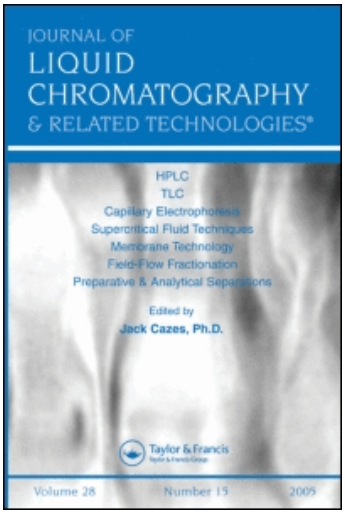

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HPLC Separation of Acyl Lipid Classes

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HPLC Separation of Acyl Lipid Classes

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Abstract: Identification of complex acyl lipids ideally includes the normal-phase HPLC to separate the acyl lipid classes followed by reversed-phase HPLC to separate the molecular species of a lipid class. Both polar lipid classes and non-polar lipid classes have been separated by normal-phase HPLC, mostly on silica and diol columns using binary gradients. The tentative identification of lipid classes can be made by HPLC retention times and the co-chromatography with lipid class standards. Various normal-phase HPLC systems separating lipid classes are reviewed here.

Keywords: Normal-phase HPLC, Lipid classes, Phospholipids, Triacylglycerols

INTRODUCTION

Fatty acids (FA) and fatty acid containing lipids (acyl lipids) are important components of biomembranes and storage oils in animals, plants, and microorganisms, and have many uses in medicine and industry. FA are structurally complicated, due to the difference in length, number of double bonds, position of double bonds, configuration of double bonds, number and position of triple bonds, number and position of substituents, e.g., hydroxyl, epoxy, hydroperoxyl, and branches on the FA chain, etc. Most acyl lipids contain a glycerol backbone with two or three FA chains attached and the structure of acyl lipids are, thus, much more complicated than that of FA.

The analysis of acyl lipids ideally includes normal-phase HPLC separation (fractionation) of lipid classes from the total lipid extract. The individual

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acyl lipid class is then separated to molecular species by reversed-phase HPLC followed by mass spectrometry (MS). Reversed-phase HPLC separation of the molecular species of various acyl lipid classes has been recently reviewed.[1–4] The normal-phase HPLC separation of phospholipid (polar lipid) classes was reviewed by Christie in 1996.[5] The present review covers the normal-phase HPLC separation of acyl lipid classes including polar and non-polar lipids. Various HPLC columns have been used for the separation of acyl lipid classes, mostly the silica and diol columns. The selectivity of both columns are similar. Since the polarities of various lipid classes vary considerably, for example the polarities of triacylglycerol (non-polar) and lysophosphatidylcholine (polar), the elution from the column of all of the lipid classes of a natural sample requires gradient eluent.

NORMAL-PHASE SILICA COLUMN HPLC

Silica Column Eluted with Ternary Gradient

Initially ternary gradient eluent was developed to elute the silica HPLC column to accommodate the wide polarity range of various lipid classes in a natural sample. Non-polar solvents such as hexane and chloroform can be used to elute non-polar lipids from the column, while polar solvents containing water can be used to elute polar lipids. The ternary gradients developed earlier were from the initial non-polar eluent to the final polar eluent containing water. Since water and non-polar solvents cannot be mixed homogeneously in one phase, intermediary eluents between the two extremes were used to make the entire HPLC eluent homogeneous in one phase. The ternary gradient eluents are more complicated than that of binary eluents.

One of the early ternary gradient eluents developed by Christie[6] used three solvents as follows: (A) hexane/tetrahydrofuran (99:1), (B) chloroform/2-propanol (1:4), and (C) 2-propanol/water (1:1). Solvent B was introduced into solvent A over a 5 min period to elute non-polar lipids, then a gradient of solvent C into a mixture of A and B was generated to elute each of the phospholipids. The HPLC chromatogram is shown as Figure 1. In a prior report,[7] isooctane was used instead of hexane. An evaporative light scattering detector (ELSD) was used and the calibration curves of ELSD were created for quantification purpose. ELSD detection is not dependent on the presence of a chromophore in the lipid molecules and, thus, enables quantification, nor is it affected by changes in different mobile phases that are often used in the separation of the lipids. However, a disadvantage of this method is that no other detector can be used for the effluent after ELSD and the effluent after ELSD cannot be fraction collected for further studies. For the theory of ELSD and the comparison to other HPLC detectors, see the chapter written by Moreau.[8]

