a central position on rods. PC serves as an analogous referent for polar lipids. The response factors with their percentage standard deviations are given in Tables II and III. The average relative responses of di- and monoglycerides in ratio to TG were 1.14 and 1.16 for 1,3- and 1,2-dipalmitins, respectively, and slightly higher, 1.21, for 2-monopalmitin (Table II). The relative response of palmitic acid averaged 0.75 with a range of 0.66–0.82 at decreasing concentrations. A lower response has also been reported for stearic acid compared to tripalmitin[10] and to tristearin[5]. In addition to the "active carbon content"[4], volatility factors may partially account for these low values. The response curve for GE closely parallels that of TG and gives a relative response of 1.00. The average relative response for SE was also 1.00. This response curve, however, did not overlap with the TG curve; the response varied with concentration ranging from 0.74 at the lowest load to 1.28 at the highest load. The same variation was found also for WE. Similar behavior of WE and SE is advantageous for regular quantitative analysis because they are often found to overlap in routine chromatographic analysis and are not separated on Chromarod S.

It has been reported that the response of cholesterol exceeds that of TG[5,11,12]. In our hands the relative response of cholesterol in ratio to TG averaged 1.38, but ranged widely (1.09–1.72). The relative response of 1-hexadecanol (FAlc) was only 0.67. In their "active carbon content" fatty alcohols are comparable to respective fatty acids and thus similar low responses are understandable. Also volatility factors are comparable.

The relative response of polar lipids was measured in ratio to PC (Table III). The response of PC was also determined in ratio to the response of TG to have a common link between neutral and polar lipids. The response of PC exceeds slightly that of TG giving an average value of 1.17 (Table II). No systematic change in this relative value was found with increase in concentration. Therefore the PC/TG ratio can be used as a factor when Iatroscan chromatograms are to be normalized for

TABLE II

STANDARD DEVIATION OF THE AREA DETERMINATION AND THE RESPONSE OF DIFFERENT NEUTRAL LIPIDS AND LECITHIN IN RATIO TO TRIGLYCERIDE AT DIFFERENT CONCENTRATIONS IN AN IATROSCAN ANALYSIS

Reference compound	*S. D. of area determination, n = 5 (%)*	*Response ± percentage standard deviation (n = 5) in ratio to TG*							*Average response*
		0.4 μg	*0.8 μg*	*1.0 μg*	*2.0 μg*	*5.0 μg*	*10.0 μg*	*15.0 μg*	
TG	6.1								
1,3-DG	9.0	1.15 ± 9.0	1.08 ± 10.0	1.17 ± 6.0	1.29 ± 4.5	1.30 ± 9.8	1.03 ± 3.2	0.94 ± 0.9	1.14
1,2-DG	8.2	1.22 ± 10.9	1.16 ± 8.8	1.24 ± 7.0	1.35 ± 8.4	1.29 ± 10.4	0.97 ± 2.9	0.88 ± 0.8	1.16
2-MG	6.1	1.24 ± 9.5	1.22 ± 9.2	1.34 ± 8.5	1.46 ± 6.4	1.42 ± 12.1	1.02 ± 7.2	0.80 ± 2.4	1.21
FA	6.0	0.82 ± 7.8	0.76 ± 3.9	0.77 ± 2.7	0.74 ± 4.5	0.71 ± 3.0	0.73 ± 6.5	0.66 ± 3.6	0.75
GE	8.7	0.91 ± 7.6	0.90 ± 9.1	1.00 ± 2.5	1.11 ± 5.9	1.07 ± 1.7	0.98 ± 5.6	1.02 ± 4.8	1.00
SE	7.5	0.74 ± 25.8	0.91 ± 15.9	0.95 ± 8.4	0.79 ± 11.8	1.18 ± 3.6	1.18 ± 6.4	1.28 ± 4.9	1.00
CHOL	7.0	1.09 ± 5.5	1.19 ± 3.1	1.15 ± 5.6	1.34 ± 3.9	1.56 ± 4.0	1.61 ± 3.5	1.72 ± 2.4	1.38
WE	8.8	0.73 ± 5.2	0.77 ± 14.3	0.83 ± 7.8	0.98 ± 4.8	1.13 ± 2.9	1.19 ± 5.3	1.28 ± 4.2	0.99
FAlc	7.4	0.59 ± 6.6	0.62 ± 4.0	0.62 ± 5.5	0.64 ± 5.4	0.70 ± 3.8	0.76 ± 9.3	0.74 ± 8.2	0.67
HC	8.6	0.62 ± 12.1	0.63 ± 19.6	0.52 ± 9.3	0.64 ± 5.3	0.58 ± 5.3	0.56 ± 16.6	0.71 ± 14.3	0.61
PC		1.15 ± 8.7	1.17 ± 10.3	1.41 ± 11.0	1.15 ± 10.7	1.27 ± 4.2	1.05 ± 8.1	0.99 ± 5.0	1.17

