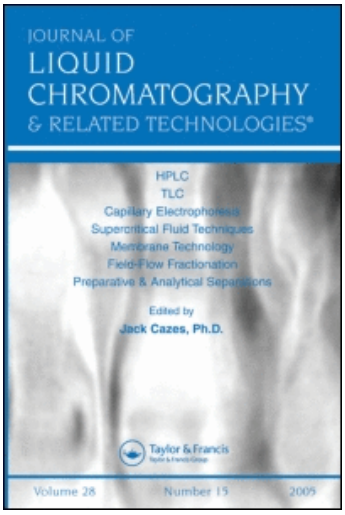

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ISOLATION OF GLYCOLIPIDS FROM BLOOD ELEMENTS

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ABSTRACT

Several chromatographic e.g. HPTLC, HPLC, etc. methods have been published in the literature for the separation, after sufficient pretreatment, of derivatized or non-derivatized glycolipid samples.

Our task is the extraction, isolation and separation of the glycolipids from different blood elements, followed by suitable fractionation methods, giving the lipid classes in sufficient purity and quantity for HPLC, HPTLC and OPTLC measurements and possibly further biochemical use.

We show the differences between the procedures commonly used and that developed in our laboratory.

The advantage of our method, which employs 3 cm long Brownlee Labs HPLC cartridges, is that it can be automated, it gives class fractionation of the lipid samples and as it is hardware compatible with HPLC equipment it can be used directly in a coupled column system for on line separation in the individual class. The development of this column coupling method for the fractionation of a given lipid class from the total lipid extract on an analytical column is under development.

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INTRODUCTION

The sample preparation for most instrumental analytical methods is a time and labour consuming process. This is really true in the case of biological samples and HPLC, as the costly HPLC columns have to be protected from the various irreversibly or near irreversibly retained components of the biological sample. The use of short preparative columns for sample preparation is getting very common and is facilitated by the numerous commercial products available (Sep-Pak, Prep Elute, Bond Elute, Extrelute from Waters, Hamilton, Analytichem, Merck - to name a few - respectively). The problem with these is that they are suitable only for manual work (1,2), as they can not be installed in a chromatograph. We decided to develop a method suitable later for column coupling. The possibility of column coupling is necessary for our analysis, as the preparation of a very small sample can be performed with less wastage in that way.

The object of our investigations, the glycolipids of blood elements, are minor components of the cell membrane. The majority of glycosphingolipids are assumed to be present at the outer leaflet of plasma membranes and play very important biological roles. The functions of glycolipids (3,4) has been increasingly examined parallel with the methodological developments (5,6,7,8) of identification during the last few years.

At first only derivatized glycolipids were separated by HPLC (9) with UV detection, later non-derivatized glycolipids without on line detection (10) monitored by TLC runs. We presented non-derivatized glycolipid separation with refractive index (RI) detection (11), on line detection of these compounds was accomplished by Handa and Kushi (8) at 206 nm.

The importance of the separation of glycosphingolipids without derivatization is that the separated components can then be directly used for further biochemical, immunological and structural investigations.

EXPERIMENTAL

Reagents and materials. All the solvents used were HPLC grade purchased from E. Merck (Darmstadt, F.R.G.). The water emplo-

yed was prepared according to Gurkin's method (12), from water double distilled from glass after ion-exchange. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Staining reagents were prepared from analytical grade chemicals. ODS-GU and SIL-GU cartridges were purchased from Brownlee Labs Inc. (Santa Clara, Calif., USA). The HPTLC plates used for monitoring HPLC fractions and purity checking were bought from E. Merck (Darmstadt, F.R.G.).
Total lipid extract was prepared from human lymphocytes.

Apparatus. Centrifuge: Janetzki K70 MLW (Leipzig, G.D.R.). Incubator: LP 507/1 Labor MIM (Esztergom, Hungary). Chromatographs: HP 1084 B with HP 79875 variable wavelength UV detector, Hewlett Packard (Palo Alto, Calif., USA), Beckman 112 solvent delivery module, Altex 156 differential refractometer, Beckman Instruments Inc. (Berkely, Calif., USA). Fraction collector: LKB Minirac 1700, LKB Produkter AB (Bromma, Sweden). Data system: HP 3354 Laboratory Automation System, Hewlett Packard (Avondale, Calif., USA).

