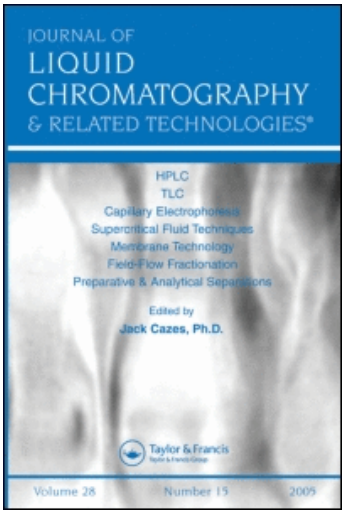

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ISOLATION AND COMPLETE SEPARATION OF LIPIDS FROM NATURAL SOURCES

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ABSTRACT

A method of isolation and complete separation of lipids from natural sources into classes and species is reported which combines our previously published techniques with new techniques described in this article for the first time. Pigments are separated from crude total lipid extracts with two successive TLC systems: a) petroleum ether/benzene/glacial acetic acid (30:70:2) and b) acetone/methanol/water (40:20:1). Pigment-free total lipids are separated on a silicic acid column into neutral, glyco- and phospholipids. Neutral, glyco- and phospholipids are separated into classes and species by suitable HPLC methods.

INTRODUCTION

The study of lipids has assumed considerable importance in recent years with the recognition that they are involved in many vital biological

processes in animals, plants and microorganisms. It is well known that lipids serve as a major storage form of energy and that they are responsible for maintaining the structural integrity of cells as the principal components of the membranes. Also, lipids are ingredients of all foods and their composition is obviously vital to good nutrition. Disturbances in lipid metabolism are known to accompany a variety of disease states and the role of lipids in heart disease remains an unresolved controversy. It has become apparent that certain lipids such as phosphatidylinositol and its metabolites are vital cellular messengers while the discovery of Platelet-Activating Factor,[1] an unusual species of phosphatidylcholine that possesses extremely potent biological activity, opened a new and unexpected chapter in the history of phospholipids.

Even though methods for the analysis of lipids are of great importance for many research, clinical and quality control applications and a lot of methods have been reported in the literature, to our knowledge the techniques provided are not appropriate for the complete separation of total lipids from natural sources.

Chromatography has been recognized as a useful separation technique in lipid analysis. Column chromatography (CC), thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) even though have served lipid analysis well over the years, do have a number of disadvantages such as poor recovery, doubtful reproducibility and unsatisfactory separation.

High performance liquid chromatography (HPLC) today offers considerable advantages as a qualitative and quantitative analytical tool. The wide range of column packing materials along with the variety of eluting systems available in HPLC permits successful separation of all the classes of lipids. In addition, resolution tends to fall off only slowly with increasing sample size, analysis time can be short, retention times of compounds under set conditions are reproducible in combination with the fact that the sample does not need to be derivatized and its lipid components are fully recovered (very important in the case where further biological and structural study has to be performed).

In the present study, a method of isolation and entire separation of lipids from natural sources into classes and species is reported which combines our previously published techniques with new techniques described in this article for the first time. These new techniques are a) a TLC system for the isolation of pigment-free total lipids b) a column chromatographic technique for separation of total lipids into neutral, glyco- and phospholipids and c) the separation of phospholipids into classes and species by HPLC. In the

discussion section some alternative techniques for the analysis of lipids are also presented which are already published.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade, purchased from Merck (Darmstadt, G). HPLC solvents were purchased from Ruthburn (Walkerburn, Peebleshire, UK). Lipid standards of HPLC grade were obtained from Supelco (Bellefone, PA, USA). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described.[1] Chromatographic material used for column chromatography was silicic acid 35-70 mesh ASTM 7733 (Merck, Darmstadt, G). Chromatoplates (20X20cm) were of analytical (0.25mm thickness) and preparative scale (1 mm thickness). Chromatographic material used for thin layer chromatography was silica gel G -Type 60 (Merck, Darmstadt, G). Blood samples were collected from patients with primary glomerular disease previously used for the determination of PAF levels in another study. Nettle (Urtica dioica) was collected from countryside (Attica, GR). Bovine brain was also used immediately after the sacrifice of the animal.