TABLE III

STANDARD DEVIATION OF THE AREA DETERMINATION AND THE RESPONSE OF DIFFERENT POLAR LIPIDS IN RATIO TO LECITHIN AT DIFFERENT CONCENTRATIONS IN AN IATROSCAN ANALYSIS

Reference compound	*S. D. of area determination, n = 5 (%)*	*Response ± percentage standard deviation (n = 5) in ratio to PC*							*Average response*
		0.4 μg	*0.8 μg*	*1.0 μg*	*2.0 μg*	*5.0 μg*	*10.0 μg*	*15.0 μg*	
PC	8.5								
LPC	8.4	0.72 ± 16.1	0.82 ± 8.2	0.96 ± 8.8	0.94 ± 13.5	0.93 ± 13.2	0.89 ± 9.7	0.75 ± 6.0	0.86
PE	10.5	0.70 ± 12.9	0.78 ± 4.9	0.79 ± 9.7	0.85 ± 13.7	0.99 ± 11.7	1.04 ± 13.0	1.06 ± 6.0	0.89
LPE	12.2	0.92 ± 13.4	0.91 ± 9.6	0.94 ± 11.1	0.93 ± 4.1	1.12 ± 5.5	0.99 ± 4.5	0.94 ± 3.5	0.96
SPH	13.5	0.75 ± 18.4	0.74 ± 12.9	0.85 ± 9.2	0.85 ± 7.4	0.97 ± 4.7	1.01 ± 5.7	1.02 ± 9.0	0.88
Cera	11.1	1.19 ± 15.4	0.96 ± 4.9	0.88 ± 11.4	1.04 ± 17.6	1.25 ± 15.8	1.39 ± 13.0	1.25 ± 5.9	1.14
Cere	13.3		1.25 ± 9.8	1.08 ± 22.3	0.93 ± 19.8	0.93 ± 14.8	1.12 ± 5.1	1.17 ± 9.3	1.08

quantitative determinations. All the phosphoglycerides gave very similar responses in ratio to PC ranging from 0.86 for LPC to 0.96 for LPE. Sphingomyelin was comparable to phosphoglycerides in its relative response. Also Tanaka *et al.*[13] have reported approximate correlation between the weight proportions and the area ratios of PE, LPC and SPH to PC in an Iatroscan analysis, but did not report specific values. During TLC of polar lipids, cerebrosides and ceramides migrate close to the solvent front and easily overlap with any non-polar material present in the sample or adsorbent. This may partially account for the high standard deviations of our determinations (Table III). The average relative responses of ceramides and cerebrosides were respectively 1.14 and 1.08.

High standard deviations at the low load levels (0.4 and 0.8 μg) and decreasing slopes of the response curves for some reference compounds when approaching the 15-μg load level suggest that 1–10 μg is a practical range for accurate determinations. This has earlier been found as a reasonable working range for cholesterol, cholesteryl palmitate, tristearin and stearic acid[5]. In routine quantitative chromatographic analyses an easily separated compound can serve as an internal standard. For Iatroscan analyses 1-octadecanol was introduced as the reference compound for the major lipid classes of plasma lipid[14] whereas lysolecithin was recommended for measuring lecithin, sphingomyelin and phosphatidylglycerol[15]. However, the increasing relative response for cholesterol, sterol ester and wax ester as compared to TG should be considered especially when one or more of these components are dominant in the sample. This can be done by the use of a quantitative standard mixture resembling the anticipated composition of the sample to be analyzed. In any case quantitative

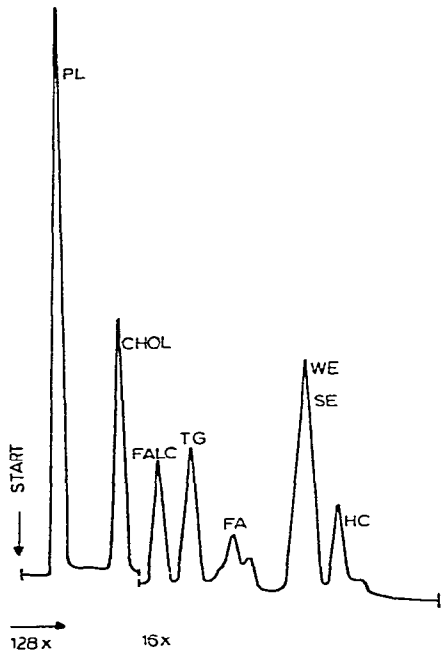

Fig. 3. Neutral lipid separation of mouse skin lipids on Chromarod S rods. PL = Polar lipids. For abbreviations see Table I. Note the attenuation change from 128 to 16 before the FAlc peak.

standard mixtures should be routinely analyzed to eliminate possible errors arising from the daily changes in the sensitivity of the analytical system.