Methods.

Total Lipid Extract (TLE) preparation. Lipid extraction was carried out similarly to the methods published previously (13, 14). The TLE was extracted from the liophilized cells at room temperature with mild sonication in chloroform : methanol mixtures in the ratios 2:1, 1:1, 1:2. The supernatant were collected together and dried in vacuo. The dried TLE was dissolved in chloroform : methanol : water = 30 : 60 : 8 mixture (100 ml) and the solution used for the isolation of the lipid classes described below.
Isolation of Acidic and Neutral Lipids (AcL, NL). The separation was performed on a ϕ12 x 70 mm DEAE Sephadex A-25 column in the acetate form. The solution of TLE (100 ml) was applied to the column, was washed with 100 ml of the same solvent, then with 50 ml of methanol. The 250 ml solution issuing from the column was used after solvent evaporation (water traces were removed with benzene), for the fractionation of neutral lipids, dissolved in chloroform, as given later.
Acidic lipids were eluted with 50 ml of 0.25 M methanolic sodium acetate. This portion of the eluent was dried in vacuo

and incubated in 15 ml 0.1 M methanolic sodium hydroxide at 37°C for 2 hours to destroy the alcali labile phospholipids (PL). We have developed a two step chromatographic method for the recovery and isolation of acidic glycolipids. The first step is similar to the method using Sep-Pak C18 cartridges (2). After incubation the sample was dried in vacuo and the residue was dissolved in cold (4°C) HPLC grade water, neutralized very carefully to $4<pH<5$ with 0.5 M HCl. The salt concentration was finally adjusted to 0.1 M by adding water. This sample solution was passed through an ODS-GU cartridge at a flow rate of 1.5 ml/min, the acidic glycolipids being collected on the column. After washing the cartridge with 30-50 ml of water, the glycolipids were eluted by 50 ml chloroform : methanol = 1 : 2 mixture.

Fractionation of Neutral Lipids. The neutral lipid portion derived from 2-8 x 10^{10} lymphocyte cells were taken up in 3 ml of dry chloroform and 5 - 30 µl aliquots were injected. Detection was at 254 : 600 nm, sample : reference wavelengths, attenuation 0.9 AUFS for the UV detector and 1 V attenuation FS for the RI detector. We employed the HP 1084 B LC equiped with UV and RI detectors coupled in series, with a fraction collector after the last detector.

Numerous elution profiles, given later, were tried, of which the selected chromatographic conditions were: flow 1 ml/min, gradient elution, solvent A was chloroform, B was methanol. The gradient started with 1 min. isocratic 100% A, followed by a linear increase to 100% B in 9 minutes, then a linear decrease to 100% A in 5 minutes. The run ended after further 5 minutes isocratic 100% A. This gradient, No.5, gave optimal results for our purposes. The separation was conducted at ambient temperature. In addition to on line detection the fractions collected during the run were also monitored by parallel TLC runs on silica plates. The empty fractions were discarded and those containing the same components (same subclass) united. Of the two chromatograms one was stained with 1 : 1 sulfuric acid : water, to visualize all of the components, giving also a specific red colour for cholesterol during heating. The other plate was stained with orcinol reagent, the neutral glycolipids appearing as purple spots.

RESULTS AND DISCUSSION

There are several methods for the separation of the total lipids, derived from biological sources such as cell membranes, in the literature. To have a comparable picture the commonly used methodologies have been summarized in flowcharts, shown in Figure 1. Comparing flowcharts 1, 2 and 3, it can be clearly seen that all these methods require a great amount of work, time and materials, especially high purity solvents. The trend of analysis of biological samples points to the importance of pretreatment and sample preparation (15). Because of the high cost of HPLC grade chemicals and analytical columns the use of guard and/or precolumns and rigorous sample pretreatment became more and more necessary, while decreasing the time and material requirements of the analysis was also needed.

