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CHROMATOGRAPHIC ANALYSIS OF BLOOD LIPIDS

COMPARISON BETWEEN GAS CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY WITH FLAME IONIZATION DETECTION

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SUMMARY

Intact human blood plasma lipids of different composition were analyzed by gas chromatography and thin-layer chromatography with flame ionization detection. The reproducibility of the results obtained by gas and thin-layer chromatography was compared. The main advantages and disadvantages of both methods for lipid analysis are discussed. Generally, the variability of the results measured by thin-layer chromatography in series and from day to day was greater than that obtained by gas chromatography.

INTRODUCTION

In the study of lipid metabolism and its disorders, a sufficiently rapid, selective and precise method is lacking for the determination of the level or composition of the individual lipid classes. At present, primarily various chro-

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matographic methods and combinations thereof are used for this purpose. The most frequently used methods are gas and thin-layer chromatography.

Gas chromatography (GC) of intact blood lipids was first described by Kuksis et al. in 1967 [1]. Since then, several other works devoted to this problem have been published and the method has been automated [2–6]. The use of flame ionization detection (FID) for detection and quantitation of substances separated by thin-layer chromatography (TLC) was first described roughly at the same time as GC of intact lipids [7]. Subsequently, similar works appeared, but broader application of FID in the detection and quantitation of substances separated on a thin layer was first described in the work of Okumura et al. [8, 9].

Both methods, GC and TLC—FID, are limited to a certain extent in practical applications. In GC, this is primarily a result of the small capacity of the examination and the impossibility of direct analysis of some polar lipids [4]. In TLC, reproducibility of measurements at low concentrations of the separated substances has so far been dubious. In addition, GC yielded separation of fractions of neutral lipids in dependence on their carbon number, while TLC in the given experimental arrangement permits only separation of lipid classes. This property, which is a drawback from the point of view of biochemical research, may be compensated in clinical biochemistry by the much greater capacity of this method compared with GC. TLC—FID has been proposed and successfully applied to the screening of lipid levels [10].

Our work was concerned with studying the reproducibility of the results of analysis of blood lipids by the two methods considering the suitability of application of TLC—FID in the study of disorders in lipid metabolism.

EXPERIMENTAL

Chemicals and instruments

All the solvents used were rectified and tested for purity before use. Pure substances were used for calibration of the gas chromatograph and to obtain suitable correction factors for TLC—FID. Purity was controlled by GC. Triglycerides with carbon numbers of 48 (tripalmitin), 50 (*rac*-glyceryl-1,3-palmitate-2-stearate), 52 (*rac*-glyceryl-1,3-stearate-2-palmitate) and 54 (tristearin) were products of Supelco, Bellefonte, PA, U.S.A. Cholesteryl esters with carbon numbers of 41 (cholesteryl myristate), 43 (cholesteryl palmitate), 45 (cholesteryl stearate) and 47 (cholesteryl arachate) and internal standards for GC (cholesteryl butyrate and tridecanoin) and free cholesterol were supplied by Applied Science Labs., State College, PA, U.S.A. Phospholipids *rac*-1,2-palmitoylglycerol-3-phosphorylcholine, *rac*-1-palmitoylglycerol-3-phosphorylcholine and *rac*-1-palmitoyl-2-oleyl-3-phosphorylethanolamine were supplied by Fluka, Buchs, Switzerland; sphingomyelin and phosphatidylserine were supplied by Koch-Light, Colnbrook, Great Britain. Alfol RD 18 (1-octadecanol) was a product of Condea, Hamburg, G.F.R.

Preparation of sample

Biological samples were obtained by extraction of 2 ml of blood plasma from persons with normal or increased blood lipid levels according to the

TABLE I

COMPOSITION OF MODEL LIPID MIXTURE FOR TLC—FID

1 μl of the solution was applied on the rod.

Substance	Concentration (μg/μl)	Weight (%)
Cholesterol	0.4	7.7
Cholesteryl palmitate	1.7	32.7
Tripalmitin	1.2	23.1
Palmitic acid	0.2	3.8
rac-1,2-Palmitoylglycerol-3-phosphorylcholine	1.7	32.7

TABLE II

COMPOSITION OF MODEL LIPID MIXTURE FOR GC

1 μl of the solution was injected into the gas chromatograph.