Preparation of Standards and Samples

All lipid standards were prepared as 5% solutions in chloroform/methanol (1:1). Homogenized nettle leaves and roots were extracted and the total lipids were obtained. Total lipids were extracted from blood, fractionated on silicic acid column chromatography and the fraction with the biologically active lipids was obtained.[2] This fraction was further separated in neutral and phospholipids with current counter distribution.[3] Total lipids were isolated from bovine brain by extraction.

Silicic Acid Column Preparation

The silicic acid, was washed with water and methanol and activated overnight at 120 °C. The glass column 13.5mm (I.D.), was slurry packed (46cm height) using chloroform. The lipid sample was dissolved in 0.5mL of chloroform/methanol (1: 1), then taken up with up to 2mL chloroform.

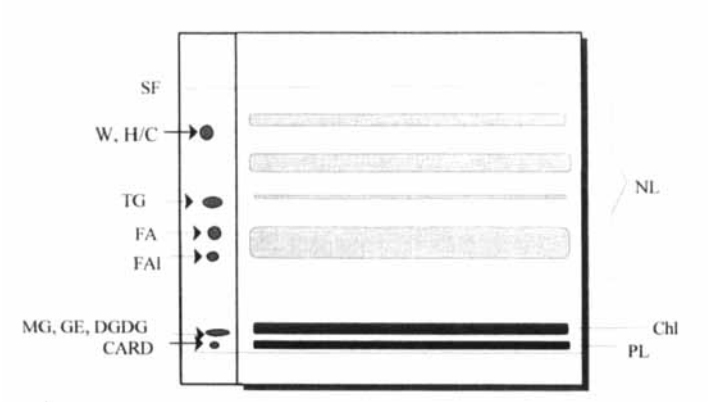

Figure 1. TLC development of standard lipids and of lipids from nettle in petroleum ether/benzene/glacial acetic acid (30:70:2).
SF: solvent front; W: waxes; HC: hydrocarbons; TG: triglycerides; FA: fatty acids; FAl: fatty alcohols; MG: monoglycerides; GE: glycerylethers; DGDG: digalactosyldiglycerides; CARD: cardiolipin; NL: neutral lipids; Chl: chlorophylles (pigments); PL: polar lipids.

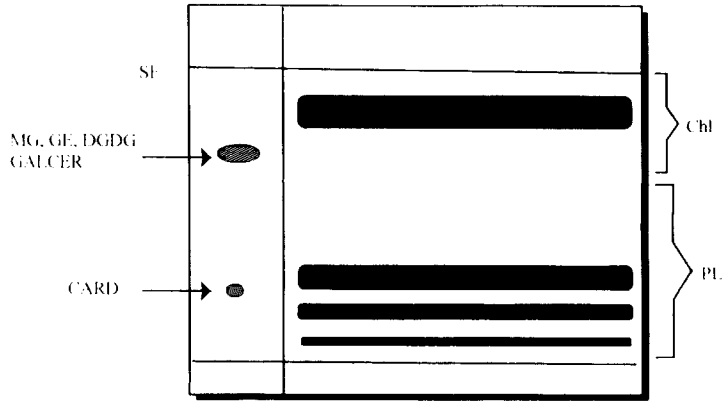

Figure 2. TLC development of standard lipids and of lipids from nettle in acetone/methanol/water (40:20:1).
SF: solvent front; MG: monoglycerides; GE: glycerylethers; DGDG: digalactosyldiglycerides; CARD: cardiolipin; GALCER: galactocerebrosides; Chl: chlorophylles (pigments); PL: polar lipids.