The separation of different neutral lipids on Chromarod S rods is demonstrated in Fig. 3 with the total lipid extract from mouse skin. With light petroleum–diethyl ether–99% formic acid (65:3:0.7) as the solvent system all the polar lipids remain as one group near the origin. Chromarod S rods cannot separate wax esters and sterol esters from each other. This separation can be accomplished on Chromarod S-II rods (Fig. 4). Although the reference compounds with C_{16} carbon chain moieties were completely resolved, only a partial separation was obtained with human meibomian gland lipids. This is apparently due to differences in chain lengths and degrees of unsaturation of natural samples. In his review on the analysis of fatty acid methyl esters by FID, Ackman[4] reports correction factors ranging from 1.10 to 0.93 for fatty chains of C_{10}–C_{24} with 0–6 double bonds. Each additional C atom and double bond

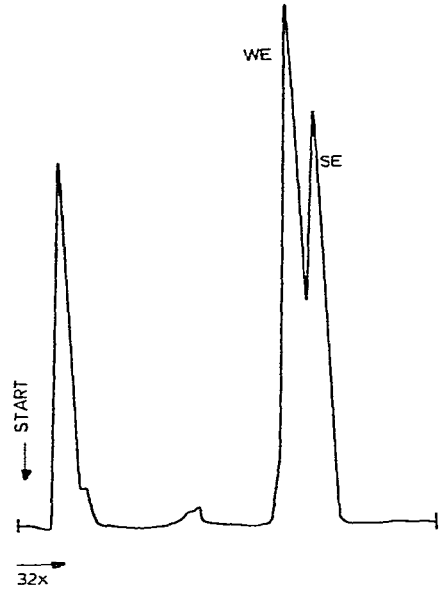

Fig. 4. Wax ester and sterol ester separation of human meibomian gland lipids on Chromarod S-II rods. For the abbreviations see Table I.

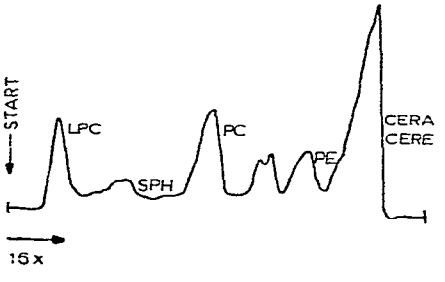

Fig. 5. Polar lipid separation of a sample of human eyelid skin on Chromarod S rods. For the abbreviations see Table I.

decreased the correction factor by approximately 1 %. These factors will automatically be taken into account when quantitating natural mixtures if standards from the same natural sources are used as references. The polar lipid separation of a sample of human eyelid skin is shown in Fig. 5. Neutral lipids were first separated from polar lipids using chloroform as the solvent. Then neutral lipids were burned off on the Iatroscan analyzer and the rods with polar lipids were redeveloped in chloroform–methanol–water (40:15:2.2) followed by the Iatroscan analysis.

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REFERENCES

1 T. Cotgreave and A. Lynes, *J. Chromatogr.*, 30 (1967) 117.
2 F. B. Padley, *J. Chromatogr.*, 39 (1969) 37.
3 T. Okumura and T. Kadano, *Bunseki Kagaku (Jap. Anal.)*, 22 (1973) 980.
4 R. G. Ackman, *Progr. Chem. Fats Other Lipids*, 12 (1972) 167.
5 J. C. Sipos and R. G. Ackman, *J. Chromatogr. Sci.*, 16 (1978) 443.
6 D. M. Bradley, C. R. Rickards and N. S. T. Thomas, *Clin. Chim. Acta*, 92 (1979) 293.
7 D. Vandamme, V. Blaton and H. Peeters, *J. Chromatogr.*, 145 (1978) 151.
8 D. Vandamme, G. Vankerckhoven, R. Vercaemst, F. Soetewey, V. Blaton, H. Peeters and M. Rosseneu, *Clin. Chim. Acta*, 89 (1978) 231.
9 E. G. Bligh and D. W. Dyer, *Can. J. Biochem. Physiol.*, 37 (1959) 911.
10 M. Tanaka, T. Itoh and H. Kaneko, *Yukagaku (J. Jap. Oil Chem. Soc.)*, 25 (1976) 263.
11 W. W. Christie and M. L. Hunter, *J. Chromatogr.*, 171 (1979) 517.
12 B. Herslöf, in L.-Å. Appelqvist and C. Liljenberg (Editors), *Adv. Biochem. Physiol. Plant Lipids*, Elsevier, Amsterdam, Oxford, New York, 1979, p. 301.
13 M. Tanaka, T. Itoh and H. Kaneko, *Yukagaku (J. Jap. Oil Chem. Soc.)*, 26 (1977) 454.
14 P. van Tornout, R. Vercaemst, H. Caster, M. J. Lievens, W. de Keersgieter, F. Soetewey and M. Rosseneu, *J. Chromatogr.*, 164 (1979) 222.
15 A. Martin-Ponthieu, N. Porchet, J.-C. Fruchart, G. Sezille, P. Dewailly, X. Codaccioni and M. Delecour, *Clin. Chem.*, 25 (1979) 31.