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# ANALYTICAL AND PREPARATIVE COLUMN CHROMATOGRAPHY OF NEUTRAL LIPIDS WITH CONTINUOUS MONITORING OF THE ELUATE BY A FLAME IONIZATION DETECTOR

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SUMMARY

A method is described for the fractionation of neutral lipids into classes by means of silicic acid column chromatography. Elution is performed with a concave gradient of ethyl ether in petroleum ether; the gradient is achieved by means of two metering pumps. This gradient is simple to obtain and can be easily modified for different requirements following our scheme of calculation. The effluent is monitored continuously by means of a hydrogen flame ionisation detector, a part of the effluent being continuously drawn off. Our method can be used for analytical purposes as well as for isolation of pure lipidic fractions.

The quantitative analysis of the components of a lipid mixture is performed following a weighing microtechnique, using a Cahn electrobalance.

Examples are reported of the separation of reference lipid mixtures of oils and tissue lipids and of oil solutions of steroids for pharmaceutical use.

Our method is also useful for detecting lipidic contaminants in solvents and materials for chromatography. The necessity of very carefully controlled solvents and materials to avoid contamination is underlined.

## INTRODUCTION

Methods for the separation of neutral lipids into classes by column chromatography have been reported by many authors, where many different elution systems, stepwise[1–21] or with a continuous gradient[5,21–25], have been used together with different techniques for the analysis of the eluates, based in most cases on colorimetric measurements, weighing[5,8,9] or radioactivity measurements[15]. In a great majority of cases the use of colorimetric techniques specific for the analysis of various classes, while adequate for analytical purposes, does not, however, always decisively show whether the procedure is suitable for the isolation of a fraction of good purity.

In our research concerning the lipid composition of organs and tissues of animals under different experimental treatments[26], we developed a method for the fractiona-

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tion of neutral lipids into classes, attempting to satisfy preparative and analytical exigencies as well as those inherent with the application of the method, which we tried to automate as much as possible.

In order to achieve this aim, we studied a procedure which had to fulfil the following conditions:

(a) That it had to have a minimum of contamination of the eluate by the elution system and the apparatus;

(b) That it permitted the separation of a lipid mixture during one working day, which limits the quantity analyzed to suit the other parameters on which the analytical procedure depends. On the other hand the quantity of mixture analysed must be such as to permit a weighing technique for the determination of the main fractions;

(c) That it guaranteed good resolution during the separation, compatible with the composition of the mixtures studied;

(d) That the development of the column was achieved in a highly efficient manner with respect to the separation effects according to LAKSHAMANAN AND LIEBERMAN[27] and to practical technical arrangements, that is by a solvent mixture of continuous varying composition following a concave concentration gradient, as studied by us;

(e) That the composition of the eluates was controlled in an as efficient and practical way as possible, that is by a continuously automated system. Owing to the nature of the components under study, this was done by using an apparatus based on the continuous transport of an aliquot of the eluate through a hydrogen flame ionization detector, with registration of the responses as described in refs. 28–31.

Such a method for the fractionation of neutral lipids from organs and for the isolation of pure lipid fractions has been developed. Its whole realization, particularly with respect to the flexibility with which gradients are obtained and to the use of an automated detector for the eluted fractions, prompted us to describe this method, which might also be of interest for the solution of analogous problems.

## EXPERIMENTAL

### *Solvents and materials for column chromatography*

Petroleum ether for chromatography, boiling range 65–75°, was twice distilled before use after filtration through a silicic acid column. This solvent is subsequently called petroleum ether (P.E.) in the text. Ethyl ether was freed of peroxides by passage over basic aluminium oxide and double distillation. Double distilled methanol was used. Silicic acid (Mallinckrodt, 100 mesh) was sieved through a 300 mesh sieve and solvent treated as described by HIRSCH AND AHRENS[5]. According to HIRSCH AND AHRENS, analogous results can be obtained using Biorad silicic acid for chromatography when prepared as described by these authors. Silicic acid selected in this way is heated to 125° for 8 h in an oven, cooled in a desiccator and 9.38 ml water are added to 100 g of the product. The water content is theoretically 8.58 % but in reality it is a little higher, because, as mentioned by BÖTTCHER *et al.*[32], silicic acid even when heated for more than 2 h at 120° retains a practically constant quantity of water, about 1.2 %, as demonstrated by titration with Fisher reagent.

### *Chromatographic columns*

LKB glass columns, type 4200, of diameter 9 and 12 mm, and 30 cm in length

are used, and columns of 6 and 25 mm diameter of the same length are also used. Analogous results are obtained with glass columns constructed in the laboratory, 11 mm in diameter, 27 cm length, terminating in two cones (No. 12) into which two teflon conical plugs designed for minimal dead volume are introduced. The connections between the columns and other apparatus are made by teflon tubes, O.D. approx. 1/16 in. (1.58 mm), I.D. 1–1.2 mm. In order to introduce the adsorbent in the columns, the latter are half filled with solvent, and silicic acid suspended in petroleum ether is gradually poured in, allowing the solvent to slowly flow out of the column. 5 g silicic acid are normally used for the 9 and 12 mm diameter columns giving a height of adsorbent in the column of 155 and 95 mm ($H/d$ 17.3; 7.9) respectively; for the 6 mm diameter columns, a maximum of 3.5 g of silicic acid is allowed ($H$ 250 mm, ratio $H/d$ 42.0); for 25 mm diameter columns, 23 g of silicic acid ($H$ 90 mm, ratio $H/d$ 3.6) are normally used.

*Sample preparation*

The following compounds are used as reference lipids: squalane; cholesterol stearate or palmitate; tripalmitin; palmitic acid; cholesterol from ASI or Calbiochem, purified by us by column chromatography as described in this paper until a single component is obtained. Glyceryl distearate (a mixture of 1,2- and 1,3-isomers) and glyceryl monostearate were prepared by us from the commercial product according to QUINLIN AND WEISER[33] and successively rechromatographed. Testosterone propionate was of analytical grade, controlled by UV spectrophotometry and thin-layer chromatography; the natural olive oil was an authentic sample from our Institute. Mixtures of the reference lipids were prepared by weighing on a Cahn electrobalance, model Gram. Total lipids of rat liver were obtained by extraction with chloroform–methanol (2:1) according to FOLCH *et al.*[34]. The total lipid content of an aliquot of the extract was determined by weighing to a constant weight in a vacuum desiccator.

