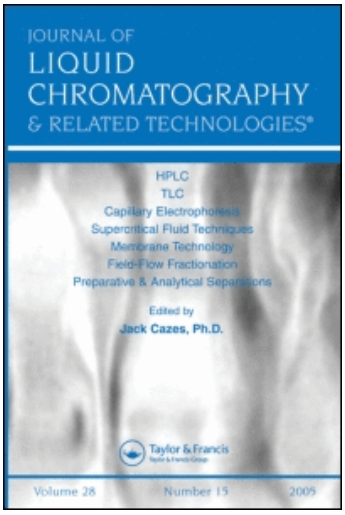

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NEUTRAL LIPID CLASS FRACTIONATION AND FURTHER SEPARATION OF SIMPLE NEUTRAL GLYCOLIPIDS BY OPTLC

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ABSTRACT

In lipid preparation from biological sources extraction and chromatographic methods are widely used. The use of the OPTLC method is introduced in the present paper. The method is suitable for the class separation of the neutral fraction of a total lipid extract with a single isocratic run. With a step or an exponential gradient the simple neutral glycolipids can be separated. Either elution can be performed on a 10 x 20 cm plate on 12 parallel samples. The chromatograms were evaluated by densitometric scanning after staining with orcinol-H_2SO_4 reagent.

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INTRODUCTION

The lipid separation procedures of a given biological sample usually start with an indispensible extraction step to prepare crude Total Lipid Extract (TLE) largely free of non--lipid contaminants (1). In the work up of TLE various chromatographic steps can be used, e.g. classical and High Performance column and layer chromatographic methods. A new layer chromatographic technique, Overpressured Thin Layer Chromatography (OPTLC) and an instrument have been developed in Hungary (2). Detailed studies of the method were published (3,4). Application areas of the technique have included a wide range of biologically important substances (5,6,7,8).

Of modern layer chromatographic techniques only HPTLC has been widely applied in lipid separations. In these applications usually a given, purified lipid class was separated on a HPTLC plate (9,10). An exception to this rule was a communication (11) which separated gangliosides of total lipid extracts. Unfortunately no sequel to this appeared, which might be attributed to that;

- only one of the most polar lipids, gangliosides, were separated, sulphatides are not mentioned in the article,
- the method is extremely time consuming (3 developments of 10 hours in all, not counting the drying periods),
- the separation on a 20 x 20 cm TLC plate does not give the possibility of the determination of other, non-separated lipid classes.

In lipid analysis the OPTLC technique has not been applied up to now. This is a report of our experiments on the separation of the Neutral Lipid Fraction (NLF) of TLE by OPTLC.

The method proved suitable for the class separation of the neutral lipids in a simple, fast isocratic run with chloroform (C). The simple neutral glycolipids can be further separated with a gradient run using either a step from pure chloroform to chloroform, methanol (M), water (W) mixture (C : M : W = 65 : 25: 4) or a delayed exponential gradient of M in C (final concentration approximately C : M = 1 : 1).

EXPERIMENTAL

Reagents and Materials

All the solvents used were HPLC grade purchased from E. Merck, Darmstadt, F.R.G., the water used was prepared according to Gurkins method (12), from water double distilled from glass and sterilized. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Staining reagents were prepared from analytical grade chemicals. The HPTLC plates used were purchased from E. Merck, Darmstadt. TLE was prepared from human lymphocytes.

Apparatus

Centrifuge: Janetzki K70 MLW, Leipzig, G.D.R.

Incubator: LP507/1 Labor MIM, Esztergom, Hungary.

Sample applicator: CAMAG Nanomat, CAMAG, Muttenz, Switzerland.

OPTLC system: Chrompres 10 Overpressured Layer Chromatograph, Labor MIM, Esztergom, Hungary. Instead of its eluent pump two HPLC pumps: Waters M-45, Waters Associates Inc., Milford, Mass., USA and Beckman 112 SDM, Beckman Instruments Inc., Berkeley, Calif., USA and a home made gradient mixing chamber were used.