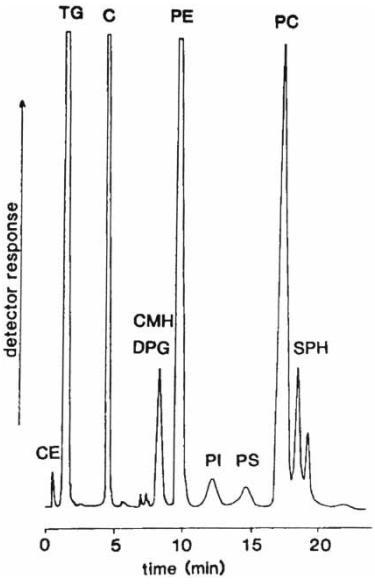

Figure 1. Separation of a lipid extract from rat kidney on a silica column using a ternary gradient of hexane/tetrahydrofuran/chloroform/2-propanol/water.[6] Abbreviations: CE, cholesterol esters; TG, triacylglycerols; C, cholesterol; DPG, diphosphatidylglycerol (cardiolipin); CMH, ceramide monohexoside; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin (usually seen as a double peak).

Moreau[9] simplified the three solvents of ternary gradient as follows: (A) isooctane/tetrahydrofuran (99:1), (B) 2-propanol, and (C) water. Subsequent studies by Letter[10] further simplified the three solvents as follows: (A) hexane, (B) 2-propanol, and (C) water. The chromatogram of six lipid classes is shown as Figure 2.

The HPLC separation of non-polar lipid classes was reported recently by Torres et al.[11] including alkoxyglycerols. The method uses a silica column and a gradient elution of isooctane, methyl *tert*-butyl ether (containing 0.01% of formic acid) and 2-propanol in different proportions. The chromatogram is shown as Figure 3. Isooctane can be replaced with hexane giving the same elution order and almost the same retention times of these non-polar lipid classes.

ELSD has been a commonly used detector in lipid class separation. It is sensitive, and a universal mass detector requiring no sample derivatization and can be used for quantitative analysis. However, for accurate quantification, calibration curves are required. Picchioni et al.[12] reported the calibration curves of nine commercially available lipid class standards. They were phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine