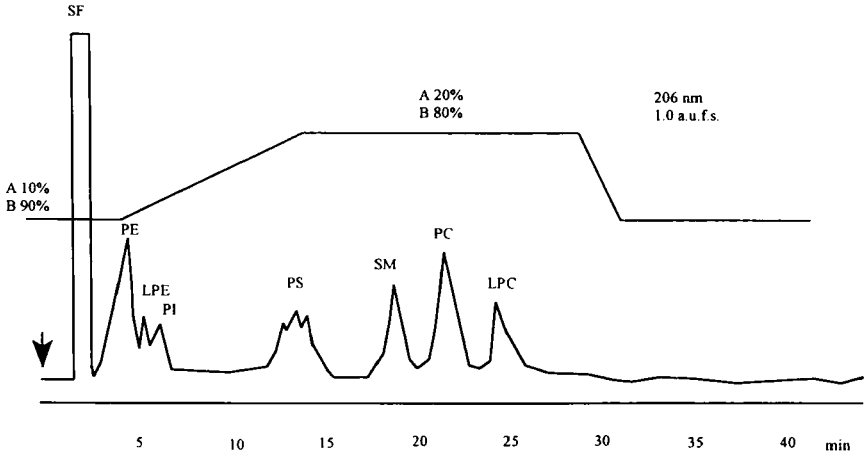

Figure 3. HPLC chromatogram of phospholipids standards on a HPLC silica B/5 column. Conditions and solvents in Results section. Gradient as indicated.
SF: solvent front; PE: phosphatidylethanolamine; LPE: lyso-phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; SM: sphingomyelin; PC: phosphatidylcholine; LPC: lyso-phosphatidylcholine.

Chromatography

HPLC was performed on a dual pump Jasko (Tokyo, Japan) model 880-PU HPLC, supplied with a 330 μL loop Rheodyne (P/N 7125-047) injector. A Jasko model 875 UV spectrophotometer was used as detector (205 - 210nm). The spectrophotometer is connected to a Hewlett-Packard (Avondale, PA, USA) model HP-3396A integrator-plotter. The following columns were used: a cation exchange column SS 10μm Partisil 25cm x 4.6mm I.D., PXS 10/25 SCX from Whatman (Clifton, NJ, USA); an absorption column Silica 25cm x 4.6 mm I.D., H5 from Hichrom (Reading, Berkshire, U.K.); an absorption column Silica 25cm x 4.6mm B/5, Perkin- Elmer, (Norwalk, CT, USA); and a reverse phase column Nucleosil-300, C_{18} column 7μ, 250 x 4mm I.D. from Analysentechnik (Mainz, G). The flow rate was 1mL /min.

RESULTS

Extraction

Total lipids were extracted from the sample according to Bligh-Dyer[4]

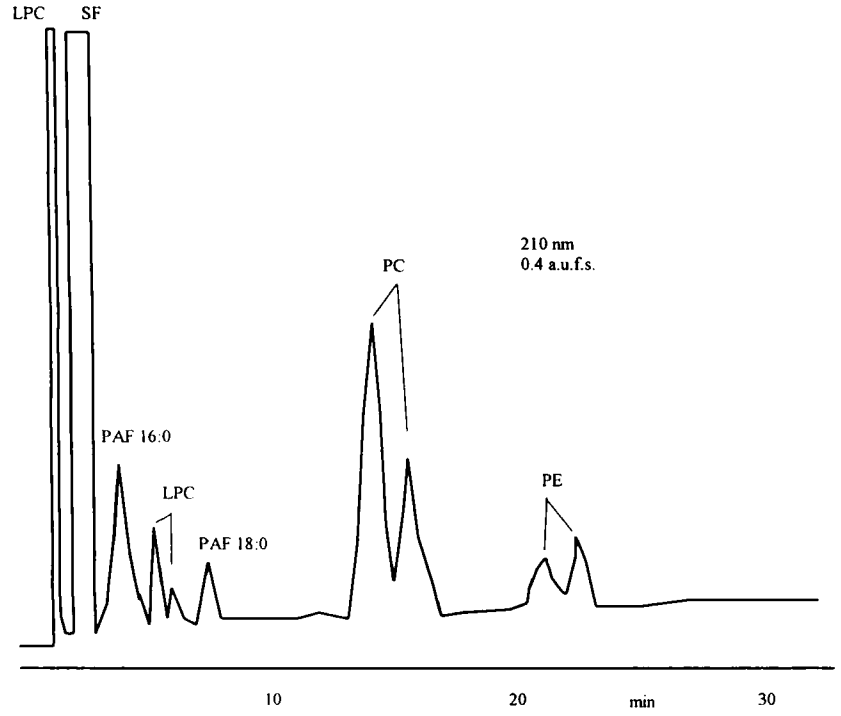

Figure 4. HPLC chromatogram of phospholipids standards on a HPLC nucleosil-300 column. Conditions and solvents in Results section.
SF: solvent front; PE: phosphatidylethanolamine; PC: phosphatidylcholine; LPC: lyso-phosphatidylcholine; PAF: Platelet-Activating Factor.