Spectrophotometric densitometer: Opton KM3, Opton Feintechnik GmbH., Oberkochen, F.R.G.

Data system: HP 3354 Lab. Automat. System, Hewlett Packard, Avondale, Calif., USA.

Methods

Sample preparation

The preparation of TLE and the isolation of the NLF was described elsewhere (13). The crude NLF was freed from alkali labile components by incubation. The dried NLF was dissolved in 15 ml 0.1M methanolic KOH and incubated at 37°C for 2 hours. The sample was then neutralized with cc.HCl, evaporated in vacuo. The lipid compounds were taken up in 2 ml of C : M = 85 : 15 mixture. Average samples were prepared from 10^{10} lymphocyte cells.

Plate pretreatment

All plates used were precleaned by running them in methanol and dried. The OPTLC technique requires that the edges of the plates should be sealed, preferably after scraping off a strip (2-4 mm) of sorbent, by impregnating with IMPRES 1. For single dimensional development 3 sides of the plate were sealed and solvent dispersion troughs were scraped into the plates 14, 17 and 20 mm from the lower edge of the plates. Three parallel lines were scraped (and impregnated) into the plates perpendicular to the development direction (from the sample application height to the top) to insure straight fronts. For two dimensional development all four sides were impregnated and teflon solvent dispersion troughs were used.

OPTLC system

The block diagram of the instrument is shown in Figure 1. This

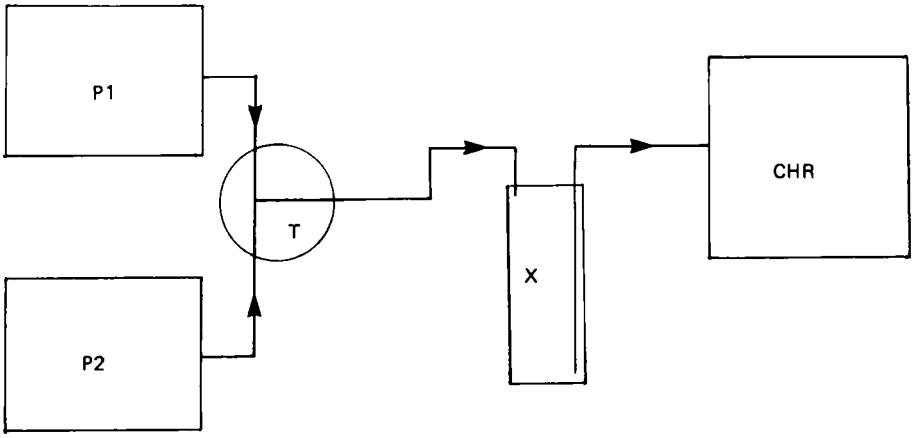


Figure 1. Block diagram of the OPTLC instrument used

P1 = pump 1

P2 = pump 2

T = T-junction

X = mixing chamber

CHR = chromatography chamber

system was used because it gives reproducible liquid flows and gradient profiles at low flowrates necessary to achieve approximately 1.00 cm/min velocity on the plate, which is optimal for elution (6,14).

For isocratic elution one pump (P1) was directly connected to the inlet valve. For the step gradient the two pumps (P1,P2) were connected with a T junction (T) before the inlet valve to make possible quick eluent change over. The exponential gradient was formed with a home made mixing chamber (X). This was a 5 ml closed container equipped with a magnetic stirrer with an inlet at the top and an outlet at its bottom. 2.5 ml of starting solvent (C) was placed in the container into which the strong

solvent was continuously pumped with vigorous stirring. To delay the start of the exponential gradient pumping was switched from C to M (P1 to P2).

OPTLC chromatography and densitometry

Sample application was 2-500 nl/spot, depending on the separation aims, with Pt-Ir capillaries.

For single dimensional development usually 12 samples were applied to a plate at 30 mm from the lower edge and at least 20 mm from the perpendicular edges of the plate.