*Charging the column*

*Direct method.* The sample, dissolved in as small a volume as possible (1–2 ml of petroleum ether), is pipetted on to the column, and washed with the same solvent (3 × 1 ml), allowing a slow efflux by gravity. The empty part of the column is refilled with the solvent and the upper part is connected to the feeding system.

*System with an injector.* A simple and efficient injector is obtained by introducing into the small feeding tube, immediately before it enters the column, a three way LKB joint, type 3065 A, modified by substituting the glass wedge and the tube lock in one arm with a teflon wedge, terminating with a flat face on the other end, on which a grey rubber septum (Perkin-Elmer for GLC) is fixed by the nut. For the injection, a Hamilton precision syringe with teflon plunger (gas tight type) for the assurance of perfect fit, is used. Normally approx. 20 mg of neutral lipids are chromatographed in columns containing 5 g silicic acid, that is 4 mg/g (1/250), rising to as much as 7.5 mg/g. In the preparative columns the sample is approx. 85–100 mg for 23 g silicic acid.

*Feeding system for the column*

The solvent is pumped into the column by a metering pump, type CH MM 1-B 24

Milton Roy, with an adjustable flow rate ($R_2$ ml/min). The gradient of ether in the petroleum ether is obtained by pumping, with another metering pump with a flow rate $R_1$, a volume $V_R$ of ether from the reservoir into a mixing chamber (equipped with a magnetic stirrer) containing a volume $V_M$ of petroleum ether. The expression which gives the shape of the gradient as a function of the flow rates $R_1$ and $R_2$ and the volumes $V_R$ and $V_M$ is derived from WREN's general equation[35] described for U-connected vessels, as results from the following development.

In the most complete form, WREN's equation is

$$C_v = C_R - (C_R - C_M) \cdot \left(1 - \frac{v}{V_R + V_M}\right)^P \tag{1}$$

where

$$P = \frac{d_R^2 \cdot \rho_M}{d_M^2 \cdot \rho_R} \tag{2}$$

were $C_R$ and $C_M$ are the initial concentrations of the solvent forming the gradient (in this case ether) in the reservoir and in the mixing chamber; $C_v$ is the concentration of the liquid which is present in $M$ at the moment when a volume $v$ has run off to feed the column; $A_R$ and $A_M$ are the areas of the reservoir ($R$) and mixing ($M$) vessels; $d_R$ and $d_M$ are the respective diameters and $\varrho_M$ and $\varrho_R$ are the densities of the liquids contained in $M$ and $R$. The simplified form where

$$P = V_R/V_M = A_R/A_M \tag{3}$$

is used instead of

$$\frac{d_R^2 \cdot \rho_M}{d_M^2 \cdot \rho_R}$$

when the liquids have the same density or, on the other hand, when the movement of both liquids can be made independent of their density. As mentioned above, we have achieved this by transferring the liquids by means of two accurately regulated metering pumps. The flow rates of the two pumps $R_1$ (transfer) and $R_2$ (feed to column) are related to the volumes to be transferred during the same time interval by the simple expression

$$P = \frac{V_R}{V_M} = \frac{R_1}{R_2 - R_1} \tag{4}$$

$P$ is the power of eqn. (1) and the gradient is characterized by its value. From eqn. (4) and the total volume of the eluate

$$V_T = V_R + V_M \tag{5}$$

it can be derived that

$$V_M = \frac{V_T}{P + 1} \tag{6}$$

and

$$V_R = P \cdot \frac{V_T}{P + 1} \tag{7}$$

For a concave gradient[27,35,36] $P < 1$*.

Applying these simple equations (1, 4, 5, 6, 7) one is able to calculate the experimental data necessary to obtain the chosen gradient.

In our experiments $P = 1/4$ was chosen, and thus $V_R = 1/5\ V_T$ and

$$V_M = \frac{4}{5} V_T \text{ and } \frac{R_1}{R_2 - R_1} = \frac{1}{5 - 1} = \frac{1}{4}$$

and $R_2 = 5$ and $R_1 = 1$. Therefore if the flow rate $R_2$ is fixed as 1 ml/min, $R_1$ is $1/5\ R_2$, *i.e.* 0.2 ml/min. For $C_M = 0$ (no solvent producing the gradient in the mixing chamber at time 0), eqn. (1) is simplified to

$$C_v = C_R - C_R \left(1 - \frac{v}{V_T}\right)^P \tag{8}$$

When the concentrations and volumes are expressed in percent,

$$C_v\,\% = 100 - 100 \left(1 - \frac{v}{100}\right)^P \tag{9}$$

from which equation, for $P = 1/4$, it is easy to draw the theoretical graphical expression of the gradient (Fig. 1a) or a table. After the definition of the gradient shape by the parameter $P$ ($R_1$ and $R_2$), it only remains to establish the total volume of the eluate $V_T$, which is dependent on the chromatographic problem, that is on the column volume; $V_R$ and $V_M$ can then be obtained from eqns. (6) and (7).