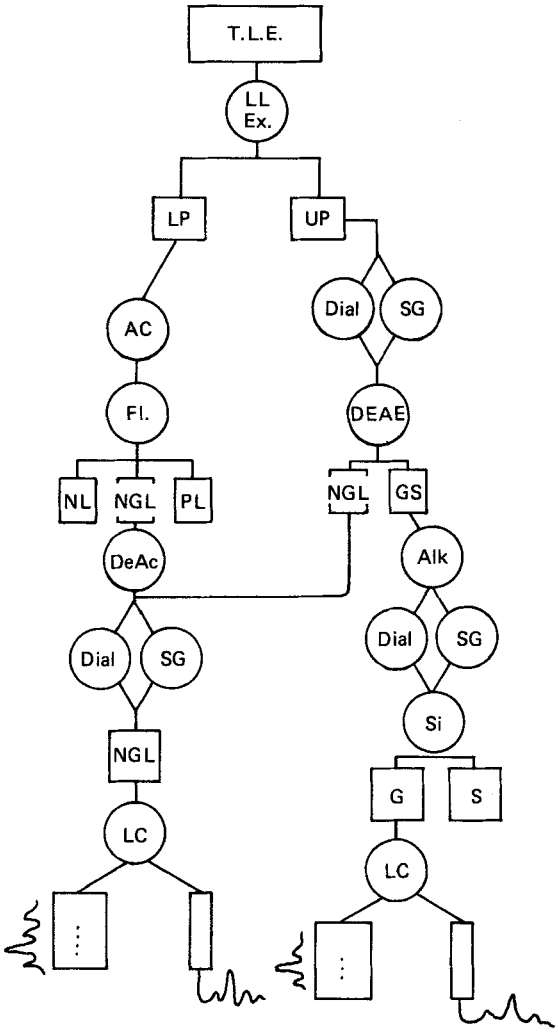
A further requirement can be inherent in the samples, like in our case. The sample can be extremely small amount and unrepeatable (for example a lymphocyte sample of a leuchemic patient before chemotherapy). For this reason the whole analytical process has to be precise, reproducible and rugged.

The method developed in our laboratory for the separation of TLE into lipid classes in purity, adequate for further fractionation into separate lipid types, has the advantage of speed, automatebility and the possibility of development into an on line complete HPLC fractionation.

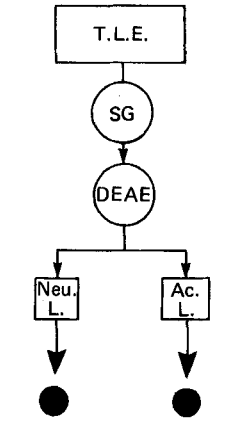
The first step in our method of TLE fractionation is the known DEAE Sephadex column chromatography for the isolation of neutral and acidic lipids. For the following steps of the separation we applied Brownlee Labs SIL-GU and ODS-GU cartridges.

Comparing the flowcharts 1, 2, 3 with 4 it can be seen (Figure 1) that the use of small cartridge columns for the class fractionation after the separation of acidic and neutral lipids is much simpler and more economic in terms of labour, solvents and time.

The acidic lipid purification with the ODS-GU cartridge described in the experimental part was checked for purity of the ganglioside class with HPTLC as shown in Figure 2. As can be seen on the chromatogram, only resorcinol positive compounds are present.