Development conditions were;

run	isocratic	step grad.	exp. grad.
eluent	C	C/C:M:W	C/C,M
eluent flow (ml/min)	0.15	0.15	0.20
start pressure (bar)	30	30	30
pillow pressure (bar)	12	12	12
development distance (cm)	17	17	17
gradient start (at cm)	-	13	7

For two dimensional development two spots were applied to a plate at 30-30 mm from the corners. The first development of both spots was 7 cm with chloroform. The plate was dried, rotated 90^{o} and one of the spots rechromatographed in the second dimension 4 cm with C : M : W = 65 : 25 : 4 eluent.

The staining was achieved by immersion of the plate into orcinol-H_2SO_4 reagent and heating at $100^{o}C$ for 2-3 minutes.

Densitometric conditions in remission mode were:

wavelength: 560 nm

slit: 3.5 x 0.1 mm

scanning speed: 50 mm/min

A/D frequency: 2 Hz

RESULTS AND DISCUSSION

Summarizing the experimental results the OPTLC technique is suitable for the separation of lipid samples. In our method the NLF of TLE can be applied and separated in a single isocratic run into lipid classes with different polarities. For qualitative analysis the migration distances (md) and Rf values can be used, which are highly reproducible, as can be seen in Figure 2. and in Table 1.

Figure 2. shows an OPTLC plate with 12 parallel runs (a) and the densitogram of one lane (b). The photograph clearly demonstrates the usefulness of the isocratic OPTLC run for the fractionation of the neutral lipid classes. These results are supported by the numerical data, migration distances (md), Rf values, their averages and the standard deviations (SD), summarized in Table 1. In the class separation of neutral lipids (NL) we aimed at leaving the NGL class at the start, while eluting all the other less polar classes. As can be seen (md of NGL) very small or no movement was achieved and caused a relatively large deviation (SD of NGL).

As sample quantity is frequently severely limited, as in our case of analysing blood cell lipids of leukemic patients, it was necessary to extract as much information as possible from one sample. For this purpose the step gradient elution was evolved making possible the separation of the simple neutral glycolipids after the above mentioned class fractionation on the same plate. Although the use of a second, water containing, eluent gives rise to severely curved β and δ fronts, they do not make impossible the evaluation of the spots in or near them. At the same time the intraclass sepa-