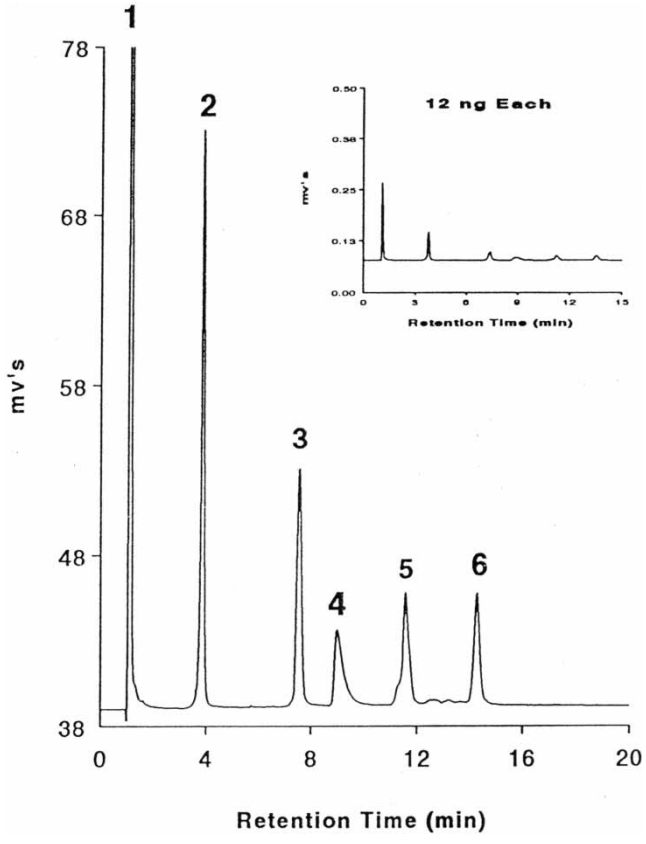

Figure 2. Separation of six lipid class standards on a silica column using ternary gradient of (A) hexane, (B) 2-propanol, and (C) water.[10] (1) cholesterol, (2) palmitic acid, (3) phosphatidylethanolamine, (4) phosphatidylserine, (5) phosphatidylcholine, and (6) sphingomyelin each 7 μg/20 μL by evaporative light-scattering detection.

(PC), lysophosphatidylcholine (LPC), phosphatidic acid (PA), digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG), sterol glycoside (SG), and acylated sterol glycoside (ASG). The ELSD responses of various lipid classes were shown to be different. LPC was the most sensitive in the ELSD response among these nine lipid classes. PA was the least sensitive and was only about one fourth of that of LPC. Later Seppanen-Laakso[13] also reported the calibration curves of cholesterol ester (CE), triacylglycerol (TAG), free cholesterol (FC), and PC. The ELSD responses of these four lipid classes varied. CE was the most sensitive in the ELSD response among these four lipid classes. PC was the least sensitive and was only about one fiftieth of that of CE. In a recent report[11] of the calibration curves of the non-polar lipid classes, ELSD responses

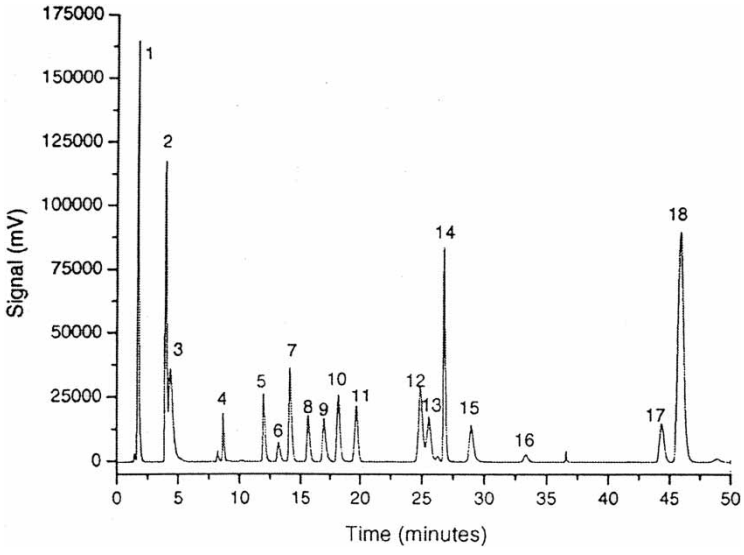

Figure 3. Separation of non-polar lipid classes on a silica column using ternary gradient of isooctane/methyl *tert*-butyl ether (contained 0.01% of formic acid)/2-propanol.[11] (1) squalene, (2) sterol esters, (3) waxes, (4) fatty acid ethyl esters, (5) diesterified alkoxyglycerols, (6) α-tocopherol. (7) triacylglycerols, (8) β-tocopherol, (9) γ-tocopherol, (10) free fatty acids, (11) δ-tocopherol, (12) non-esterified dialkoxyglycerols, (13) monoesterified alkoxyglycerols, (14) sterols, (15) 1,3-diacylglycerols, (16) 1,2-diacylglycerols, (17) non-esterified alkoxyglycerols, (18) monoacylglycerols. Micrograms injected 10–20.

were shown to be pretty similar including squalene, free fatty acids, monoesterified alkoxyglycerols, sterols, TAG, sterol esters, 1,3-diacylglycerols, fatty acid ethyl esters, α-tocopherol, monoacylglycerols, and non-esterified alkoxyglycerols. For the quantitative analysis of natural samples, the HPLC conditions (including ELSD) for the sample should be the same as those of the calibration curves of standards. Ideally, the standards used for calibration curves should be the same as those of the lipid classes in the natural samples.

