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FAECAL LIPID CHROMATOGRAPHY

I. QUANTITATIVE DETERMINATION WITH CHROMARODS

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SUMMARY

A new and original method is proposed for the qualitative and quantitative analysis of faecal lipids by thin-layer chromatography and detection through the flame ionization detector of an analyser (the Iatroscan TH 10). This method enables the rapid quantification of the different faecal lipid classes, including cholesterol, with great accuracy and reproducibility. In-series operations are possible with easy manipulation.

INTRODUCTION

Faecal lipid measurement is of the greatest interest in clinical biology for the appreciation of assimilation diseases [1,2]. Several techniques have been used for this analysis: gravimetric [3,4], volumetric [5,6], spectrophotometric [7—9], chromatographic [10—12] and colorimetric [13—15]. These methods are time-consuming, impractical, unsuitable for serial analysis and involve manipulations with offensive material. The method proposed here eliminates certain difficulties by using Folch reagent [16] for extraction, thin-layer chromatography (TLC) on Chromarods for the separation of different lipid classes and flame ionization detection (FID) for quantification.

MATERIALS

Reagents

All reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). The other reagents, all analytical grade, were obtained from E. Merck (Darmstadt, F.R.G.).

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Apparatus and operating conditions

An Iatroscan TH 10 Mark II analyser (Iatron Labs., Tokyo, Japan), described by Sipos and Ackman [17], was used equipped with a recorder electronic stepping integrator IRC 1 B (Intersmat, France). FID was performed with a hydrogen flow-rate of 160 ml/min. Clean outside air was supplied by an aquarium pump at a flow-rate of 2100 ml/min. The intensity of the current proportional to the organic compounds during pyrolysis was recorded. The recorder chart speed was proportional to the Chromarod scanning speed in the flame which was 0.32 cm/sec. The recorder speed was 0.20 cm/sec and the attenuation rate was 14 mV.

METHODS

Extraction procedure

The faecal lipid extract was recovered by extraction from 1 g of homogenized faeces to which 50 ml of Folch reagent (chloroform—methanol, 2:1, v/v) had been added. Two sonifications made optimal extraction possible. A 0.9% sodium chloride solution (10 ml) was added to the mixture obtained to eliminate the impurities in the upper phase. The lower phase containing the total lipids was separated from the upper phase by decantation, and then filtered. An aliquot (20 ml) was evaporated under reduced pressure and the dry residue was dissolved in 2 ml of chloroform.

Chromatography

The Chromarods used for TLC were cylindrical quartz rods 152 × 0.9 mm, covered with a sintered silica gel, type S II (5 μm particle size). Prior to use, the Chromarods were activated in the oven at 110°C for 2 h and then were passed through the FID. A 1 μl volume of faecal extract was spotted on the Chromarod 1 cm from one of the ends. In order to ensure identical conditions, another rod was spotted with a standard mixture containing a known amount of each lipid class. The two rods were then placed in a special rod holder frame. This frame was then placed in a glass tank lined with filter paper which had been saturated for 20 min with a solvent mixture benzene—chloroform—formic acid (35:15:1, v/v/v). The migration time was 30 min at 22—25°C. On removal from the tank, the rods were dried at 60°C for 5 min and transferred to the scanning frame of the analyser. Every peak was identified on the basis of retention time and in comparison with the standard mixture analysed under the same conditions. The detector response for the standard mixture was used to calculate the concentration of lipids in the biological sample.

RESULTS

Linearity of detector response

The study was performed using a standard mixture containing increasing amounts from 1 to 10 mg/ml of each lipid: L-α-phosphatidylcholine, monostearyl-*rac*-glycerol, D-1,2-dipalmitin, cholesterol, stearic acid, tristearin and cholesteryl stearate. Each standard mixture was analysed with the Iatroscan ten times during one week. The mean of the peak squares and the

extreme values calculated by the integrator that were obtained from the different concentrations of each lipid fraction, led us to examine the function $Y = f(X)$ where Y represents the concentration (mg/ml) and X the square (μV/sec), in order to find the best correlation coefficient (R). The quality of the linear relationship between the theoretical values and those calculated by the integrator was thus obtained using this function. As shown in Table I, the function $Y = A + BX + CX^2$ gave the best correlation coefficient.

Fig. 1 shows the quadratic regression lines obtained for each lipid fraction. Since the value of C is very small (for example, $20 \cdot 10^{-11}$), the detector response must be considered as linear, and thus suitable for analysing small amounts of lipids similar to those found in faecal extracts.