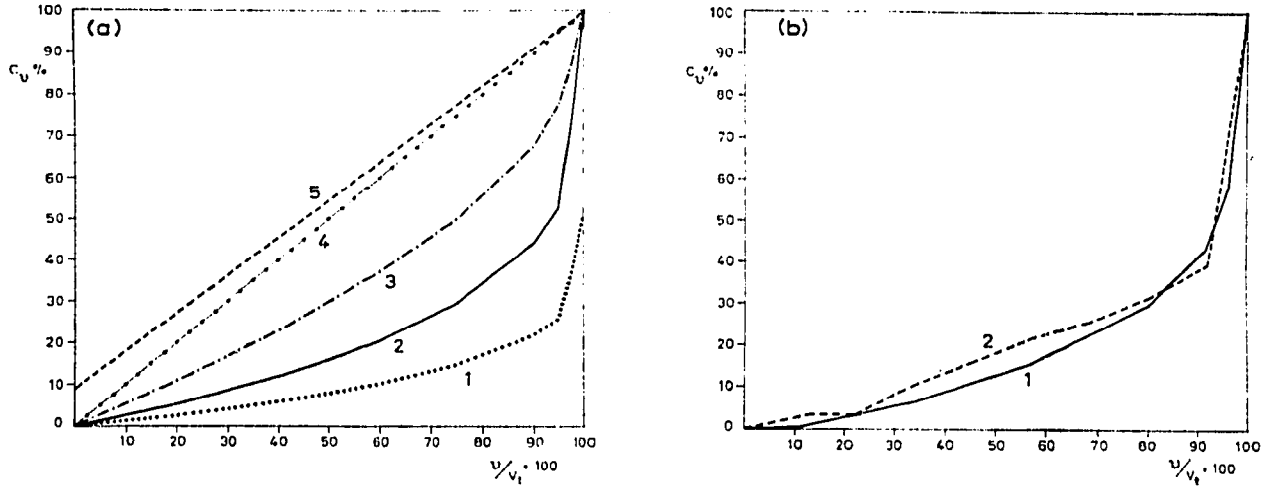


Fig. 1 (a) Theoretical gradient curves: (1) Concave $P = 1/4$, $C_R$ 50 % ethyl ether (e.e.) in petroleum ether; (2) concave $P = 1/4$, $C_R$ 100 % e.e.; (3) concave $P = 1/2$, $C_R$ 100 % e.e.; (4) linear, $C_R$ 100 % e.e.; (5) linear, $C_R = 100$ % e.e., from $C_M = 9.5$ % e.e. in petroleum ether. (b) Experimental gradient curves: (1) concave, $P = 1/4$, $C_R = 100$ % e.e. with pump, as in the text; (2) concave $P = 1/4$, $C_R = 100$ % e.e. with siphon communicating bottles.

* The concavity is further accentuated for $P \to 0$.

In most of our experiments, $V_T$ was 430 ml, $V_R$ 86 ml of ethyl ether, $V_M$ 344 ml of petroleum ether, $R_2 = 1$ ml/min and $R_1 = 0.2$ ml/min. These volumes were chosen so as to obtain a gradient which permits a separation analogous to that obtained by stepwise feeding of the column, with mixtures of petroleum ether–ethyl ether (v/v) in the following proportions and amounts: 99:1, 80 ml; 96:4, 70 ml; 92:8, 150 ml; 75:25, 60 ml; ethyl ether 70 ml. To obtain a better separation of the hydrocarbon fraction, our gradient is preceded by a short elution with 30 ml of petroleum ether. Also a final elution with 60 ml of ethyl ether shortens the recovery time for the monoglycerides.

For columns of 25 mm diameter and approx. 25 g of silicic acid, the gradient is of the same type, with $V_T = 1865$ ml, $V_R = 373$ ml of ether and $V_M = 1492$ ml petroleum ether, preceded by an elution with 50 ml petroleum ether and followed by an elution with 260 ml ethyl ether (total development time 2175 min, *i.e.* 36 h and 15 min).

The experimental confirmation of the gradient curve is carried out by measuring the concentration of ethyl ether in petroleum ether at various points of the elution (at various values of $v/V_T \cdot 100$). In order to measure the concentration of ethyl ether it is coloured with Sudan III red and this permits an easy spectrophotometric determination of $C_v\%$ ($C_v\% = E/E_{tot} \cdot 100$)*.

A gradient, obtainable with two vessels communicating at the bottom so that the respective areas were $A_R/A_M = 1/4$ ($d_R/d_M = 1/\sqrt{4}$) ($\varrho_R = \varrho_M$ approx.) was also studied, but we did not obtain regular or reproducible results (Fig. 1b).

*Analysis of eluates*

In order to monitor the various fractions eluted from the column as concentration peaks, a part of the eluate from the column is transferred onto a chain conveyor, which, after the solvents have been evaporated to dryness, introduces the solid residues into a hydrogen flame ionization detector. A Liquid Chromatography Detector (LCD) Mod. 5400 of Barber Colman, constructed according to principles described in refs. 28–30 is used. The operating conditions for the instrument are as follows: attenuation × 100 (input $3 \times 10^{-10}$ A), with a 5 mV Leeds and Northrup Speedimax W recorder; nitrogen, hydrogen and air pressures 1.6, 2.2 and 3.8 kg/cm$^2$ respectively; chain speed regulator 9.7.

The splitting ratio was adjusted to 5–6 %, and this was obtained by a glass overflow splitter of the type described by WOODS[37] but was of a smaller size and covered at the top by a teflon plug. Our design of the splitter assures much less contamination of the last fractions of the chromatogram, reducing evaporation and the deposition of a residue that remains as a ring in the central part of the splitter cup. The needle used was a Hamilton No. 726, with a Kel-F hub. Apart from changing the needle, the splitting rates can be varied by varying the height of the splitter in respect to the chain; this has also been reported by LUND AND KUNSMAN[38], who described a different type of overflow splitter

The main part of the eluate is directed to a time controlled fraction collector (Radirac LKB 3400) which is synchronised to start with the start of the chromatography; every fraction is normally regulated to 10 min; the flow $R_2$ being 1 ml/min,

* The values of $C_v\%$ are reported graphically on the ordinate, with respect to $v/V_T \cdot 100$ on the abscissa (Fig. 1b).

the corresponding whole fraction volume $V_F$ is 10 ml; but since $V_S$ is the volume removed by the splitter, $V_F' = V_F - V_S$ where $V_F'$ = the actual fraction volume, collected in the tubes. $V_F'$ and $V_S$ are determined before the start of the experiment by a series of exact measurements, excluding losses by evaporation in the test tubes. In cases when a chromatogram lasts several hours and such losses cannot be avoided, the volume of the fraction is adjusted to the theoretical value $V_F'$ with petroleum ether before quantitative and qualitative measurement are carried out on the fractions.

The qualitative control of the eluates is done by thin-layer chromatography with convenient aliquots of $V_F'$, normally 1 ml per fraction or less for the main peak. This volume is evaporated to dryness in conical test tube and transferred, in a minimum volume of solvent, by a Hamilton microsyringe onto a plate, 20 × 20 cm, covered with Silica Gel G, thickness 0.5 mm.

The solvents used are (for those not previously specified, of analytical grade):

(a) Petroleum ether–ethyl ether–acetic acid (95:5:1) for less polar lipids (hydrocarbons and esters of sterols);

(b) Petroleum ether–ethyl ether–acetic acid (70:30:1) for neutral lipids;

(c) Chloroform–methanol–water (65:25:4) for more polar lipids (phospholipids).

Details of the technique are reported elsewhere[39].

The analysis of the eluates is performed by weighing. After confirmation of the identity of each fraction, 8 ml or less of each fraction volume $V_F'$, corresponding to a single peak are pooled, evaporated to dryness under nitrogen in a water bath at 40° or, for bigger volumes, in a rotating evaporator under vacuum or in a nitrogen atmosphere. The residues are quantitatively transferred with 3–4 aliquots of 100 $\mu$l petroleum ether into tared teflon microcups of approx. 50 mg weight (teflon weighing cups No. 2034 of Cahn Instruments) and after evaporation of the solvent on a heated thermoregulated plate at 40°, they are stored for 24 h in a desiccator and then weighed on a Cahn electrobalance (Gram model). After keeping the samples for a further 24 h in the desiccator, the weight, which generally remains constant, is recorded.