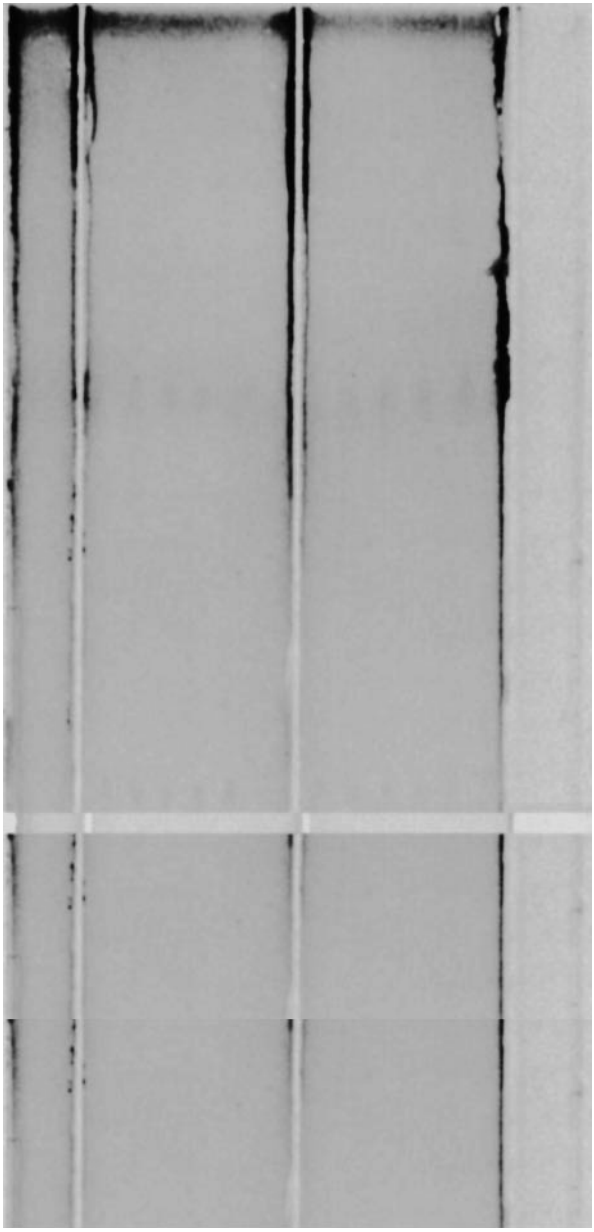


Figure 2. OPTLC separation of the neutral lipid classes. 2.a plate, 2.b densitogram of one lane, NGL = neutral glycolipids, Ch = cholesterol, Glyc = glycerides, ChE = cholesterol esters. Plate Merck HPTLC Si 60, 17 cm elution with chloroform.

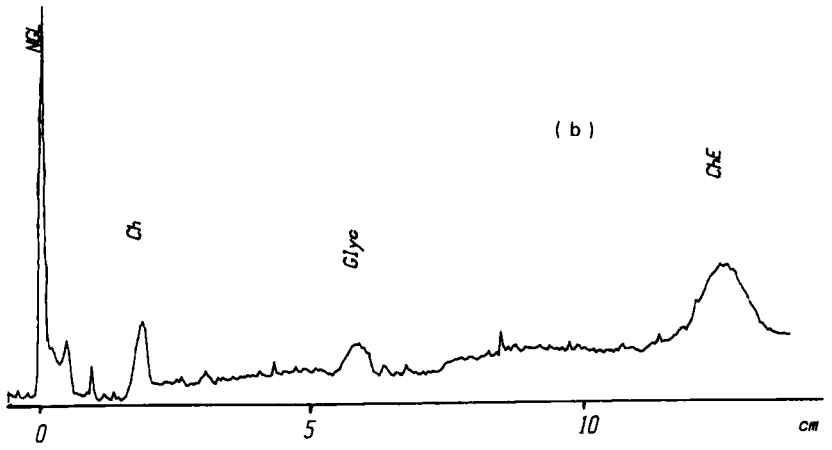


Figure 2 (continued)

Table 1. Migration distances (md in cm) and Rf values of neutral lipid classes separated by OPTLC.
NGL= neutral glycolipids, Ch= cholesterol, Glyc= glycerides, ChE= cholesterol esters, SD= standard deviation

Class	NGL		Ch		Glyc		ChE	
No.	md	Rf	md	Rf	md	Rf	md	Rf
1.	0.05	0.003	0.37	0.109	1.12	0.329	2.31	0.688
2	0.00	0.000	0.35	0.109	1.09	0.329	2.33	0.688
3	0.00	0.000	0.34	0.100	1.09	0.321	2.31	0.679
4	0.05	0.003	0.35	0.103	1.10	0.318	2.31	0.679
5	0.05	0.003	0.38	0.115	1.13	0.332	2.31	0.682
6	0.05	0.003	0.37	0.112	1.15	0.338	2.34	0.688
7	0.05	0.003	0.36	0.109	1.12	0.329	2.35	0.691
8	0.00	0.000	0.36	0.106	1.13	0.332	2.36	0.694
9	0.05	0.003	0.34	0.100	1.15	0.338	2.50	0.735
10	0.05	0.003	0.37	0.109	1.14	0.335	2.53	0.744
11	0.05	0.003	0.38	0.112	1.17	0.344	2.48	0.729
12	0.05	0.003	0.38	0.112	1.20	0.353	2.46	0.723
Average	0.04	0.002	0.36	0.110	1.13	0.333	2.38	0.700
SD	0.023	0.0014	0.015	0.0048	0.033	0.0095	0.084	0.024