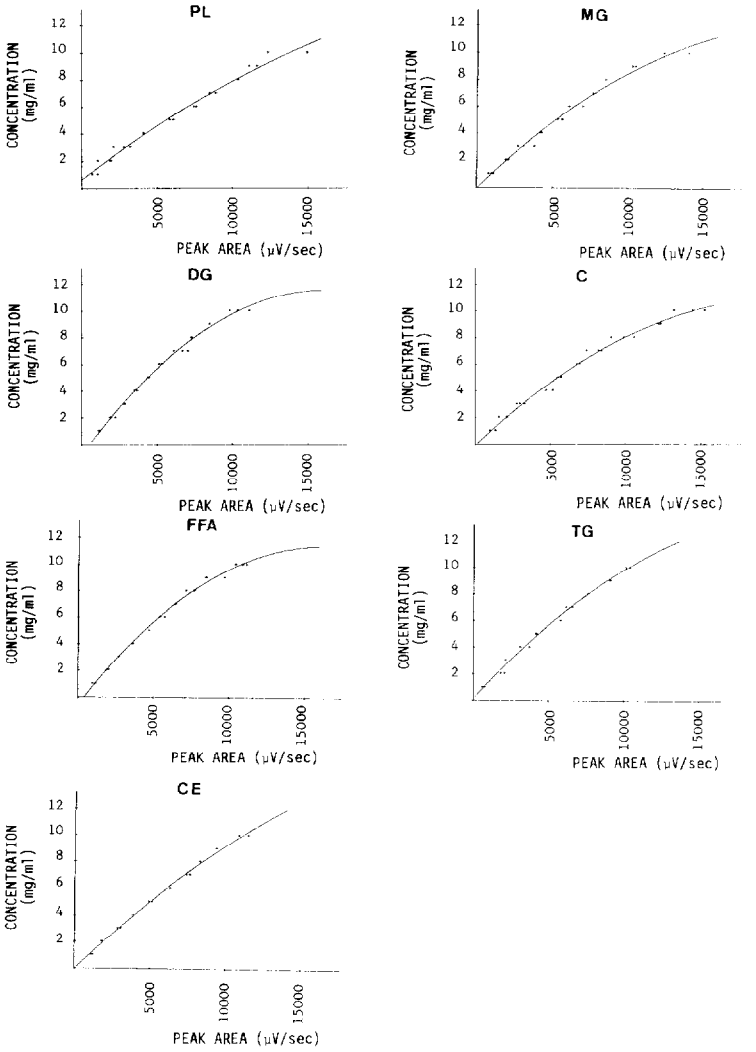

Fig. 1. Quadratic regression lines obtained for each lipid fraction by plotting concentrations versus peak areas.

TABLE I

DETECTOR RESPONSE: STUDY OF DIFFERENT FUNCTIONS $Y = f(X)$ GIVING THE BEST CORRELATION COEFFICIENT FOR EACH LIPID FRACTION

Lipid fraction*	Correlation coefficient (R)							
	$Y = A+BX$	$Y = A + \frac{B}{X}$	$\frac{1}{Y} = A + \frac{B}{X}$	$Y = A+B\sqrt{X}$	$Y = Ae^{BX}$	$Y = AX^B$	$Y = A+(B \log X)$	$Y = A+BX+CX^2$
PL	0.98	0.58	0.89	0.97	0.85	0.97	0.89	0.99
MG	0.98	0.59	0.96	0.98	0.84	0.99	0.90	0.99
DG	0.97	0.73	0.99	0.99	0.83	0.98	0.95	0.98
C	0.97	0.70	0.93	0.99	0.81	0.97	0.94	0.99
FFA	0.98	0.73	0.98	0.99	0.85	0.99	0.95	0.99
TG	0.99	0.56	0.96	0.98	0.85	0.98	0.89	0.99
CE	0.99	0.73	0.98	0.99	0.87	0.99	0.94	0.99

*For abbreviations see Fig. 6.

TABLE II

REPRODUCIBILITY OF MEASUREMENTS OF THE LIPID CLASSES IN A STANDARD MIXTURE CONTAINING 5 mg/ml OF EACH LIPID FRACTION AND IN A BIOLOGICAL SAMPLE

Results are given by peak area values expressed as mean ± S.D. and C.V. of thirty determinations over three days.