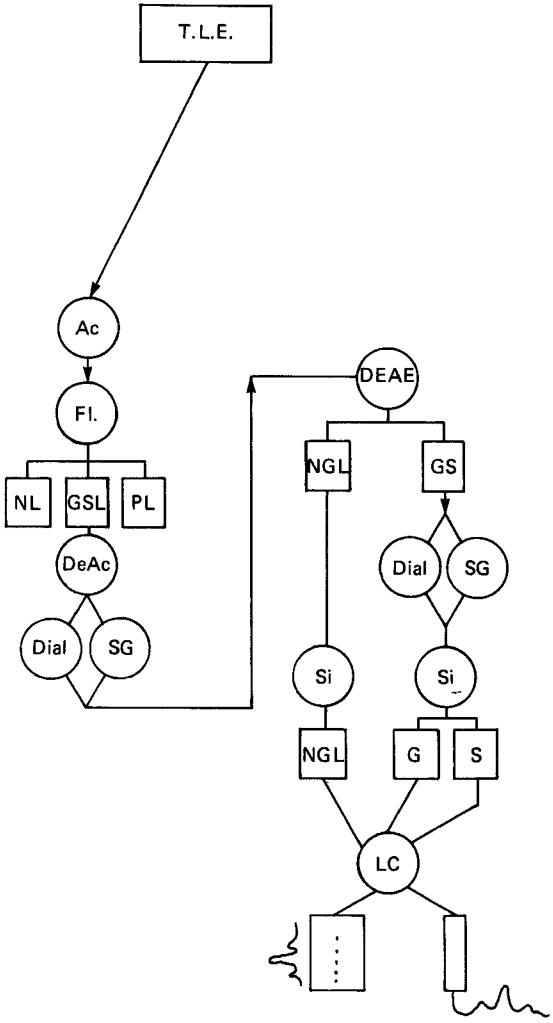


FLOWCHART # 1

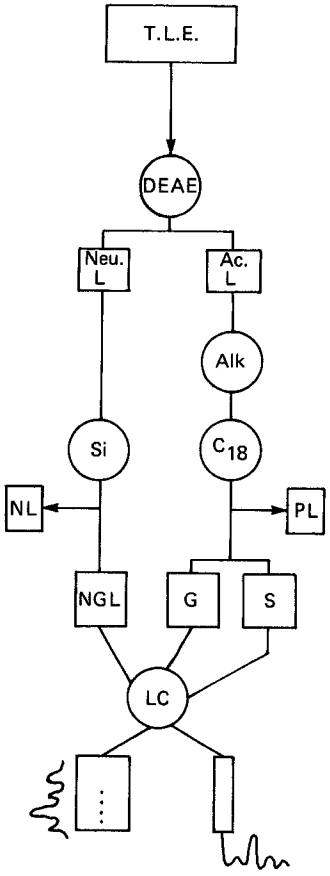


FLOWCHART #2

Figure 1. Possible work up routes of TLE.
Flowcharts #1 - 3 from literature data, #4 showing our method.
Symbols used: LL EX.: liquid-liquid extraction, LP: lower phase, UP: upper phase, Ac: acetylation, Fl: Florisil col.chrom., DeAc: deacetylation, Dial: dialysis, SG: Sephadex G col.chrom., G: gangliosides, S: sulphatides, Alk: alcalization, Si: silicic acid col.chrom., SI: SIL-GU col.chrom., C_{18}: ODS-GU col. chrom.