except that distilled water was used instead of saline. The chloroform phase from the extraction procedure was evaporated to dryness at 35 °C in a flash evaporator. The dry residue was transferred into a test tube using small volumes of chloroform/methanol (1:1), and the sample of crude total lipids was dried under a stream of nitrogen.

Isolation of Pigment-Free Total Lipids

In the case of samples of plant origin, the pigments were separated from total lipids with the aid of two successive TLC systems as follows: Crude

total lipids are redissolved in a small volume of chloroform/methanol (1:1) and applied to an appropriate number of preparative TLC plates. The chromatogram is developed in petroleum ether/benzene/glacial acetic acid (30:70:2). As shown in Figure 1 polar lipids (phospholipids and glycolipids) along with the pigments remain in the origin while neutral lipids mainly migrate along the plate with the exception of monoglycerides (MG) and glycerylethers (GE) which comigrate with glycolipids. The bands of neutral lipids as well as the band of polar lipids and pigments are scrapped off separately, extracted according to Bligh-Dyer, centrifuged and the organic solvents are phased by adding appropriate volumes of chloroform and water to arrive to a final chloroform/methanol/water ratio of 1: 1:0.9. The extract that contains polar lipids, small amount of neutral lipids and pigments is evaporated to dryness and redissolved in a small volume as above. Polar lipids and pigments are rechromatographed on an appropriate number of preparative TLC plates, using acetone/methanol/water (40:20:1) as developing system. As shown in Figure 2 pigments migrate near the solvent front while polar lipids migrate along the plate. Glycolipids as well as MG and GE migrate below pigments. The fraction of pigments is discarded while the polar lipids along with the small amount of neutral lipids are recovered as above. The fraction of polar lipids and neutral lipids are pooled together to reconstitute the total lipid extract free from pigments. The last step is necessary because glycolipids are partitioned in both fractions of neutral and phospholipids in the first developing system and polar lipids contain also of a small amount of neutral lipids (MG, GE) in the second development system.

Application of the described procedure was successfully performed to nettle leaves and roots (Figure 1, 2).

Separation of Total Lipids in Neutral, Glyco- and Phospholipids

An amount of 0.8g of pigment-free total lipids is redissolved in 0.5mL of chloroform/methanol (1:1), then taken up with up to 2mL chloroform and fractionated on a silicic acid column. Neutral lipids are eluted with 8 bed-volumes of chloroform followed by 2 bed-volumes of chloroform/acetone (19:1). Glycolipids are eluted with 2 bed-volumes of chloroform/acetone (2:1) followed by 17 bed-volume of acetone. Finally, phospholipids are eluted with 10 bed-volumes of methanol. The fractions of neutral, glyco- and phospholipids are evaporated to dryness and redissolved in a small volume of chloroform/methanol (1:1) as described above.

Application of the described procedure was successfully performed in the total lipids from bovine brain and from nettle leaves and roots.

Separation of Neutral Lipids into Classes and Species

Neutral lipids are separated into classes and species by HPLC[5] using a nucleosil-300, C_{18} column and a stepped gradient elution with the following solvents: A, methanol/water (80:20); B, acetonitrile/methanol (60:40); C, acetonitrile/tetrahydrofuran (99.5:0.5); and D, isopropanol/acetonitrile (99:1). A linear gradient from solvent A to solvent B in 10 min, a hold for 5 min in B and then a linear gradient to solvent C in 10min followed by a second hold in C for 15min results in a total separation within 40min (see Figure 6). By introducing a third linear gradient step from solvent C (decreasing the hold step to 5min) to solvent D in another 10min and holding D for 15min, a more distinct separation of triglycerides can be achieved. The flow rate is 1mL/min and detection is achieved at 206nm with UV detector.