Lipid fraction*	Standard mixture		Biological sample	
	Mean ± S.D. (mV/sec)	C.V. (%)	Mean ± S.D. (mV/sec)	C.V. (%)
PL	56.25 ± 0.86	1.53	72.42 ± 1.03	1.42
MG	54.11 ± 0.95	1.76	2.77 ± 0.53	2.10
DG	45.19 ± 0.87	1.93	5.03 ± 0.09	1.79
C	57.06 ± 0.88	1.54	136.49 ± 1.14	0.84
FFA	46.24 ± 0.92	1.99	70.89 ± 1.25	1.76
TG	41.05 ± 0.86	2.10	6.39 ± 0.13	2.03
CE	47.07 ± 0.96	2.04	2.59 ± 0.06	2.32

*For abbreviations see Fig. 6.

Reproducibility

The reproducibility of the measurement of all lipid classes was expressed by the standard deviation (S.D.) and the coefficient of variation (C.V.) calculated from the analysis of a pure standard mixture and a sample of faecal lipid extract. Three series of ten rods were analysed over a three-day period according to the method described. From the results presented in Table II it can be seen that the accuracy and the reproducibility of the method are satisfactory.

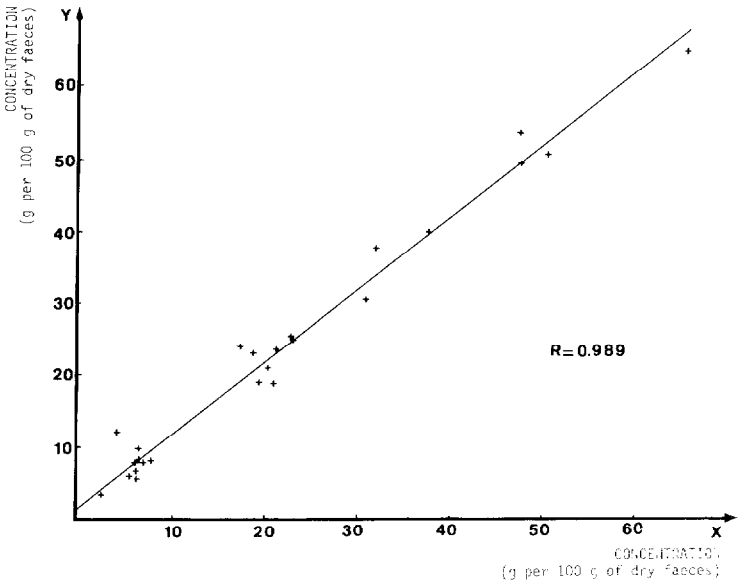

Fig. 2. Analysis of total lipids (PL, MG, DG, FFA, TG, CE) using the Van den Kamer method (abscissa) and chromatography with Chromarods: correlation between the methods.

Comparison between Iatroscan and Van den Kamer's techniques

The results obtained by the proposed method and those obtained with a classical acidimetric method, such as Van den Kamer's, on faecal extracts from hospitalized subjects were compared. The weights of total lipids, expressed in g per 100 g of dried stool, analysed by the two techniques are compared in Figs. 2 and 3. The measurement of dried stool was obtained using the

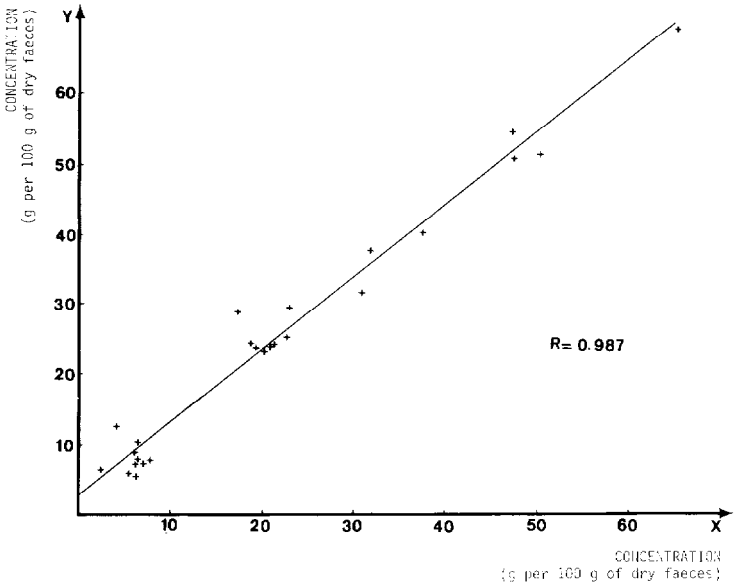

Fig. 3. Analysis of total lipids by both methods: the chromatography method including cholesterol in total lipids as opposed to the acidimetric (Van den Kamer) method (abscissa).