Substance	Concentration (μg/μl)	Weight (%)
Cholesterol	0.300	9.8
Cholesteryl myristate	0.025	0.8
Cholesteryl palmitate	0.250	8.2
Cholesteryl stearate	1.200	39.4
Cholesteryl arachate	0.150	4.9
Cholesteryl esters	1.625	53.3
Tripalmitin	0.025	0.8
rac-Glyceryl-1,3-palmitate-2-stearate	0.200	6.6
rac-Glyceryl-1,3-stearate-2-palmitate	0.600	19.7
Tristearine	0.300	9.8
Triglycerides	1.125	36.9

method of Folch et al. [11]. The composition of samples prepared from pure substances is given in Tables I and II.

Thin-layer chromatography

TLC—FID was carried out on an Iactroscan TH-10 (Iatron Labs., Tokyo, Japan) using silica gel S-I (10 μm particle size) and S-II (5 μm) Chromarods. Scanning speed was 3.2 cm/sec, air flow through the detector 2100 ml/min, hydrogen flow 180 ml/min for all samples.

Analysis of neutral lipids and free fatty acids. The internal standard for TLC—FID was 1-octadecanol. Each sample was dissolved in 0.5 ml of a 1% solution of the internal standard in chloroform—methanol (2:1, v/v) and 1 μl was applied on the rod. The developing chamber was saturated for 20 min;

the rods were activated by being passed through the FID prior to use. The samples of blood lipids were separated using hexane—diethyl ether—formic acid (90:10:1, v/v) at 22—25°C. The system was developed to a height of 10 cm. After drying, the rods were scanned in FID. The biological samples were analyzed on S-I rods, while the model synthetic ones were analyzed on both S-I and S-II rods to check for effect of particle size.

Analysis of phospholipids. The blood phospholipids as well as model synthetic samples were analyzed on S-II rods. The sample volume was in all cases 1 μl.

A model mixture of phospholipids was separated using chloroform—methanol—water (45:26:2.5, v/v) at 22—25°C. The chromatogram was developed to a height of 8 cm. The biological sample of human blood phospholipids was first developed in acetone to move the neutral lipids and free fatty acids (FFA) to the front of the rods, and after scanning in FID (from R_F = 0.1), the phospholipids remaining at the start were then developed in the same system as the model mixture. The second scanning was carried out under the conditions described above.

Simultaneous analysis of neutral lipids and phospholipids. Both polar and neutral lipids can be separated and assayed simultaneously according to the method of Iatron Laboratories [12]. The solution of plasma lipids prepared by the method mentioned above was spotted on Chromarod S-II and developed in chloroform—methanol—water (40:20:2.5, v/v) to a height of 5 cm (two times) and then in *n*-hexane—light petroleum—diethyl ether—formic acid (30:24:6:0.08, v/v). The scanning conditions were the same as mentioned above.

Integration and quantitative evaluation was carried out using an Autolab System I Computing Integrator (Spectra Physics, Mountain View, CA, U.S.A.).

Gas chromatography

GC analyses were carried out using the Perkin-Elmer F-30, F-17 and Sigma 1 instruments under conditions described earlier [5, 6]. The sample composition for GC is given in Table II. Prior to analysis, the samples were dissolved in a solution of cholesteryl butyrate (200 ng/μl) in chloroform—isooctane solution (20:80, v/v); 1 μl of this sample was injected into the gas chromatograph.

TABLE III

C.V. VALUES FOR THE DETERMINATION OF THE CONTENT OF THE LIPID CLASSES IN THE MODEL SYNTHETIC SAMPLE BY TLC—FID

Concentrations of individual compounds are given in Table I.

	C.V.	Cholesteryl esters	Triglycerides	Fatty acids	Free cholesterol	Phospho-lipids
S-I rods	In series*	5.5	6.0	12.1	4.6	3.8
	From day to day**	6.3	8.1	—	4.2	6.2
S-II rods	In series*	1.1	1.4	15.4	4.4	3.9
	From day to day**	3.2	3.1	30.7	4.9	5.9

*Calculated from ten runs analyzed during a single day.
**Calculated from six series of four runs analyzed over three weeks.

Quantitative data were obtained by a computer after integration by means of a Perkin-Elmer Sigma 10 Data System, as described earlier [5].