Separation of Glycolipids

Glycolipids are separated into classes and species by HPLC[6] using a nucleosil-300, C_{18} column and a stepped gradient elution, which starts with a linear gradient from 100% methanol/water (4:1) to 100% acetonitrile/methanol (7:5) in 10min and then hold for 15min. A 25min elution is sufficient for the separation of four glycolipid classes (gangliosides, sulfatides, digalactosyl-diglycerides and galactosyl-cerebrosides) which are studied. The flow rate is 1mL/min and a UV detector is used at 206nm.

Separation of Phospholipids:

a) Into classes. Phospholipids are fractionated (Figure 3) on a Silica B/5 column with a stepped gradient elution with the following solvents: A, water; B, hexane/isopropanol (43:57). A hold for 5min in A 10%, a linear gradient from A 10% to A 20% in 10min, a hold for 15min in A 20% and then a linear gradient to A 10% in 2min and hold for 10min in A 10%. During this separation the flow rate is 2mL/min and phospholipids are detected at 206nm with UV detector. By this method phospholipids are satisfactorily separated within 25min.

b) Into species. Each class of phospholipids can be further fractionated into species with a nucleosil-300, C_{18} column and isocratic elution system of methanol/water/acetonitrile (63:7:30). The flow rate is 1mL/min and the detection was achieved at 210nm with UV detector.

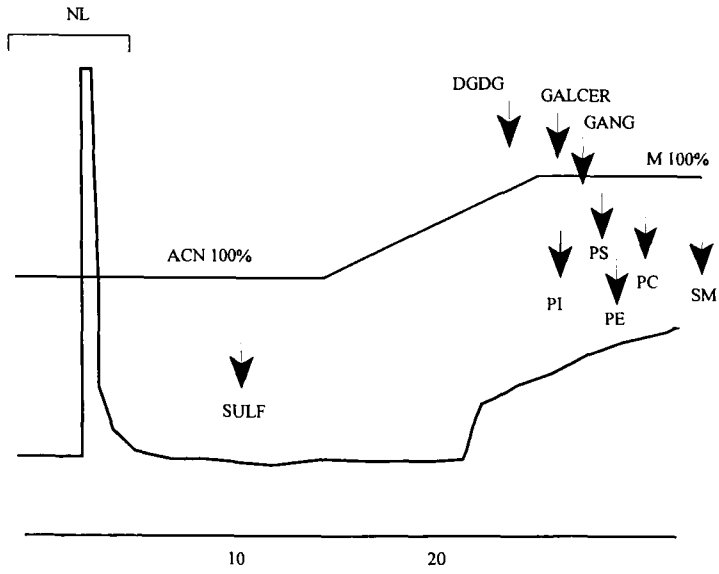

Figure 5. Separation of total lipids into neutral, glyco- and phospholipids on a HPLC silica column. Conditions and solvents in Discussion section. Gradient as indicated. NL: neutral lipids; SULF: sulfatides; DGDG: digalactosyldiglycerides; GALCER: galactocerebrosides; GANG: gangliosides; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; SM: sphingomyelin; PC: phosphatidylcholine.

The separations of phosphatidylethanolamine (PE), phospatidylcholine (PC), lysophosphatidylcholine (LPC) from human blood as well as the separation of semisynthetic PAF into species are shown in Figure 4. This method has the advantage of separating the more polar phospholipids (e.g. LPC, PAF) as well as the less polar ones (e.g. PE) into species within a reasonable time (25min).