The results are calculated for the total volume $V_F$ of the fractions. The amount obtained for each single peak is referred to the total weight of the chromatographed substance, thus obtaining the percent composition of the mixture.

## RESULTS AND DISCUSSION

The pattern of a chromatographic separation performed under the conditions described in the text is shown in Fig. 2. The graph is a reproduction of a record obtained by the liquid chromatography detector (LCD). The resolution is optimal for hydrocarbons, esters of sterols, and triglycerides. The fatty acid peak is also separated from triglycerides and cholesterol, but the resolution of the cholesterol and diglyceride peaks is incomplete. In spite of this, the separation obtained by us can be favourably compared with those of other authors[5,9,10,22].

The pattern of the separation is not essentially modified when a column of 12 mm is substituted by one of 9 or 6 mm diameter. With this last type of column the ratio $H/d$ is about 42 instead of 7.9 for the 12 mm diameter column but there is no evidence of the advantages that are associated with the lengthening of the column[23,40]. Probably a higher $H/d$ ratio or a higher flow ratio might be used; certainly the choice of the best conditions for a chromatographic separation is not a

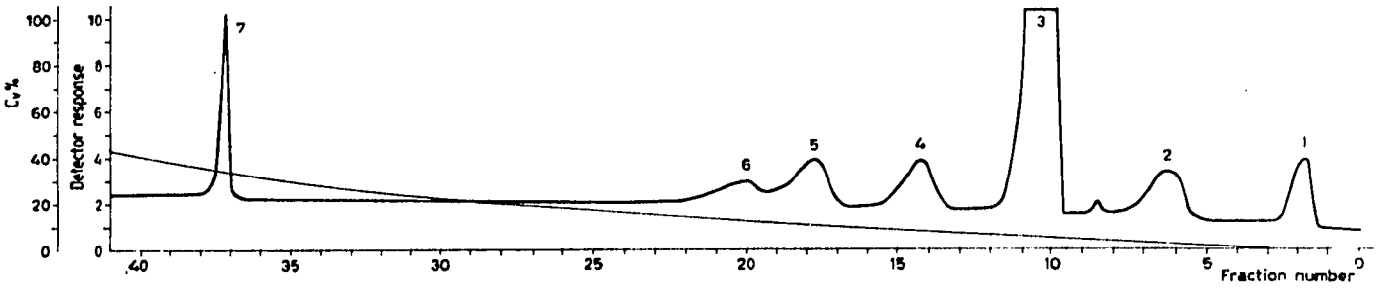


Fig. 2. Chromatogram of a reference lipid mixture. Column 12 mm diam. × 95 mm high, charged with 5 g silicic acid. Sample 20.23 mg. Detector sensitivity 3 × $10^{-10}$ A. Concave gradient ($P$ = 1/4), $C_R$ = 100 % ethyl ether in petroleum ether; column flow rate 1 ml/min. Splitting ratio: 6 % of the column flow by-passed to detector. Peaks 1 = squalane; 2 = cholesterol stearate; 3 = tripalmitin; 4 = palmitic acid; 5 = cholesterol; 6 = glycerol distearate; 7 = glycerol monostearate. The gradient curve is shown.

simple problem as discussed by HANAHAN *et al.*[41] in the case of the use of stepwise or continuous gradients in the column chromatography of phospholipids.

For example, a modification of the gradient, where it is subdivided into two parts, one concave of $V_T$ = 215 ml utilized for 56 %, with $C_R$ 50 % in ether and a successive linear gradient between 9 and 100 % of ether of $V_T$ = 190 ml, utilized for 63 % to $C_v$ = 66.5 %, does not improve the resolution of cholesterol and the diglycerides, but it does permit the elution of the monoglycerides in the gradient without the necessity of using pure ether at the end of the gradient (Fig. 3). It is