Evaluation of results

Reproducibility of the determination of all lipid classes by both methods was expressed by means of coefficients of variation (C.V.). For the neutral lipids the serial C.V. were calculated from ten measurements of each biological

TABLE IV

REPRODUCIBILITY OF THE DETERMINATION (C.V.) OF THE LIPID CLASSES IN MODEL SYNTHETIC SAMPLE BY GC

Concentrations of individual lipid classes are given in Table II.

C.V.	Free cholesterol	Cholesteryl esters	Triglycerides
In series*	0.51	1.31	0.89
From day to day**	1.33	2.75	2.73

*Calculated from ten runs analyzed during one day.
**Calculated from seven series of four samples analyzed over seven weeks.

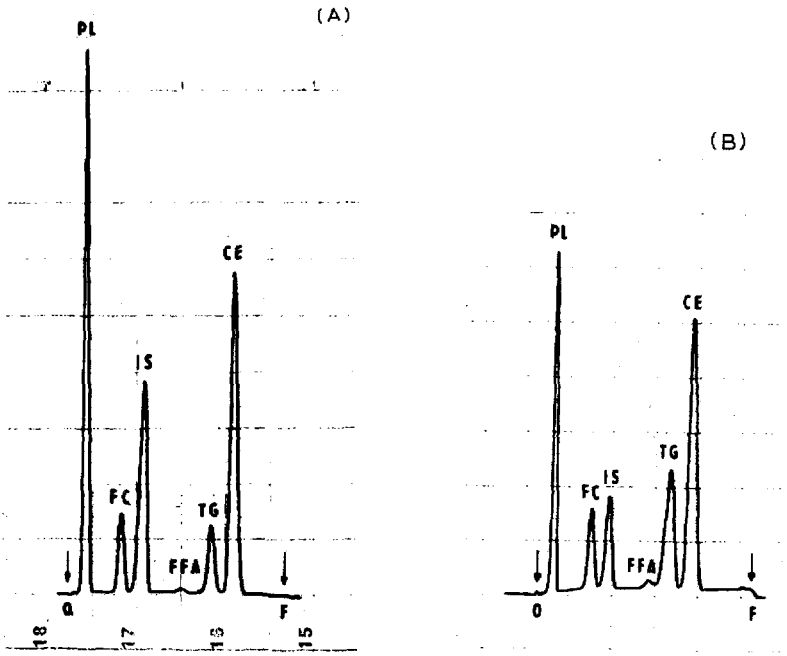

Fig. 1. Analysis of neutral lipids and free fatty acids by TLC—FID on Chromarods S-I. (A) Model sample prepared from pure saturated substances. Peak designation: PL = *rac*-1,2-palmitoylglycerol-3-phosphorylcholine, FC = cholesterol, IS = internal standard (1-octadecanol), FFA = palmitic acid, TG = tripalmitin, CE = cholesteryl palmitate. (B) Biological sample isolated from human blood plasma. Peak designation: PL = phospholipids, FC = cholesterol, IS = internal standard (1-octadecanol), FFA = free fatty acids, TG = triglycerides, CE = cholesteryl esters. 0 = start, F = front. Analytical conditions are given in the text.

TABLE V

C.V. VALUES FOR THE DETERMINATION OF THE CONTENT OF THE LIPID CLASSES IN BIOLOGICAL SAMPLES WITH VARIOUS COMPOSITION BY TLC–FID

Lipid class	Sample I			Sample II			Sample III
	Content*	Series** (C.V.)	Day*** (C.V.)	Content	Series (C.V.)	Day (C.V.)	Content
Cholesteryl esters	6.3	8.7	21.6	41.1	5.2	10.0	27.2
Triglycerides	68.0	4.3	6.4	9.4	8.7	12.4	34.5
Fatty acids	1.0	37.1	51.5	2.0	29.2	37.7	0.7
Free cholesterol	3.2	3.5	4.6	7.3	6.1	8.8	5.1
Phospholipids	21.5	4.8	6.5	40.2	4.9	7.5	32.4

*Weight per cent of the individual lipid classes in the sample.
**C.V. calculated from ten runs of the same sample measured during a single day.
***C.V. calculated from six series of four runs of the same sample measured over three weeks.