DISCUSSION

The extraction of lipids can be performed by several conventional procedures. According to our experience, extraction according to Bligh-Dyer using distilled water instead of saline results in the recovery of gangliosides in the chloroform phase along with total lipids. Gangliosides can be separated from total lipids by washing the above chloroform phase with equal volume of aqueous phase (chloroform/methanol/saline, 3:48:47) where gangliosides are

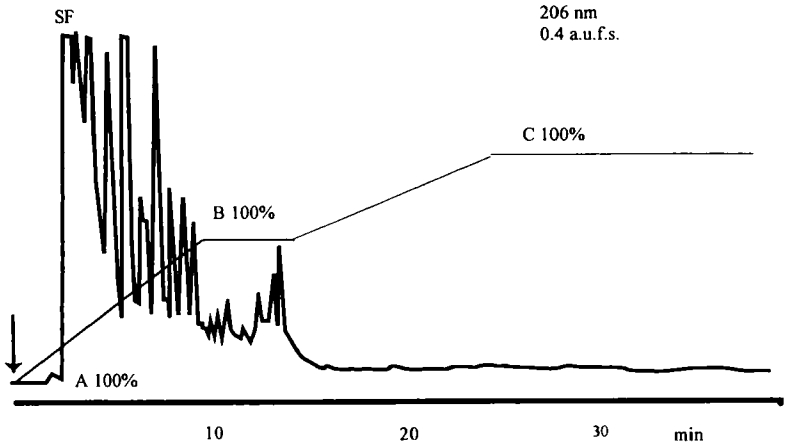

Figure 6. HPLC chromatogram of a neutral lipid fraction from nettle extracts on a HPLC nucleosil-300 column. Conditions and solvents as indicated in the Results section. SF: solvent front

partitioned in the water phase. By the addition of one volume of saline, one volume of ethanol and two volumes of chloroform in the water phase which contains gangliosides, in order to obtain a final ratio of chloroform/methanol/ethanol/saline (2:1:1:2), gangliosides, after two to four hours in 4 °C, are partitioned in the organic phase.[7] The last procedure has the advantage of obtaining gangliosides using mild techniques in an organic phase which is easily handled.

The existence of pigments in the sample which are usually isolated along with the total lipids is a problem that can be faced with the aid of the proposed new two-step TLC separation. By this way the fraction of pigments does not contain any lipids and can be discarded.

As already mentioned, neutral and glycolipids are present in both lipid fractions extracted from TLC. Therefore it was preferred to reconstitute the total lipid mixture free of pigments and then separate the pure neutral, glyco- and phospholipid fractions by the silicic acid column chromatographic technique. This technique is a useful procedure for fractionation of lipids mixtures in a preparative scale. The modified bulk elution system proposed here in comparison with the already published ones,[8] has the advantage of obtaining pure neutral, glyco- and phospholipid fractions with significantly

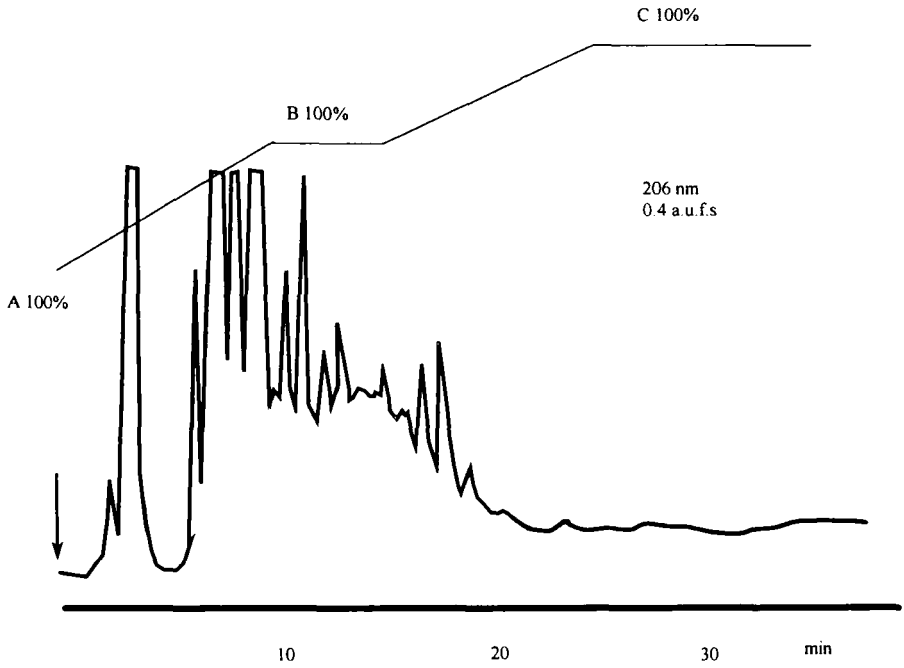

Figure 7. HPLC chromatogram of a neutral lipid fraction from human blood on a HPLC nucleosil-300 column. Conditions and solvents as indicated in the Results section. SF: solvent front.