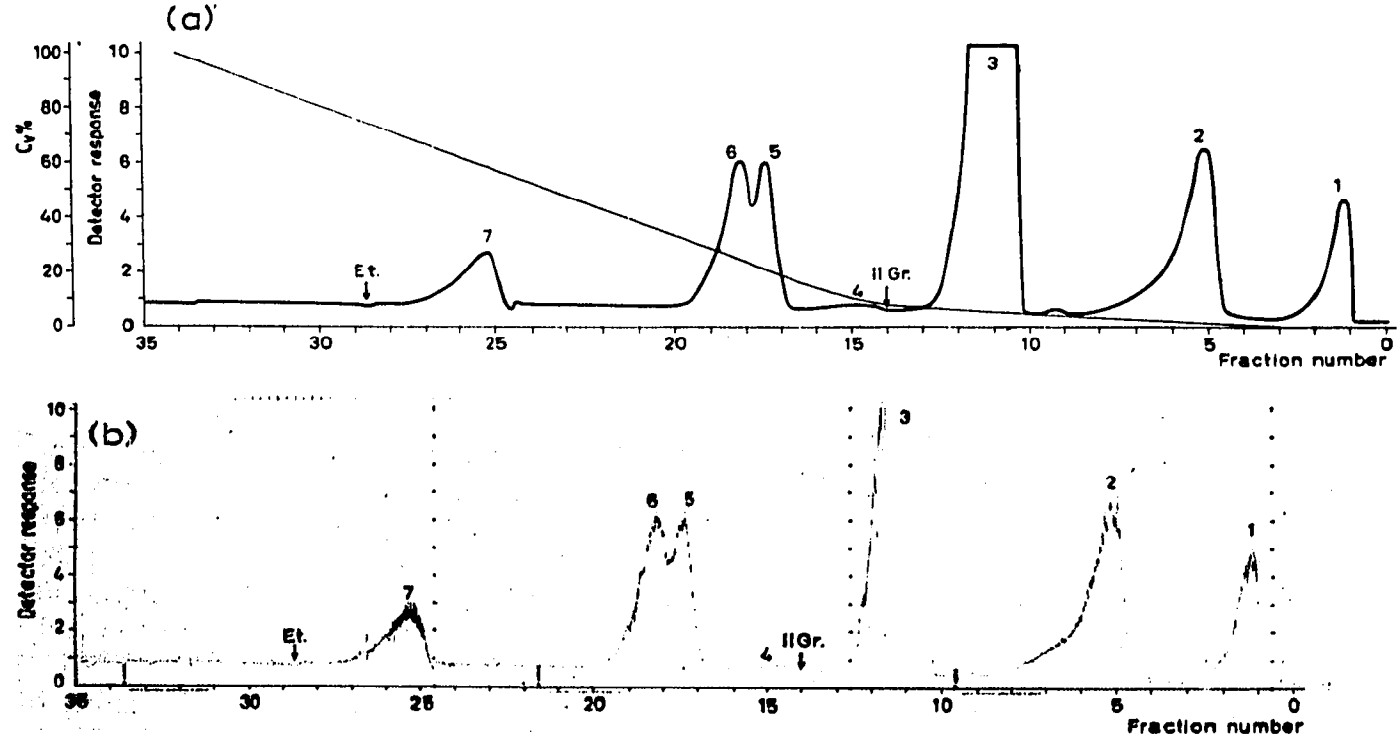


Fig. 3 (a) Chromatogram of a reference lipid mixture. Column 9 mm diam. × 160 mm high, charged with 5 g silicic acid. Sample 16.62 mg. Detector sensitivity 3 × $10^{-10}$ A. Concave gradient ($P$ = 1/4) $C_R$ = 50 %, ethyl ether in petroleum ether, 56 % used until $C_v$ = 9.5 %, plus linear gradient $C_R$ = 100 % ethyl ether in $C_M$ = 9.5 % ethyl ether in petroleum ether. Peak order as in Fig. 2. The gradient curves are reported. (b) Direct reproduction of the recording obtained with the LCD for the chromatogram described in (a).

thus clear that for the isolation of certain fractions present in a certain quantity of eluate in more or less complex mixtures of lipids, more convenient gradients or combinations of gradients can be used for each separate case, thanks to the simplicity and flexibility of the system for the gradient preparation.

Our gradient permits, under conditions only requiring programming at the start, a better separation than was obtained in our preceding work[39] where a stepwise succession of solvents (ether–petroleum ether) analogous to that described by HIRSCH AND AHRENS[5] was used. The separation as far as the diglycerides are concerned is obtained with columns consisting of 5 g silicic acid, with 260–300 ml of the gradient, corresponding to 61–70% of its total volume: when substances of a polarity lying between that of di- and monoglycerides are not expected, the final elution with ether can be started after the diglycerides, stopping the gradient at whatever point desired.

The quantitative evaluation of this separation procedure was carried out by chromatographing pure lipids individually or in mixtures and determining them by weighing the corresponding fractions obtained from the chromatogram. The results are given in Table I, where the total and relative recoveries of the various components are reported with respect to the quantity of the mixtures of pure lipids and individual lipids chromatographed.

Commenting on these results it can be generally said that the total recovery is very good and higher than 93–95%. This is also valid for the greater part of the individual components of the mixture. The use of a weighing procedure for the determination of the recovery and of the composition of the analysed mixture coupled with the control of purity of the fractions done by TLC demonstrates how, in a chromatographic procedure for the separation of lipids, it is extremely important to use highly purified solvents and accurately cleaned laboratory glassware. We found that solvents that were not purified (even chromatographic grade) contain lipid impurities (triglycerides, hydrocarbons, fatty acids and other products) in appreciably quantity. They are retained by the column when at low solvent polarity and are subsequently eluted by the gradient (Fig. 4). In many cases the contamination of the solvents, even of the purified ones, can be as much as 1–3 $\mu$g/ml (p.p.m.) and, when accurately evaluating the results, it is necessary to take into consideration the elution volume of a fraction and subtract from the total weight the amount calculated for the

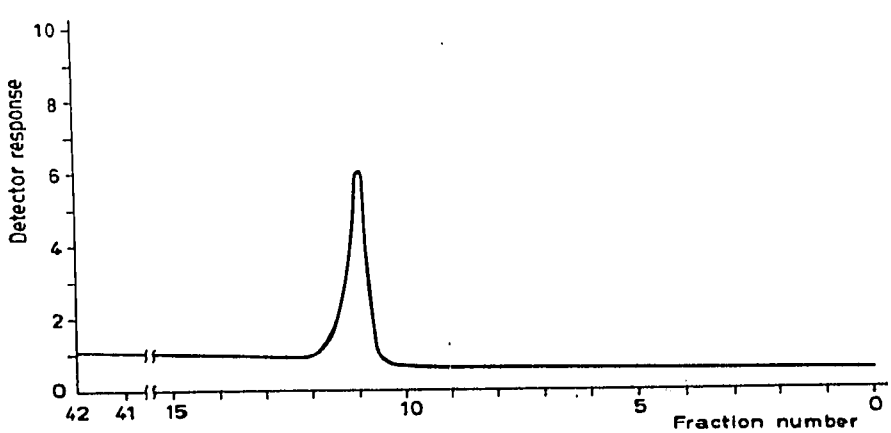


Fig. 4. Chromatogram of non-purified solvents: a peak of triglycerides is visible. Operating conditions as in Fig. 2.

TABLE I

RESULTS OF CHROMATOGRAPHIC ANALYSIS OF REFERENCE AND IMPURE LIPIDS

Recovery is reported as per cent of the total chromatographed amount. In the second line theoretica in brackets.

| *Sample* | *No.* | *Chromato-graphed amount mg* | *Hydro-carbons* | *Sterol esters* | *Triglyce-rides* | *Fatty acids* |
|---|---|---|---|---|---|---|
| Mixture of reference lipids | 1 | 20.23 | 4.60 | 17.84 | 52.89 | 6.52 |
| | | | 4.84 | 19.92 | 57.96 | 6.65 |
| | | | (95.04) | (89.56) | (91.25) | (98.04) |
| Mixture of reference lipids | 2 | 18.17 | 4.57 | 4.62 | 61.47 | 6.55 |
| | | | 4.95 | 5.01 | 63.99 | 6.62 |
| | | | (92.32) | (92.21) | (96.06) | (98.94) |
| Mixture of reference lipids | 3 | 17.44 | 6.44 | 5.01 | 57.53 | 7.73 |
| | | | 5.90 | 5.22 | 64.16 | 7.22 |
| | | | (109.15) | (95.98) | (89.67) | (107.06) |
| Mixture of reference lipids | 4 | 20.26 | 4.83 | 7.10 | 62.20 | 4.87 |
| | | | 4.70 | 7.80 | 63.15 | 4.78 |
| | | | (102.76) | (91.92) | (98.49) | (101.88) |
| Rat liver lipids | 5 | 39.94 | 0.09 | 5.19 | 18.20 | 1.05 |
| | | | — | — | — | — |
| Rat liver lipids | 6 | 39.94 | 0.07 | 5.24 | 18.79 | 1.53 |
| | | | — | — | — | — |
| *Single reference and impure lipids* | | | | | | |
| Tripalmitin | 7 | 12.02 | | | 95.42 | 2.49 |
| | | | | | 100.00 | |
| | | | | | (95.42) | |
| Tripalmitin | 8 | 12.21 | | | 91.98 | 2.84 |
| | | | | | 100.00 | — |
| | | | | | (91.98) | |
| Tripalmitin | 9 | 15.64 | | | 91.05 | 2.56 |
| | | | | | 100.00 | — |
| | | | | | (91.05) | |
| Pure tripalmitin + palmitic acid | 10 | 12.37 | | | 74.83 | 15.27 |
| | | | | | 82.69 | 17.31 |
| | | | | | (90.49) | (88.21) |
| Pure tripalmitin | 11 | 10.90 | | | 93.50 | 0.05 |
| | | | | | 100.00 | — |
| | | | | | (93.50) | |
| Impure cholesterol stearate | 12 | 50.00 | | 94.98 | 0.26 | 1.02 |
| | | | | 100.00 | — | — |
| | | | | (94.98) | | |
| Impure mono- and di-glycerides | 13 | 5.99 | | | 3.42 | 3.49 |
| | | | | | — | — |
| Natural olive oil | 14 | 19.94 | 0.92[a] | [a] | 85.50 | 4.44 |
| | | | — | — | — | — |