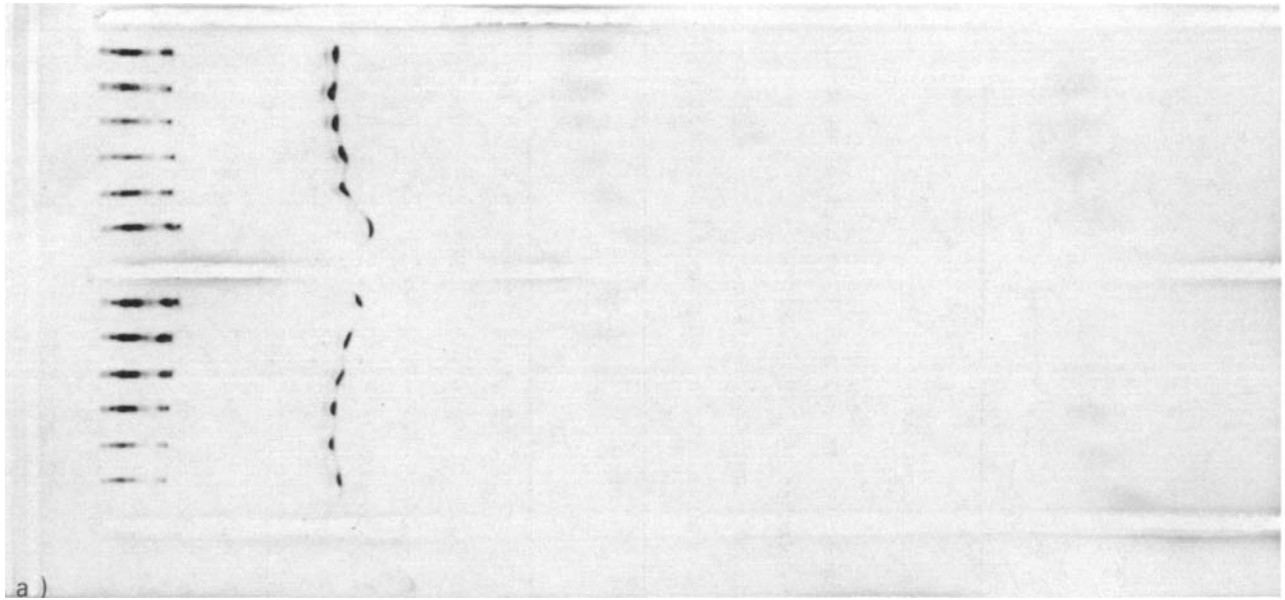
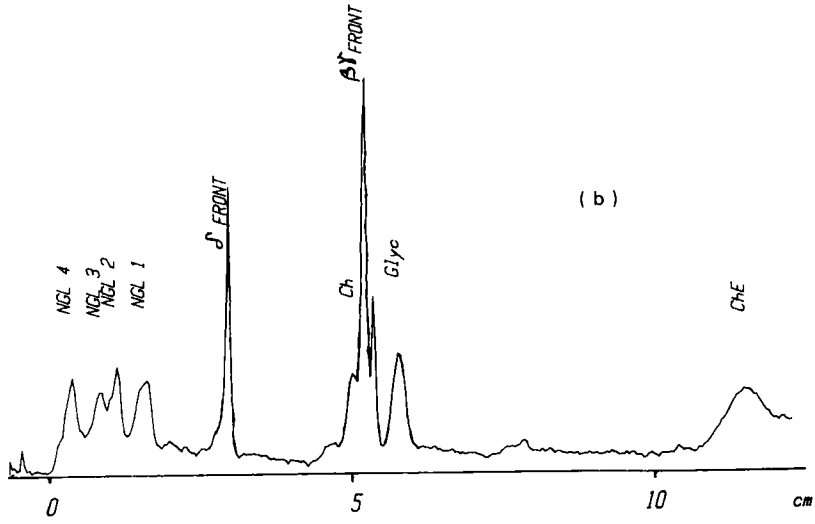


Figure 3. OPTLC separation of simple neutral glycolipids. 3.a plate, 3.b densitogram of the neutral glycolipids. Plate Merck HPTLC Si 60, 13 cm elution with chloroform, then 4 cm with chloroform:methanol:water=65:25:4. NGL 4 = tetrahexosyl ceramide, NGL 3 = trihexosyl ceramide, NGL 2 = dihexosyl ceramide, NGL 1 = monohexosyl ceramide, Ch = cholesterol, Glyc = glycerides, ChE = cholesterol esters.