TABLE VI

REPRODUCIBILITY OF THE DETERMINATION (C.V.) OF THE LIPID CLASSES IN BIOLOGICAL SAMPLES BY GC

C.V.	Free cholesterol	Cholesteryl esters	Triglycerides
In series*	0.62	1.52	1.13
From day to day**	1.21	2.13	2.99

*Calculated from ten runs of the same sample analyzed during one day.
**Calculated from seven series of four samples analyzed over seven weeks.

sample and model synthetic sample as well. For the phospholipids only serial C.V. were calculated from five measurements of the model and biological samples. Day-to-day variation was calculated similarly from the means of four serial determinations of each sample analyzed six or seven times during three weeks (TLC) and seven weeks (GC).

RESULTS

The reproducibility of measurements of synthetic samples by TLC on S-I and S-II rods is given in Table III. The results of the analyses of comparable model samples by GC are listed in Table IV.

Biological samples were analyzed on S-I rods. The reproducibility of the results of analysis of biological samples is given in Table V. Corresponding results obtained by GC are given in Table VI.

Typical separation for model and biological samples using TLC–FID is depicted in Fig. 1A and B; separation of synthetic and biological samples by GC is given in Fig. 2A and B.

In addition to analysis of cholesterol, cholesteryl esters and triglycerides, TLC can be used to separate and determine phospholipids quantitatively and also to separate phospholipid classes. The reproducibility of the analysis of

		Sample IV			Sample V			Average	
Series (C.V.)	Day (C.V.)	Content	Series (C.V.)	Day (C.V.)	Content	Series (C.V.)	Day (C.V.)	Series (C.V.)	Day (C.V.)
4.9	8.1	37.9	4.3	7.9	36.8	2.9	3.4	5.2	10.2
3.7	6.8	14.0	5.1	8.4	3.8	3.1	4.8	5.0	7.4
32.6	41.1	1.2	36.5	57.9	2.1	10.7	16.9	29.2	41.0
5.9	10.5	5.6	6.2	13.0	6.2	4.3	7.5	5.2	8.9
6.1	9.5	41.3	5.1	7.8	51.0	3.2	5.8	4.8	7.4