[a] These two peaks were not completely resolved in this experiment: recovery is calculated togethe

impurities in the solvent. The results reported in Table I have been calculated taking, when necessary, this observation into consideration.

The use of the chromatographic procedure for the analysis and purification of single lipids as described is shown in experiments carried out with tripalmitin defined as 99 % pure. As shown in Fig. 5, in fact a small but well separable peak of fatty acids is present. Thin-layer chromatography reveals these to be associated with another,

nposition of mixtures is reported when it is known. The per cent recovery for each component is given

| *rols* | *Diglycerides* | *Mono-glycerides* | *Phospho-lipids* | *Other fractions* | | | *Recovered %* | *Amount mg* |
|---|---|---|---|---|---|---|---|---|
| ).64 [a] | [a] | | | 3.56 | — | — | 95.06 | 19.23 |
| ).39 | 5.24 | | | | | | | |
| ).69) | | | | | | | | |
| ).13 [a] | [a] | 9.08 | | 0.88 | 1.65 | — | 98.95 | 17.98 |
| ).39 | 5.83 | 8.20 | | | | | | |
| ).28) | | (110.73) | | | | | | |
| ).33 | 6.45 | 6.44 | | | | | 94.95 | 16.56 |
| ).45 | 6.31 | 5.73 | | | | | | |
| 7.80) | (102.22) | (112.29) | | | | | | |
| ).64 | 5.36 | 7.13 | | 0.82 | 0.70 | | 99.66 | 20.19 |
| 7.01 | 6.14 | 6.41 | | | | | | |
| 4.72) | (87.30) | (111.23) | | | | | | |
| 4.02 | 0.88 | 1.13 | 56.50 | | | | 87.06 | 34.77 |
| | — | — | — | | | | | |
| 3.81 | 0.75 | 0.95 | 56.08 | | | | 87.22 | 34.83 |
| | — | — | — | | | | | |
| | | | | | | | 97.92 | 11.77 |
| | | | | | | | 94.82 | 11.58 |
| | | | | | | | 93.61 | 14.64 |
| | | | | | | | 90.11 | 11.15 |
| | | | | | | | 93.56 | 10.19 |
| | | | | 0.70 | | | 96.97 | 48.48 |
| | | | | — | | | | |
| | 41.18 | 47.69 | | | | | 95.79 | 5.73 |
| | 50.62 | 49.38 | | | | | | |
| | (81.35) | (96.58) | | | | | | |
| 4.22 | 3.01 | 1.43 | — | — | — | — | 99.52 | 19.85 |
| — | — | — | — | — | — | — | | |

not identified, component of a polarity lying between that of triglycerides and fatty acids. These impurities are present to the extent of 2.5–2.8 % and they are no longer observed, either as a single component (Fig. 6) or in a mixture with fatty acids (experiments No. 10 and 11), in chromatograms of tripalmitin purified by us. Equally good results are obtained for the purification of mixtures of mono- and diglycerides (Fig. 7), where small quantities of triglycerides and fatty acids are revealed. The

purity of the fractions is in every case confirmed by thin-layer chromatography.

In certain cases it can be advantageous to use columns of larger dimensions for preparative purposes. The graph in Fig. 8 shows the separation of a complete mixture of neutral lipids on a column of 2.5 cm diameter. The gradient elution was carried out for approx. 24 h. The main fractions are well separated and the recovery is of the same order as that obtained on the 5 g column.

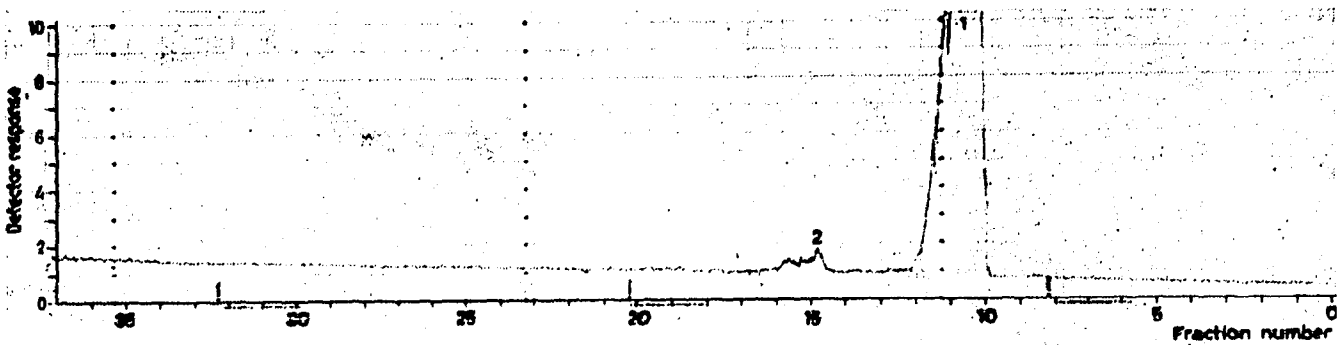


Fig. 5. Chromatogram of tripalmitin erroneously claimed as 99% pure. Direct reproduction of the recording obtained with the LCD. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 12.21 mg. Peaks 1 = pure tripalmitin; 2 = palmitic acid + unknown compound.