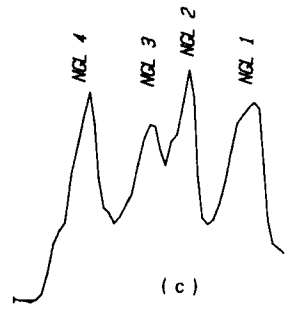


Figure 3 (continued)

Table 2. Migration distances (md in cm) and Rf values of simple neutral glycolipids separated by OPTLC
NGL 4= tetrahexosyl ceramide, NGL 3= trihexosyl ceramide, NGL 2= dihexosyl ceramide, NGL 1= monohexosyl ceramide, SD= standard deviation

NGL / No.	NGL 4		NGL 3		NGL 2		NGL 1	
	md	Rf	md	Rf	md	Rf	md	Rf
1	0.25	0.088	0.80	0.281	1.05	0.368	1.50	0.526
2	0.25	0.088	0.70	0.246	1.00	0.351	1.50	0.526
3	0.25	0.078	0.70	0.219	1.00	0.313	1,50	0.469
4	0.25	0.086	0.70	0.241	1.00	0.345	1.50	0.517
5	0.30	0.102	0.70	0.237	1.00	0.339	1.55	0.525
6	0.30	0.100	0.75	0.250	1.05	0.350	1.60	0.523
7	0.25	0.088	0.75	0.263	1.00	0.351	1.50	0.526
8	0.20	0.071	0.65	0.232	1.00	0.357	1.45	0.518
9	0.25	0.089	0.70	0.250	1.05	0.375	1.50	0.536
10	0.30	0.107	0.70	0.250	1.05	0.375	1.55	0.554
11	0.30	0.107	0.75	0.268	1.10	0.393	1.55	0.554
12	0.30	0.107	0.80	0.286	1.15	0.411	1.55	0.554
Average	0.267	0.0926	0.725	0.2520	1.038	0.3607	1.521	0.528
SD	0.033	0.0119	0.045	0.0197	0.048	0.0158	0.040	0.023