reduced volumes of eluting solvents. Alternatively, when the sample contains small amount of lipids and at the same time our interest is focused on one of the lipid classes (either neutral, glyco- or phospholipids), or in the case we want to see a general pattern of total lipids (Figure 5), separation of total lipids can be performed on a HPLC absorption column, Silica from Hichrom H5 using a gradient elution system consisting of acetonitrile and methanol. A hold of acetonitrile 100% in 15min, followed by a linear gradient to methanol 100% in 10min and a last hold of methanol 100%. Neutral lipids are eluted along with the solvent front while phospholipids are eluted with 100% methanol. The elution of glycolipids begins approximately 3min after the elution of neutral lipids and is completed 3min after introduction of 100% methanol.

Several procedures for the separation of neutral lipids have previously been reported.[5] The method of the fractionation of neutral lipids proposed here,

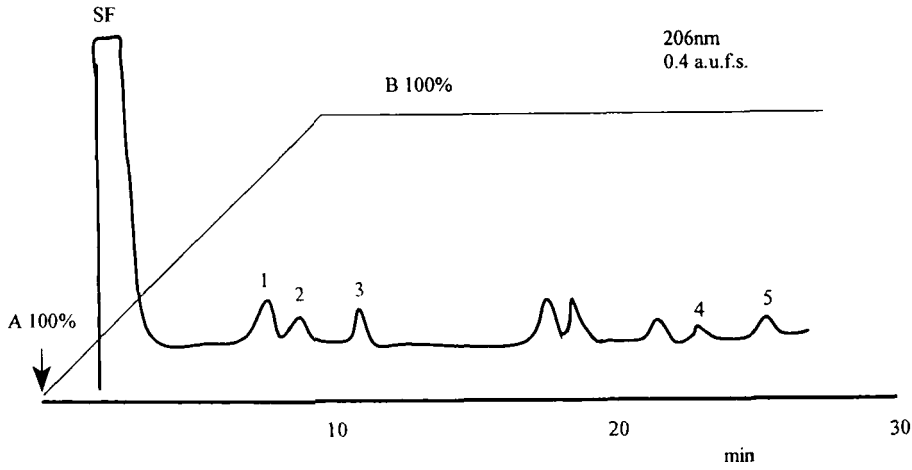

Figure 8. HPLC chromatogram of a glycolipid fraction from bovine brain on a HPLC nucleosil-300 column. Conditions and solvents as indicated in the Results section. SF: solvent front. 1: gangliosides; 2 and 3: sulfatides; 4 and 5: digalactosyl- diglycerides.

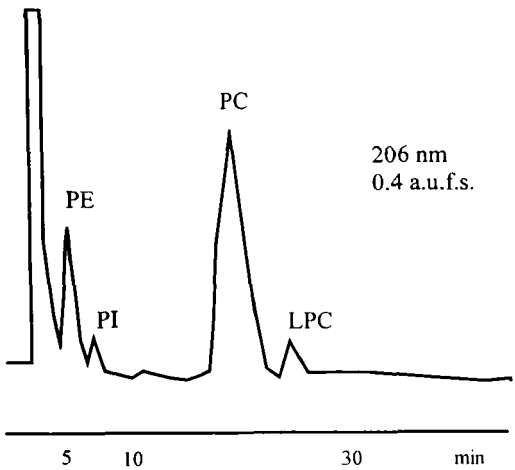

Figure 9. HPLC chromatogram of a phospholipid fraction from nettle extracts on a HPLC silica B/5 column. Conditions and solvents as indicated in the Results section. PE: phosphatidylethanolamine; PI: phosphatidylinositol; PC: phosphatidylcholine; LPC: lyso-phosphatidylcholine.