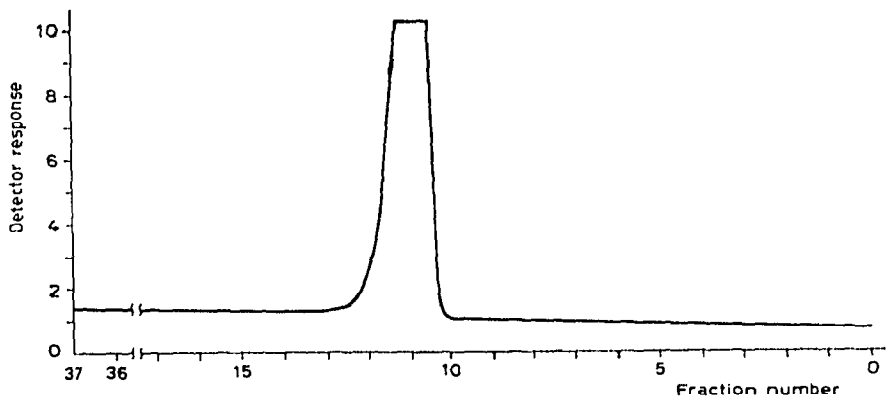


Fig. 6. Chromatogram of pure tripalmitin. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 10.89 mg. A single peak is present.

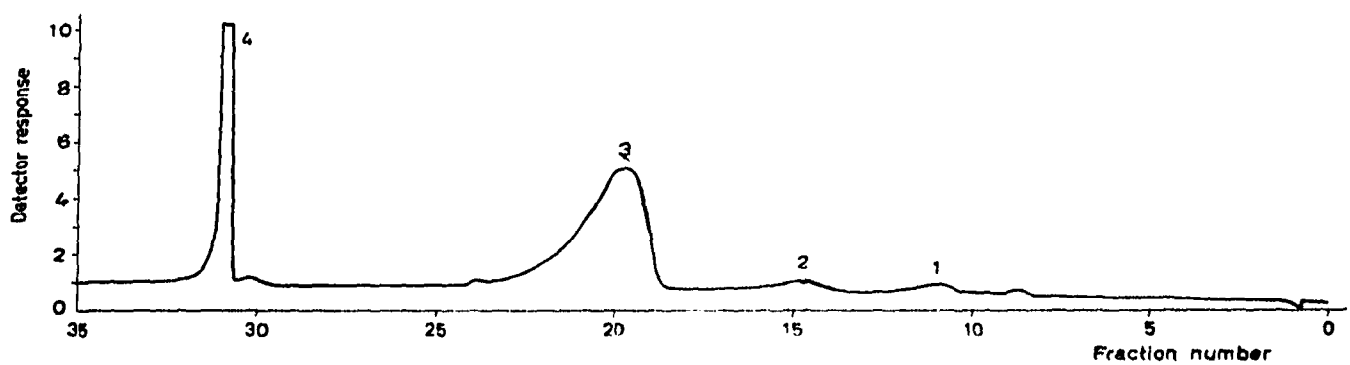


Fig. 7. Chromatogram of a mixture of glyceryl di- and monostearate. Operating conditions as in Fig. 2 except column 9 mm diam., 160 mm high; sample 5.98 mg. Peaks: 1 = tristearin; 2 = stearic acid; 3 = glyceryl distearate; 4 = glyceryl monostearate.

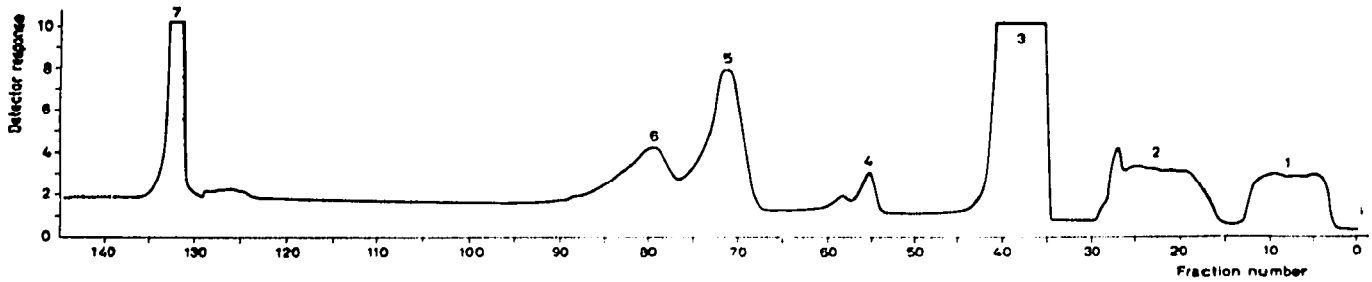


Fig. 8. Chromatogram of a reference lipid mixture. Column 25 mm diam. × 90 mm high, charged with 23 g silicic acid. Sample 85.64 mg. Detector sensitivity 3 × $10^{-10}$ A. Concave gradient ($P$ = 1/4) $C_R$ = 100% ether (373 ml) in petroleum ether ($C_M$ = 1492 ml). Column flow rate 1 ml/min. Splitter ratio: 6% of the column flow to the detector. Peak order as in Fig. 2.

Application of this technique to the analysis of total lipids in rat liver, obtained by chloroform–methanol (2:1) extraction according to FOLCH *et al.*[34], is reported in Table I (experiments No. 5 and 6). The graph obtained for the separation is shown in Fig. 9. It is interesting to note the presence of 3 peaks containing esterified cholesterol in these natural lipids. We plan to study the components of these peaks and to try to establish a relation between the separation and the nature of the components. The load capacity is higher (about 40 mg of total lipids) as approximately half the weight is present as phospholipids, which are eluted at the end by methanol. The reproducibility of the data of the percent composition of the twice chromatographed extract is very good and the recovery can be considered as satisfactory, considering the fact that the extract investigated may contain some non-lipid components.