FLOWCHART #3



FLOWCHART #4

Figure 1 - continued

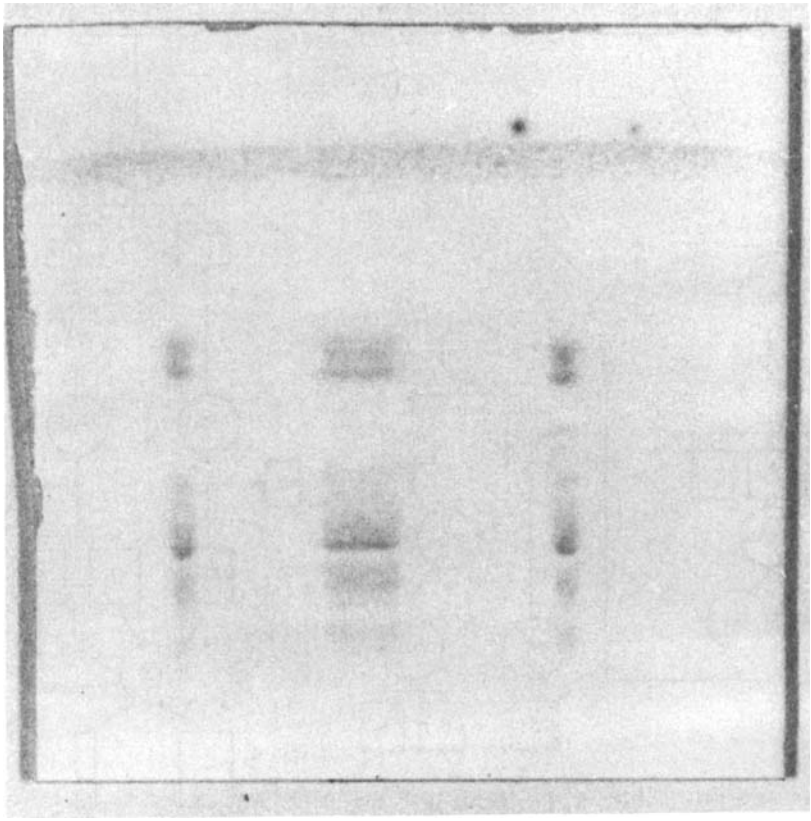


Figure 2. HPTLC separation of lymphocyte gangliosides to show their purity. Sample applications of 0.5, 1.5, 1.0 µl of ganglioside solution of 8 x 10^9 lymphocytes in 1.0 ml chloroform : methanol = 1 : 2 .
Plate: 5 x 5 cm Merck HPTLC Si 60, developing system: $CHCl_3$: CH_3OH : 0.025M KCl/H_2O = 60 : 40 : 9 , staining with resorcinol-HCl, 100°C, 20 min.

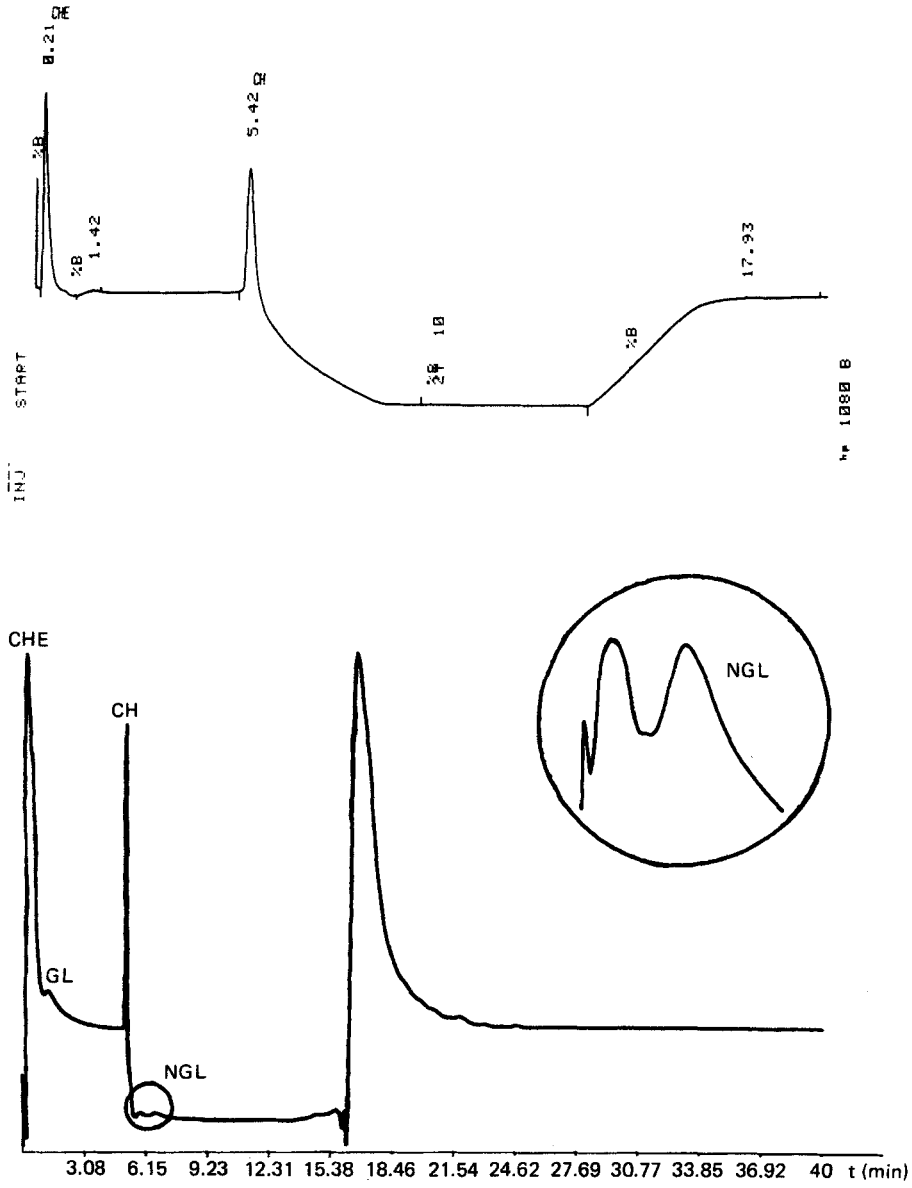
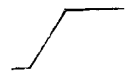
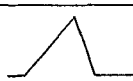


Figure 3. Chromatograms of lymphocyte neutral lipid fraction with elution profile No. 5.
1. UV detector signal, 2. RI detector signal.
CHE: cholesterol esthers, CH: cholesterol, NGL: neutral glycolipids.