Silica Column Eluted with Binary Gradient

The use of binary gradient allows for a simpler system of HPLC elusion compared to those of ternary gradients. It is possible to add the intermediary polar solvents in both eluent A and eluent B, allowing the gradient mixture to be homogeneous in one phase. Becart et al.[14] separated various phospholipids and sphingolipids standards well, using the binary gradient of (A) chloroform/methanol/ammonium hydroxide at 30% (80:19.5:0.5), and (B)

chloroform/methanol/water/ammonium hydroxide at 30% (60:34:5.5:0.5). Melton[15] separated soybean lecithins and beef phospholipids using the binary gradient of (A) isooctane/tetrahydrofuran/2-propanol/chroloform/water (415:5:446:104:30), and (B) isooctane/tetrahydrofuran/2-propanol/chroloform/water (216:4:546:164:80). In both studies ELSD was used.

Detection by UV absorbance is a sensitive method and has been commonly used at 205 nm for observing lipid classes containing at least one double bond. However, the solvents absorbing at 205 nm, e.g., chloroform and tetrahydrofuran, must be avoided. A second detector may also be used in series after UV detection of the effluent. In a report by Singleton and Stikeleather[16] a UV (205 nm) detector with the solvents (A) 2-propanol/hexane (4:3), and (B) 2-propanol/hexane/water (8:6:1.5) was used. Phospholipids were separated with a gradient starting at 100% solvent A to 100% solvent B in 20 min, isocratic with 100% solvent B for 15 min. We have used this HPLC system to separate acyl lipid classes using a flow scintillation analyzer to detect the radioactivities of the metabolites of radiolabelled phosphatidylcholine in castor microsomal incubation as shown in Figure 4.[17] In this study, both UV detector and flow scintillation analyzer were used. Co-chromatography with lipid class standards was used to confirm the radioactive peaks, while the lipid class standards were detected by UV detector.

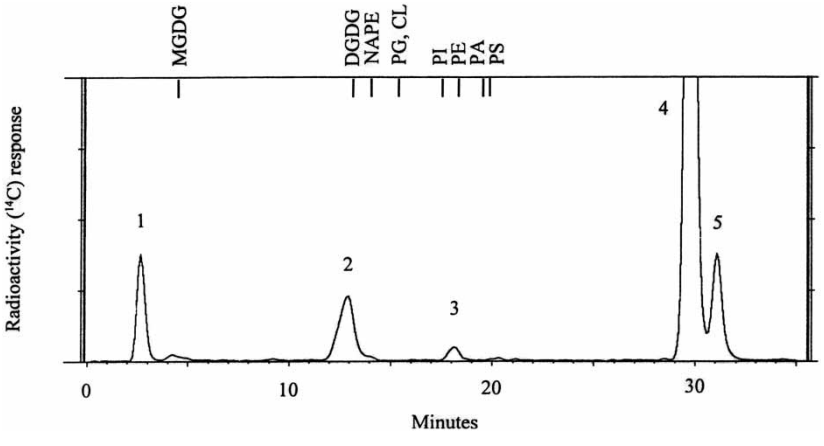

Figure 4. Separation of acyl lipid classes of total lipid extract from the castor microsomal incubation with 2-[^{14}C]oleoyl-phosphatidylcholine (PC) on a silica column using a binary gradient of (A) 2-propanol/hexane (4:3), and (B) 2-propanol/hexane/water (8:6:1.5).[17] The radioactive peaks were: (1) acylglycerols and free fatty acids, retention time 2.7 min; (2) unknown, 13.0 min; (3) PE, 18.2 min; (4) PC, 29.8 min; (5) 2-ricinoleoyl-PC, 31.1 min. Rertention times of other acyl lipid classes in this HPLC system are also shown on the top of this chromatogram. For abbreviations see Figures 1. MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; NAPE, *N*-acyl-phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid.

Lipid classes shown on the top of chromatogram (Figure 4) were the retention times of the lipid class standards. Guan et al.[18] used a similar HPLC system to separate phospholipids with the inclusion of 5 mM ammonium sulfate in the aqueous phase to improve the resolution and obtain complete separation of the phospholipids as shown in Figure 5.