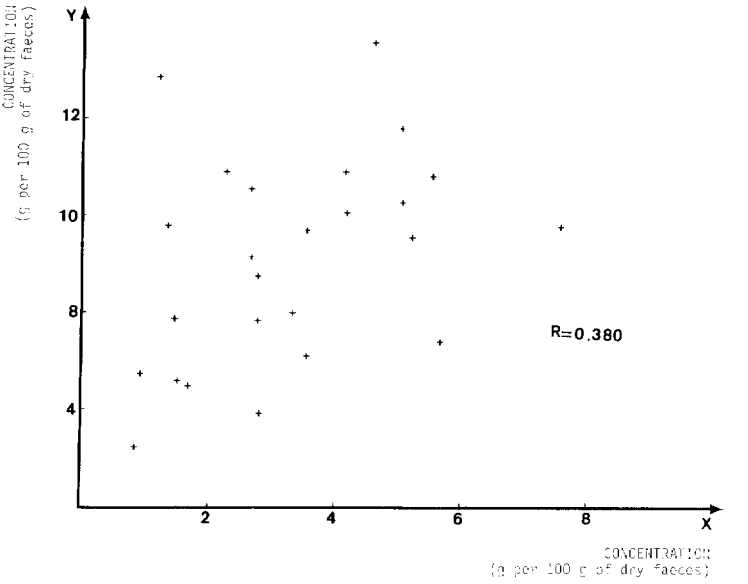

Fig. 4. Study of the correlation between both methods of analysis of polar and neutral fats. Van den Kamer's method: abscissa.

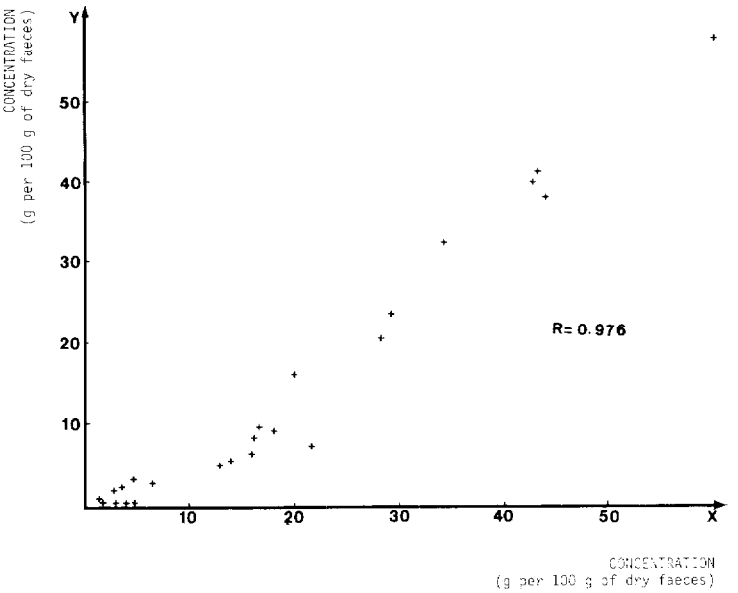

Fig. 5. Study of the correlation between both methods of analysis of free fatty acids. Van den Kamer's method: abscissa.

gravimetric method after infrared treatment or with the Karl Fisher method [18], water being extracted with Folch reagent. The correlation of the analysis of the different lipid classes between these two techniques is presented in Figs. 4 and 5.

DISCUSSION

For development, all other authors have used a solvent composed of diethyl ether, hexane and an organic acid such as formic acid [19,20] in different proportions. The effects of the proportion of this solvent were very noticeable on the resolution of some lipid classes, in particular free fatty acids and triglycerides [21], separation of which was very difficult to perform. As Fig. 6 shows, the migration solvent proposed in the proportion described above enables total separation of faecal lipids.