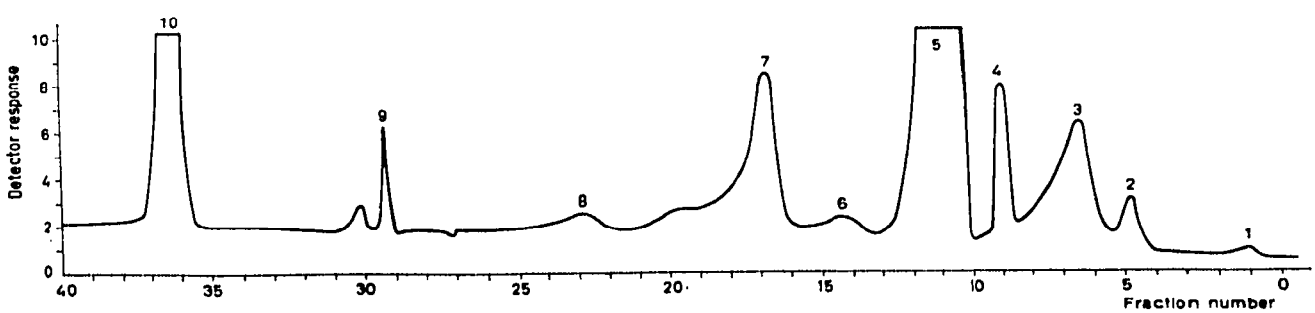


Fig. 9. Chromatogram of rat liver lipids. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 39.94 mg. Peaks: 1 = hydrocarbons; 2,3,4 = cholesterol esters; 5 = triglycerides; 6 = fatty acids; 7 = cholesterol; 8 = diglycerides; 9 = monoglycerides; 10 = phospholipids.

Examples of other important applications of this technique are given by experiment No. 14, concerning the separation of natural olive oil. The corresponding graph is reported in Fig. 10.

Fig. 11 shows the graph for a solution of testosterone propionate in neutral oil for pharmaceutical use (conc. 25 mg/ml). Clear resolution of the steroid, giving a well shaped peak lying between the di- and the monoglyceride peaks is shown. This permits a UV spectrophotometric or GLC determination. This application of our technique will be described in detail in another paper.

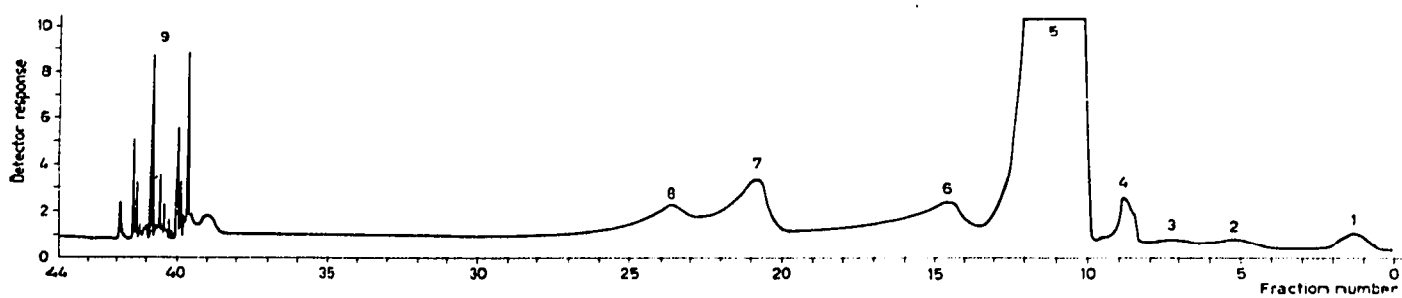


Fig. 10. Chromatogram of a natural olive oil. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 19.94 mg. Peaks: 1 = hydrocarbons; 2,3,4 = probably sterol esters; 5 = triglycerides; 6 = fatty acids; 7 = sterols; 8 = diglycerides; 9 = monoglycerides.

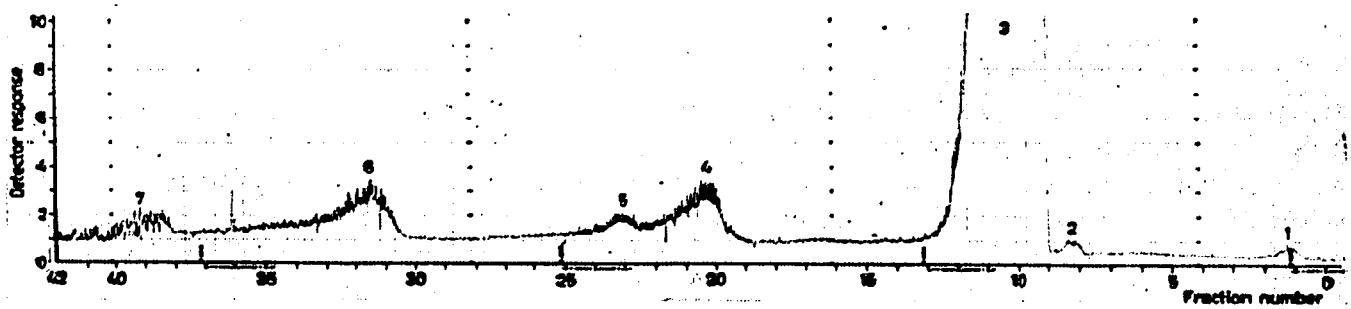


Fig. 11. Direct reproduction of the recording obtained with the LCD for chromatography of a solution of testosterone propionate in neutralised olive oil. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 40 $\mu$l oil solution. Peaks: 1 = hydrocarbons; 2 = sterol esters; 3 = triglycerides; 4 = sterols; 5 = diglycerides; 6 = testosterone propionate; 7 = monoglycerides.

## CONCLUSIONS

A method of this type is useful for the purification of single classes of neutral lipids in small quantities, for preparative purposes (for use as standards and for the preparation of derivatives for GLC etc.), and for the fractionation of lipid mixtures for analytical purposes, if one considers that colorimetric methods, as already reported in our preceding paper[39] may be employed in addition to the gravimetric method described. In any case contamination of the fractions with impurities from the solvent or the apparatus must be avoided. We think that we have demonstrated that by the use of an LCD and a weight microtechnique it is easily possible to detect impurities both qualitatively and quantitatively and abolish them if necessary. In our opinion this aspect of lipid chromatography has only been briefly considered by some authors, as for example by HORNING *et al.*[8] and has not been treated as extensively as it deserves.

Moreover a method of this type permits a chromatographic separation of lipids into classes under well standardized and reproducible conditions, with a minimum of assistance from the analyst, and a record is also obtained which permits easy appraisal of the quality of the separation. It is possible to observe the form of the peaks, the resolution, the column efficiency, in a manner similar to that done normally with

gas–liquid chromatography, applying the general considerations of liquid chromatography to lipid chromatography as described in refs. 40, 42–45.

Our results offer a useful contribution with respect to the results obtained by other authors[30,31] who have attempted to solve the difficult problem of using the LCD with microcolumns for quantitative analysis of lipids. It is one of the best real examples of the use of the LCD technique, for the analytical and preparative chromatography of lipids. The extensive use of one efficient splitter also permits one to envisage the possibility of further quantitative applications in the future.

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