Lipid classes of various phospholipid and lysophospholipid standards were separated by Lesnefsky et al.[19] on a silica column using a mobile phase of hexane/2-propanol/25 mM potassium acetate (pH 7.0)/ethanol/glacial acetic acid (367:490:62:100:0.6) with a gradient to hexane/2-propanol/25 mM potassium acetate (pH 7.0)/acetonitrile/glacial acetic acid (442:490:62:25:0.6), at 30°C with a UV detector (206 nm). Lysophospholipids eluted much later than the corresponding phospholipids.

Genge et al.[20] separated polar lipid (phospholipid) standards on a silica column using a binary gradient of (A) chloroform/methanol/0.1% formic acid (80:20:0.1), and (B) methanol/water (80:20) as shown in Figure 6. ELSD was used, however a UV (205 nm) detector could not be used because the eluent contained chloroform. Non-polar lipid standards were also separated on a silica column with a binary gradient of (A) hexane/2-propanol (95:5), and (B) chloroform/methanol/2-propanol/water/formic acid (40:40:10:10:0.2) as shown in Figure 7.[20] The complete separation of both polar and non-polar lipid classes in one HPLC run is difficult.

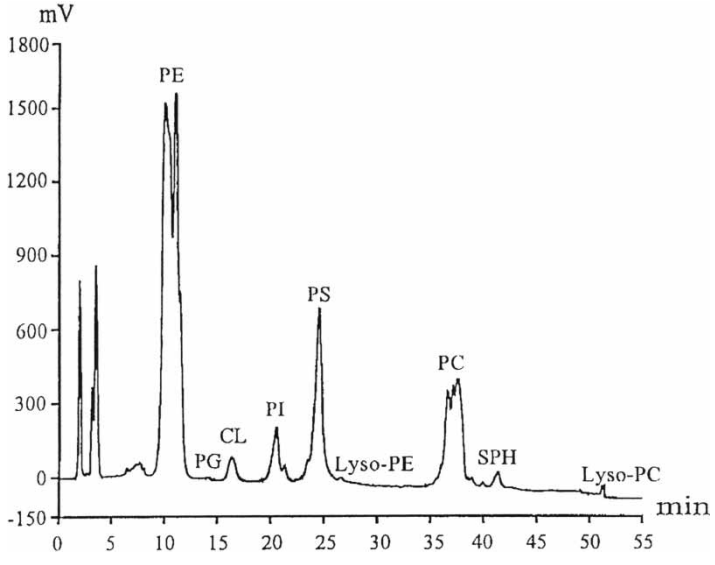

Figure 5. Separation of rat brain phospholipids by HPLC on a silica column using a binary gradient of hexane/2-propanol/water (5 mM ammonium sulfate) and UV (205 nm) detector.[18] For abbreviations see Figures 1 and 4.

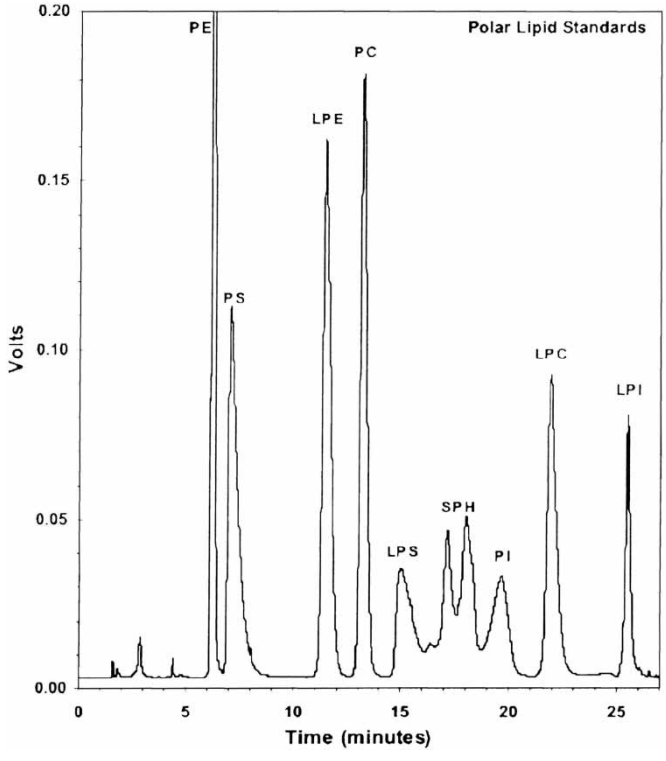

Figure 6. Separation of phospholipid (polar lipid) standards on a silica column using a binary gradient of chloroform/methanol/water/formic acid and ELSD.[20] For abbreviations see Figure 1. LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; LPC, lysophosphatidylcholine.