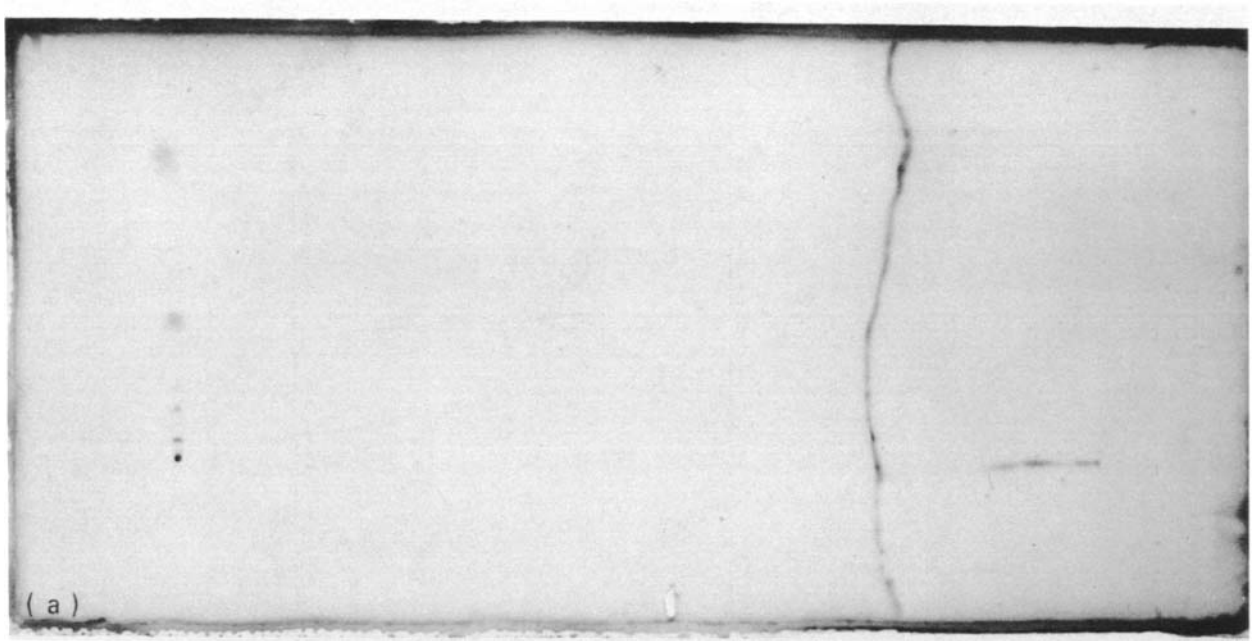


Figure 4. Two dimensional OPTLC separation of neutral lipid classes and simple glycolipids on one plate. 4.a plate 4.b the densitogram of the class separation, 4.c the densitogram of the glycolipid separation. Plate Merck HPTLC Si 60, elution: in the first dimension 7 cm with chloroform, the second dimension 4 cm with chloroform:methanol:water=65:25:4 for one spot.

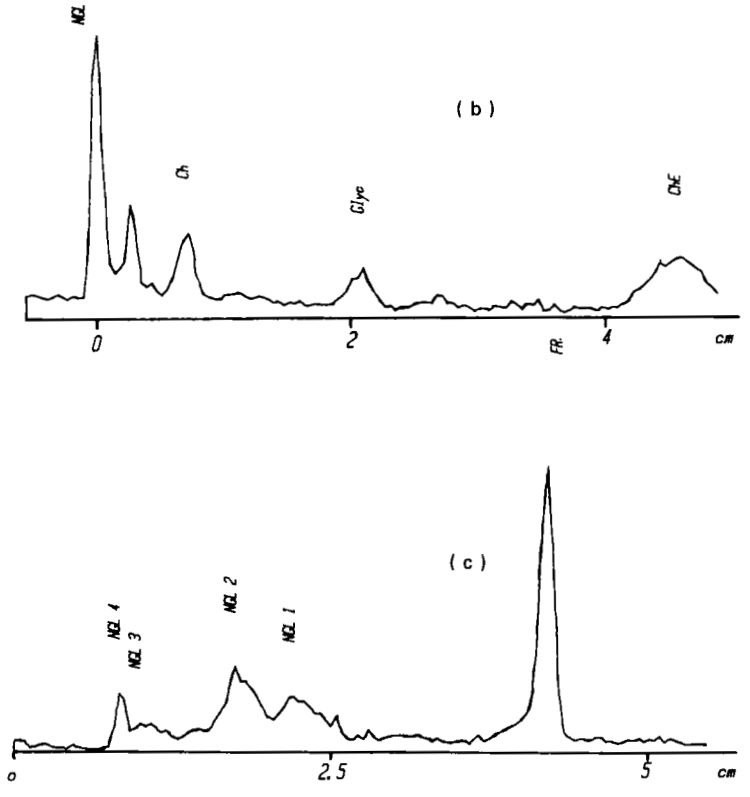


Figure 4 (continued)