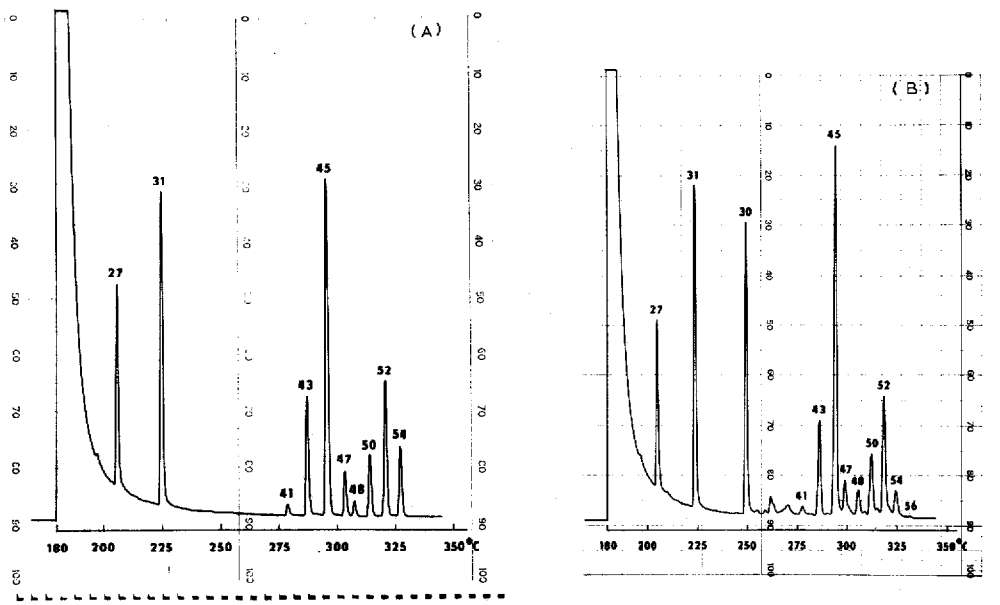

Fig. 2. Analysis of neutral lipids by GC. (A) Model sample prepared from pure saturated substances. Peak designation: 27 = cholesterol; 31 = cholesteryl butyrate (internal standard); 41 = cholesteryl myristate; 43 = cholesteryl palmitate; 45 = cholesteryl stearate; 47 = cholesteryl arachidate; 48 = tripalmitin; 50 = *rac*-glyceryl-1,3-palmitate-2-stearate; 52 = *rac*-glyceryl-1,3-stearate-2-palmitate; 54 = tristearin. (B) Biological sample isolated from human blood plasma. Peak designation: 27 = cholesterol; 31 = cholesteryl butyrate; 30 = tridecanoin (both internal standards); 41, 43, 45, 47 = cholesteryl ester fractions with carbon numbers of 41, 43, 45 and 47; 48, 50, 52, 54, 56 = triglycerides with carbon numbers of 48, 50, 52, 54 and 56. Analytical conditions: gas chromatograph Perkin-Elmer F-17, column glass 0.5 m × 1.75 mm I.D., packing 1% OV-1 on Gas-Chrom Q 100—120 mesh. Temperatures: injector and detector 340°C, oven programme from 180 to 350°C (rate 5°C/min), detector FID, attenuation 1 × 64. Carrier gas helium, flow-rate 80 ml/min. Carbon number for cholesteryl esters is defined as the sum of carbon atoms in the molecule, but for triglycerides it represents only the sum of the carbon atoms in the fatty acid moieties.

TABLE VII

REPRODUCIBILITY OF THE DETERMINATION (C.V.) OF THE INDIVIDUAL CLASSES OF PHOSPHOLIPIDS BY TLC—FID

Sample	Phosphatidyl-ethanolamine		Phosphatidyl-inositol		Phosphatidyl-serine		Phosphatidyl-choline		Sphingomyelin		Lysophosphatidyl-choline	
	Content*	C.V.**	Content	C.V.	Content	C.V.	Content	C.V.	Content	C.V.	Content	C.V.
Model mixture***	18.4	5.4	—	—	—	—	43.7	3.3	25.6	4.8	7.8	10.0
Blood Phospholipids	14.9	5.4	3.4	27.6	3.1	23.8	57.7	1.8	15.1	4.4	5.7	15.1

*Mass per cent.
**Calculated for a series of five determinations of the same sample carried out in a single day.
***The model sample also contained 4.5% lysophosphatidylethanolamine.

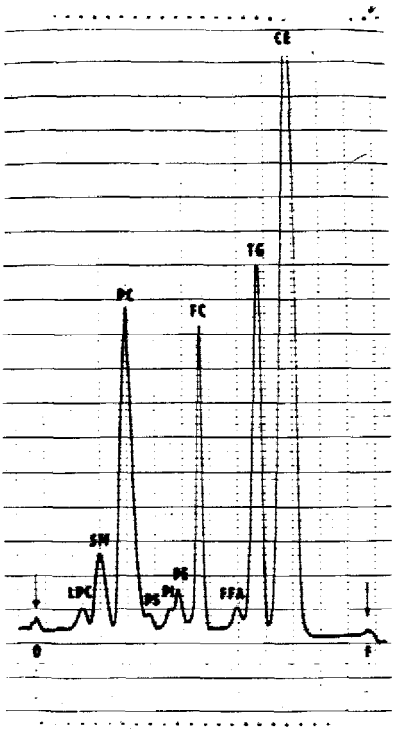

Fig. 3. Analysis of human blood plasma lipids by TLC—FID on Chromarods S-II. Peak designation: LPC = lysophosphatidylcholine, SM = sphingomyelin, PC = phosphatidylcholine, PS = phosphatidylserine, PI = phosphatidylinositol, PE = phosphatidylethanolamine, FC = cholesterol, FFA = free fatty acids, TG = triglycerides, CE = cholesteryl esters. 0 = start, F = front. The detailed analytical conditions are given in the text.

phospholipids is basically not different from the reproducibility of the determination of other components, and the results obtained here are in agreement with the literature data [13]. A survey of the results obtained is given in Table VII. The separation ability of Chromarods is clearly demonstrated in Fig. 3.

DISCUSSION

The detection limit for TLC—FID is about 50 ng per component, for GC of neutral lipids about 5 ng (12.5 μg/ml plasma for TLC—FID and 1.25 μg/ml plasma for GC under the given experimental conditions). The linear range for both methods is similar, about 0.5—20 μg. The main difference between the methods is the possibility to use GC for quantitative analysis in the non-linear range of calibration (up to 10 ng), whereas for TLC—FID such a possibility is not yet described.