Fig. 2 shows that, for total lipid analysis, the correlation between the classical technique and the proposed method is quite satisfactory. Moreover, cholesterol contained in the unsaponifiable fraction was not analysed with the acidimetric method and so was not included in total faecal lipids. The correlation of the total faecal lipids between the Van den Kamer method and the proposed method, where cholesterol is included, can be seen in Fig. 3. The correlation coefficient can be seen to decrease slowly; therefore, cholesterol represents a substantial fraction because a solid food diet can produce as much as 3 g of cholesterol excreted in the faeces. To compare the different lipid fractions, the proposed method was used to calculate hydrolysed fats corresponding to free fatty acids, and both polar and neutral fats corresponding to phospholipids, glycerides, and cholesteryl esters; on the other hand, the acidimetric technique allows the evaluation of only the two latter

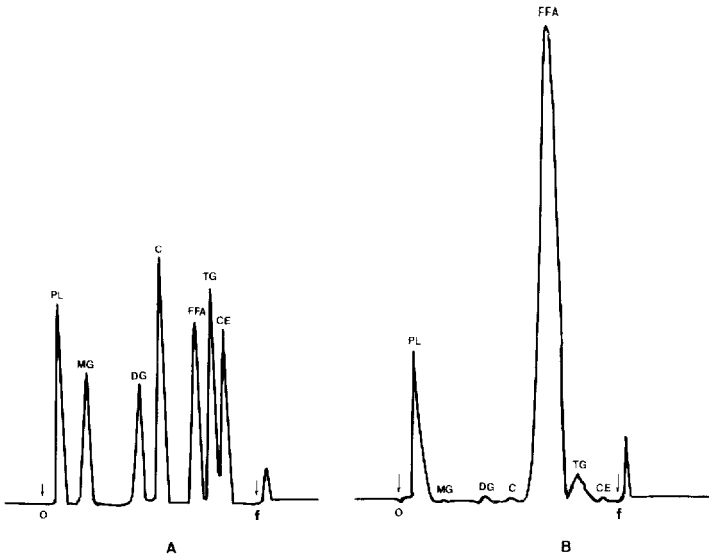

Fig. 6. Chromatograms showing the separation of neutral lipid classes on Chromarods with solvent benzene—chloroform—formic acid (35:15:1). (A) Separation of standard mixture. Peak designation: PL = L-α-phosphatidylcholine; MG = monostearyl-*rac*-glycerol; DG = D-1,2-dipalmitin; C = cholesterol; FFA = stearic acid; TG = tristearin; CE = cholesteryl stearate. (B) Separation of a human faecal extract. Peak designation: PL = phospholipids; MG = monoglycerides; DG = diglycerides; C = cholesterol; FFA = free fatty acids; TG = triglycerides; CE = cholesteryl esters. o = start; f = front.

lipid fractions. Unlike total lipids, the correlation of the results obtained for the analysis of polar and neutral fats on the two methods is very poor (Fig. 4). This is for three reasons. First, extractions performed with the classical method are not homogeneous for the different lipid fractions because of their various amphipathic properties. The water in faeces modifies the separation coefficient, so our procedure extraction mobilizes all the water while making all the lipids soluble. Secondly, when acid hydrolysis is being performed, some esterified lipids are hydrolysed into free fatty acids, and this overestimates their values. Thirdly, the quantification process used in the classical method is debatable. The polar and neutral fats have very different molecular weights and are expressed in tristearin equivalents; this tends largely to undervalue them.

The technique proposed here eliminates these disadvantages by individualizing every lipid fraction representing polar and neutral faecal fats. However, the correlation of the free fatty acid analysis between the two methods is quite sound (Fig. 5). These two methods may therefore be used to analyse molecules with identical molecular weights expressed by the same unit (stearic acid equivalent). The only difference is that the classical method does have a tendency to overestimate the free fatty acid values.

Lipid chromatographic analysis is of interest for analytical and diagnostic reasons. As regards the analytical aspect, this method gives more exact and more realistic results than the classical acidimetric methods: every lipid fraction can be analysed separately, in particular cholesterol, which is something that many classical methods cannot do although it represents a fraction that is not negligible.

Moreover, ten different stools can be analysed at the same time. This makes in-series operation possible.

The resulting method is very quick, effective, and does not need any manipulation other than extraction with Folch reagent and the spotting of the lipid extract on the Chromarods. The study of the reproducibility in the biological sample indicates that it is as good for the low amounts of separated lipids as for the high.

This method has such a degree of precision that it could be used as a reference technique to calibrate apparatus like the Infraalyzer (Technicon), which performs total faecal lipid analysis by near infrared reflectance analysis (NIRA) [22,23]. The quality of the results obtained by the NIRA technique depends only on the precision of the calibration set [24] that the TLC—FID technique permits. As for biological diagnosis, the quality of the results obtained and the ease of their interpretation could be of valuable assistance to clinicians.

CONCLUSION

A simple, rapid and accurate technique used for in-series operation is proposed for the analysis of the different faecal lipid classes. The great precision of this method means that it could be used as a reference technique to create a calibration set.

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