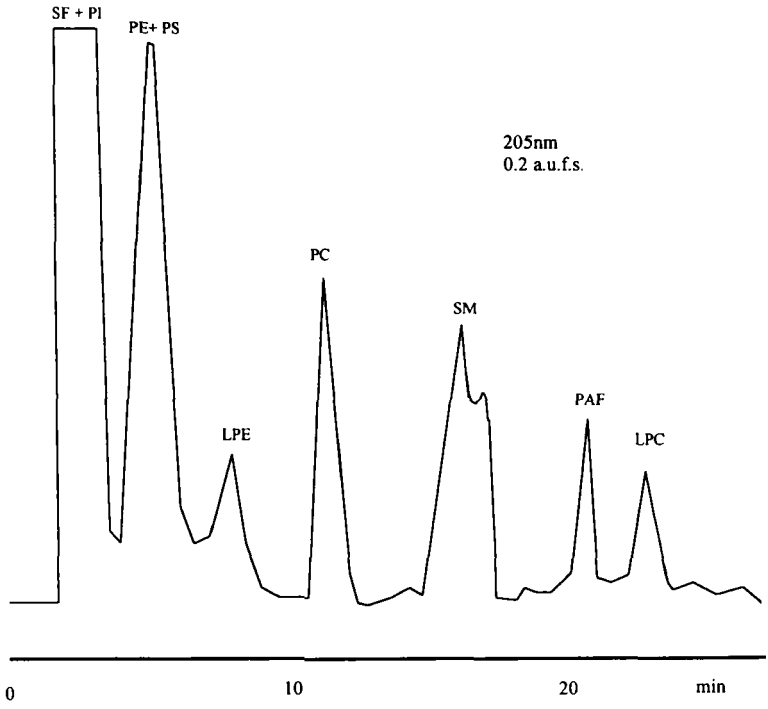

Figure 10. HPLC chromatogram of phospholipid standards on a HPLC cation exchange column. Conditions and solvents as indicated in the Discussion section.
SF: solvent front; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; LPE: lyso-phosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin; PAF: Platelet-Activating Factor; LPC: lyso-phosphatidylcholine.

has the advantage of separating them simultaneously into classes and species, within the reasonable time of approximately lhr and with UV detection which has low cost, easy operation and is compatible with gradient elution. In addition, peak(s) of the same or different classes can be collected either for investigation of the structure and the possible biological activity or for further fractionation by conventional methods suitable for each class. Application of the described method was succesfully performed in neutral lipids derived from nettle (Figure 6) and in neutral lipids derived from human blood (Figure 7).

Glycolipids consist of molecules with a wide spectrum of polarity and for this reason the methods reported focus on the separation of individual

classes from different sources.[6] The recommended method is superior since it permits simultaneously the sufficient separation of glycolipids classes and species. Application of the described method in glycolipids derived from bovine brain is illustrated in Figure 8.

A number of methods concerning the separation of phospholipids into classes has been reported during the last decade.[9] Most of these methods permit a satisfactory separation of phospholipids. The only reason we recommend our method is that on one hand the reported methods do not have additional advantages compared to the proposed one and on the other hand this method has been well tested and used on a routine basis in our laboratory. Application of the described separation in phospholipids from nettle is presented in Figure 9. The most satisfactory separation of polar phospholipids such as sphigomyelin, PAF, lysophosphatidylcholine and lyso- PAF[10] can be accomplished by using a cation exchance column which permits an alternative method of phospholipid separation. In this method (Figure 10) an isocratic elution system is used consisting of acetonitrile/methanol/water (300:150:35).

Methods for the separation of an individual phospholipid class to its species have evolved rapidly in recent years.[9] In most cases a reverse phase HPLC is used with an elution system that consists of combinations of methanol, water, acetonitrile, acetic acid, chloroform etc. In the present method the achieved separation of the phospholipids into species is comparable to the previously reported ones.

In some cases, the separation of phosphonolipids from their structurally related phospholipids is necessary, in order to investigate phosphonolipids from different natural sources. This separation has been achieved by a HPLC method[11] which presents not only a satisfactory separation of two standard synthetic phosphonolipids from other structurally related phospholipids (PE, PC, SM, etc.) but also presents a method for the separation of phosphonolipids where phospholipids are not present.

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