Table 1. Elution profiles tested for the separation of neutral lipid classes on SIL-GU column, eluents; A:chloroform, B:methanol.

Gradient No.	Gradient profile	SEGMENTS %B at start / %B at end / duration min. 1	2	3	4	5	Flow ml/min	No. of fractions
1		0 0 1	0 100 10	100 100 15	-	-	1.0	26
2		0 10 10	10 100 5	100 100 1	100 0 4	0 0 20	0.5	60
3		0 0 3	0 100 9	100 100 3	100 0 4	0 0 20	0.5	20
4		10 10 20	100 100 10	10 10 10	-	-	0.5	20
5		0 0 1	0 100 9	100 0 5	0 0 25	-	0.5	5
6		7 7 1	7 100 9	100 7 5	7 7 20	-	0.5	4
7		30 30 40	-	-	-	-	0.5	6

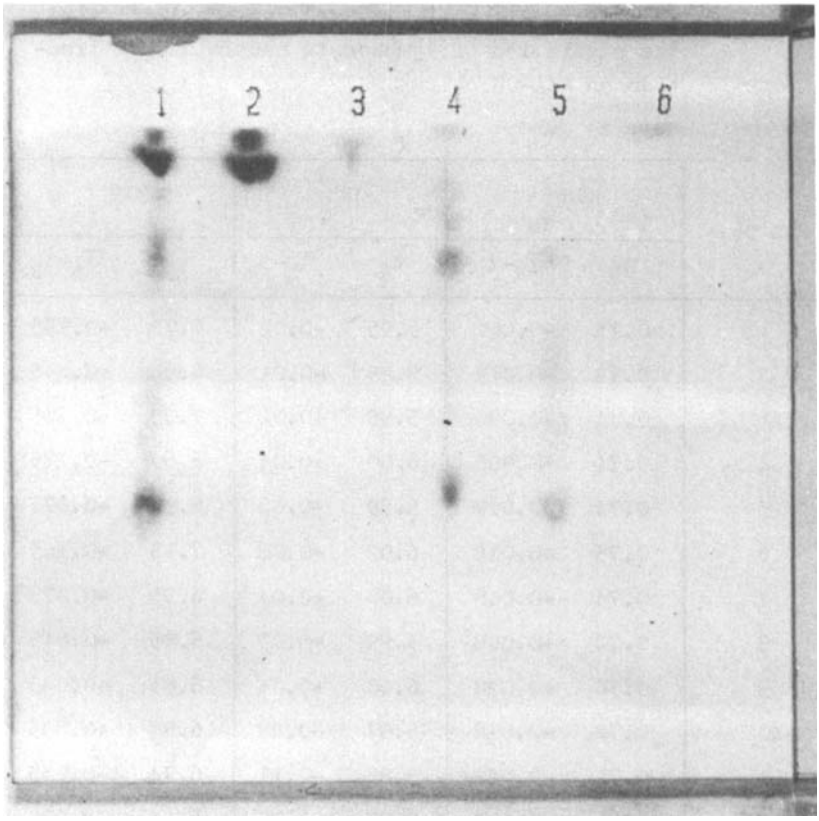


Figure 4. General monitoring plate of the separation of lymphocyte neutral lipid fraction on SIL-GU column.
Plate: 5 x 5 cm Merck HPTLC Si 60, developing system: $CHCl_3$: CH_3OH : H_2O = 65 : 25 : 4 , staining with H_2SO_4 : H_2O = 1 : 1 , 100°C, 2 min.
Lanes: 1. TLE, 2., 3. neutral lipids (CHE, CH, glycerides etc.) 4.,5. neutral glycolipids, 6. background of solvents evaporated.

Table 2. Retention time stability and reproducibility of the separation of lymphocyte neutral lipid fraction on SIL-GU column.