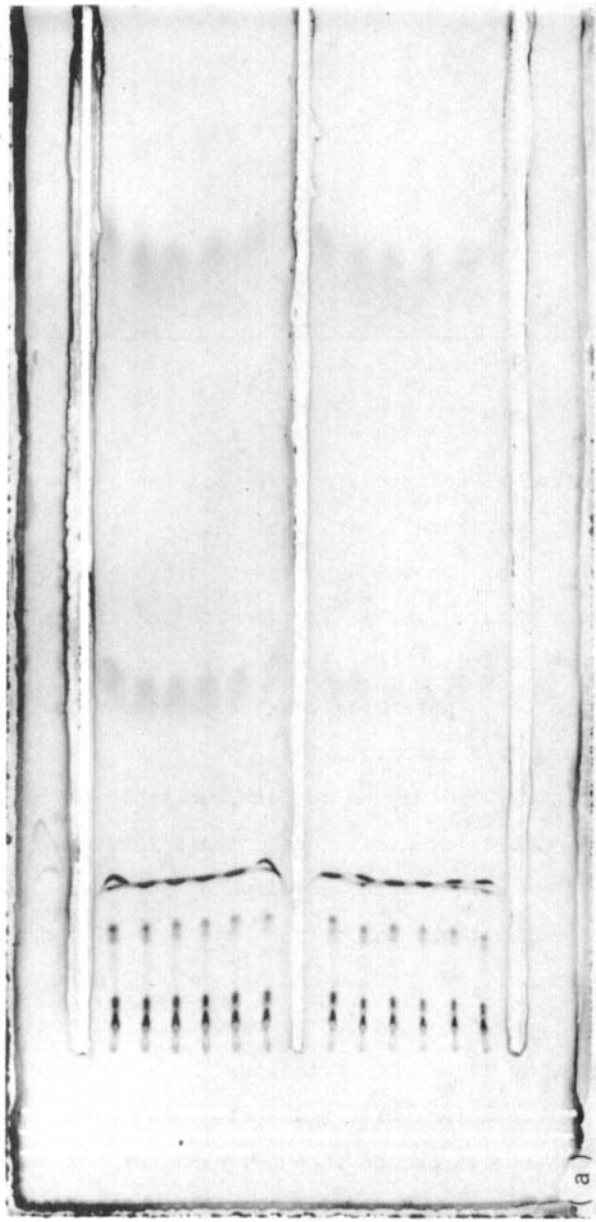


Figure 5. Gradient OPTLC separation of neutral lipids. Plate Merck HPTLC Si 60, elution 7 cm chloroform, then 7 cm exponential gradient of methanol in chloroform to 50 % methanol.

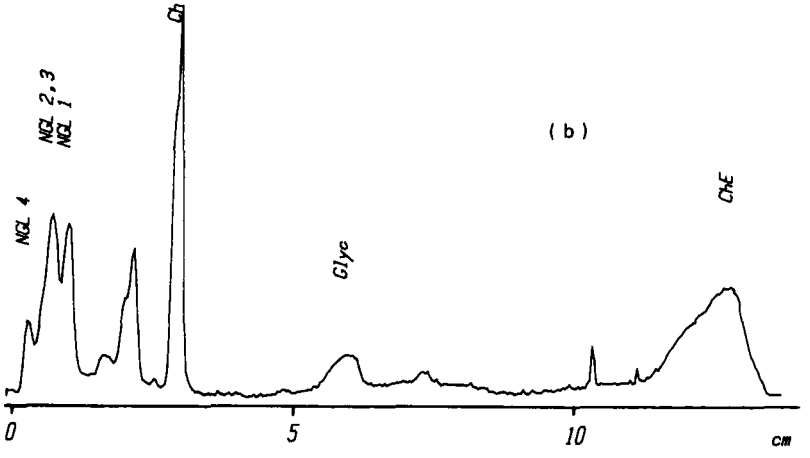


Figure 5 (continued)

ration of simple neutral glycolipids is very good and reproducible as can be seen in Figure 3. and Table 2.

The plate (Fig.3.a.) shows the complete class and NGL intraclass separation. Figure 3.b. is the densitogram of one lane of this plate, while insert (Fig.3.c.) shows the densitogram of the NGL separation. Table 2. gives the corresponding data for the intraclass separation.

A more frequently used way to perform two separations of a single sample is two dimensional development. Figure 4. shows how it can be done using OPTLC. Development for both intra and interclass separations can only be achieved if two spots are applied, and only one sample can be analysed per plate. As this method does not give better resolution but has significant drawbacks, so it was discarded.

The exponential gradient with methanol, which gave good results on normal silica plates in our preliminary experiments (15) was also examined (Fig.5.). This gradient gives good separation in the NGL class but coelutes the classes with

medium polarity.

In conclusion the adaptation of the new lipid separation with the step gradient OPTLC method, affords a rapid, labour and cost effective way to separate the neutral lipid classes from the neutral lipid fraction of the total lipid extract with the possibility of simultaneously separating the simple neutral glycolipids too of very small unique samples.

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