It is apparent from the results given that the variability of the determination of cholesterol, cholesteryl esters, triglycerides and phospholipids by TLC—FID depends on a number of factors: quality of the separation rods, relative content and chemical composition of individual lipid classes in the sample analyzed, and also some other factors which affect separation, detection and quantitation of analyzed compounds. The dependence on the quality of the separation rods

is demonstrated in Table III. The effect of the relative content and chemical composition of the sample is apparent from comparison of the results in Tables III and V. For the biological samples higher values of the day-to-day coefficients of variation were observed in comparison with the corresponding values measured for the model synthetic sample. A possible explanation of this difference is the lower chemical stability of the unsaturated substances contained in the biological samples compared with the model samples. A similar effect was not observed in GC. However, it is not possible to make a simple conclusion from the given data, because of the simultaneous effect of several factors including the actual amount of all components analyzed. Study of an isolated effect of the individual factors was not the aim of this paper.

In agreement with other authors [14], we found that the optimal reproducibility in TLC—FID can be obtained in the range 1—10 μg of separated substance. When the sample contains smaller amounts of components, the reproducibility of the determination of these substances decreases very rapidly. The dependence of the reproducibility of the measurement on the sample size is depicted in Fig. 4. There is a similar dependence in GC in the region of non-linear calibration dependences [4, 5]. Ionization of the separated substances occurs during the GC detection process, so that loss of the separated substances results primarily from irreversible sorption or thermal decomposition during the analysis [5, 15]. In TLC—FID, the principle of detection is based on the pyrolysis of the substances that are adsorbed on the carrier, resulting in the effect of the material of thin layer and its structure. A further factor that has a negative effect on the reproducibility of analysis by TLC—FID is the reproducibility of the integration. As the whole detection in the flame ionization detector takes of the order of tens of seconds per rod, the peaks of all the

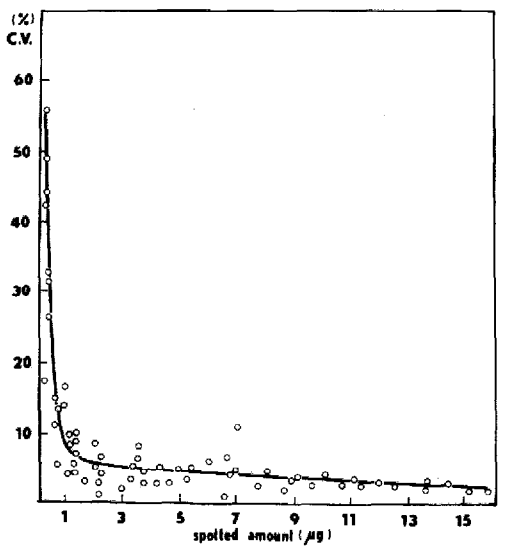

Fig. 4. Dependence of the reproducibility of the results of blood lipid analyses by TLC—FID on Chromarods S-I on the amount of substance analyzed. The dependence was measured for cholesteryl esters, triglycerides, cholesterol and phospholipids. No substantial differences between individual blood lipid classes were observed at comparable concentrations.

components are quite sharp, leading to a negative effect on the integration using common integrators, which are not constructed for such rapid processes. In spite of this lower reproducibility attained so far, TLC—FID is finding ever wider application in lipid analysis. Lower reproducibility can frequently be compensated by a greater number of determinations on a single sample, which is not a drawback, considering the high capacity of this method. A further advantage of TLC for routine clinical biochemistry of lipids is the fact that this method in the usual arrangement does not separate lipid classes into further fractions according to the molecular weight or other criteria. Together with the high capacity, this property makes TLC—FID useful for study of large sets of samples where further separation of the lipid classes is not required. The ability of TLC to analyze phospholipids and even to separate them according to class without separation of neutral lipids from the sample prior to the analysis is a further advantage (see Fig. 3).

On the other hand, GC has been found useful where high reproducibility and precision are required, or more detailed separation of lipid classes where the duration of the analysis is not an important factor. However, the development of TLC—FID suggests that further improvements in the integration could rapidly lead to a great increase in the reproducibility of measurements with this method. Some results obtained on selected rods confirm this suggestion [16, 17]. If it is possible to obtain a reproducibility in the determination of lipids comparable to that of GC, then TLC—FID will become an irreplacable tool in clinical biochemistry and in the study of lipid metabolism.

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