Run No.	CHE		CH		NGL	
	t_R	$\bar{t}_R - t_R$	t_R	$\bar{t}_R - t_R$	t_R	$\bar{t}_R - t_R$
1	0.73	-0.002	5.95	-0.01	6.72	-0.155
2	0.72	-0.012	5.95	-0.01	6.92	+0.045
3	0.74	+0.008	5.98	+0.02	7.08	+0.205
4	0.74	+0.008	6.00	+0.04	6.60	-0.275
5	0.74	+0.008	5.99	+0.03	6.95	+0.075
6	0.75	+0.018	6.02	+0.06	7.13	+0.155
7	0.74	+0.008	6.00	+0.04	6.95	+0.075
8	0.74	+0.008	5.99	+0.03	6.83	-0.045
9	0.76	+0.028	6.00	+0.04	6.83	-0.045
10	0.72	-0.012	5.87	-0.09	6.88	+0.005
11	0.71	-0.022	5.85	-0.11	6.74	-0.135
12	0.72	-0.012	5.83	-0.13	6.84	-0.035
13	0.72	-0.012	5.98	+0.02	6.79	-0.085
14	0.72	-0.012	5.99	+0.03	6.90	+0.025
15	0.76	+0.028	6.00	+0.04	6.97	+0.095
16	0.71	-0.022	6.01	+0.05	6.87	-0.005
$\bar{t}_R$	0.732		5.96		6.875	
SD	0.016		0.06		0.130	

The neutral lipid class fractionation was carried out on a SIL-GU column. The dried sample was dissolved in chloroform, as the use of chloroform-methanol mixtures for sample introduction interfered with the separation of cholesterol esthers and cholesterol, although it dissolves the sample much better. After isocratic exploratory experiments, gradient runs were tried. The elution profiles tested and relevant data are summarized in Table 1. Of the several gradients tested for neutral lipid class fractionation, No.5, the best, was selected in the end. The corresponding chromatograms are shown in Figure 3. The aim was sufficient separation between classes and if possible, negligable separation in the classes themselves. This was to make possible the collection of small fractions for total recovery, or column coupling with the heart-cut method for analytical separation of the lipids of a selected class. As shown on a general monitoring plate (Figure 4) the class separation is adequate.

The stability and reproducibility of the method can be seen from Table 2, giving the retention times, their average values and standard deviation from 16 runs. It can also be seen from the table, that column performance is stable, there was no unidirectional drift in the $\bar{t}_R - t_R$ data. Cholesterol esthers and cholesterol retention times are from the UV signal, those for neutral glycolipids from the RI signal. The retention times are corrected for the delay caused by the connecting tubing between the detectors.

As shown by the standard deviations the reproducibility is very good. Numerous samples can be analysed on a small cartridge without any significant change in system behaviour, as can be seen in the random character of the variation of $\bar{t}_R - t_R$ values.

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REFERENCES

1. M. Williams, R. McCluer, J. Neurochem., 35, 266 (1980).
2. S.K. Kundu, A. Suzuki, J. Chromatogr., 224, 249 (1981).
3. S-I. Hakomori, Annual Rev. Biochem., 50, 733 (1981).

4. H. Wiegand, Advances in Neurochem., 4, 149 (1982).
5. A. Suzuki, S. Handa, T. Yamakawa, J. Biochem., 82, 1185 (1977).
6. T. Yamazaki, A. Suzuki, S. Handa, T. Yamakawa, J. Biochem., 83, 1101 (1979).
7. P. Hanfland, H. Egge, U. Dabrowski, D. Roelke, J. Dabrowski, Biochemistry, 20, 5310 (1981).
8. S. Handa, Y. Kushi, Adv. Exp. Med. Biol., 152, 23 (1982).
9. R. McCluer, J. Evans, J. Lipid Res., 14, 611 (1973).
10. K. Watanabe, Y. Arao, J. Lipid Res., 22, 1020 (1981).
11. J. Vajda, J. Pick, L. Leisztner, Spec. FEBS Meeting, Athens, Greece, 1982.
12. M. Gurkin, J. Ripphahn, Int. Lab., May/June, 63 (1980).
13. R. Yu, R. Ledeen, J. Lipid Res., 13, 680 (1972).
14. L. Svennerholm, P. Fredman, Biochim. Biophys. Acta 617, 97 (1980).
15. V. Iyengar, Anal. Chem., 54, 554A (1982).