Genge et al.[20] also reported the ELSD calibration curves using sixteen lipid class standards for quantification. Since the ELSD response is different not only from various lipid classes but also from various molecular species (different in fatty acid chains) of each lipid class. The accurate quantification of lipid classes in natural samples using these calibration curves was difficult, because these curves were derived from one molecular species of each lipid class, whereas natural lipid classes are mixtures of some molecular species. We have reported that the ELSD responses of different molecular species of acylglycerols differ as much as 30%,[21] while the variance of ELSD responses among the various lipid classes could be as much as three times.[9,10,20]

Silica HPLC (normal-phase) alone can not be used to positively identify the molecular species of a lipid class. The fractionated lipid class from normal-phase HPLC can be further separated into molecular species by reversed-phase HPLC. The identification of the molecular species of a lipid

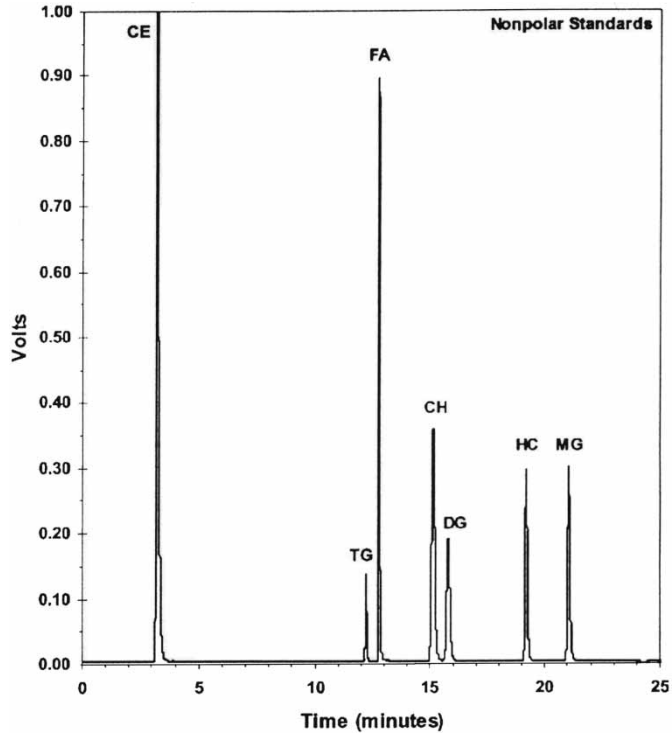

Figure 7. Separation of non-polar lipid standards on a silica column using a binary gradient of chloroform/methanol/water/formic acid and ELSD.[20] Abbreviations: CE, cholesterol oleate; TG, triolein; FA, oleic acid; CH, cholesterol; DG, diolein; HC, 25-hydroxycholesterol; MG, mono-olein.

class can be made by co-chromatography with standards on a reversed-phase HPLC.[22,23] The identification of the molecular species of a lipid class can also be made by silica HPLC coupled on-line with mass spectrometry. Pacetti et al.[24] identified the molecular species of lipid classes using silica HPLC coupled on-line with electrospray ionization ion-trap tandem mass spectrometry (ESI-MS-MS) as shown in Figure 8. The mobile phase used was a gradient of (A) chloroform/methanol/ammonium hydroxide (30%) (80:19.5:0.5), and (B) chloroform/methanol/water/ammonium hydroxide (30%) (60:34:5.5:0.5).

NORMAL-PHASE DIOL COLUMN HPLC

The diol column was available later than the silica column. Since separation of lipid classes on a silica column using a binary gradient has been about the same as that of a ternary gradient, the ternary gradient on the diol column

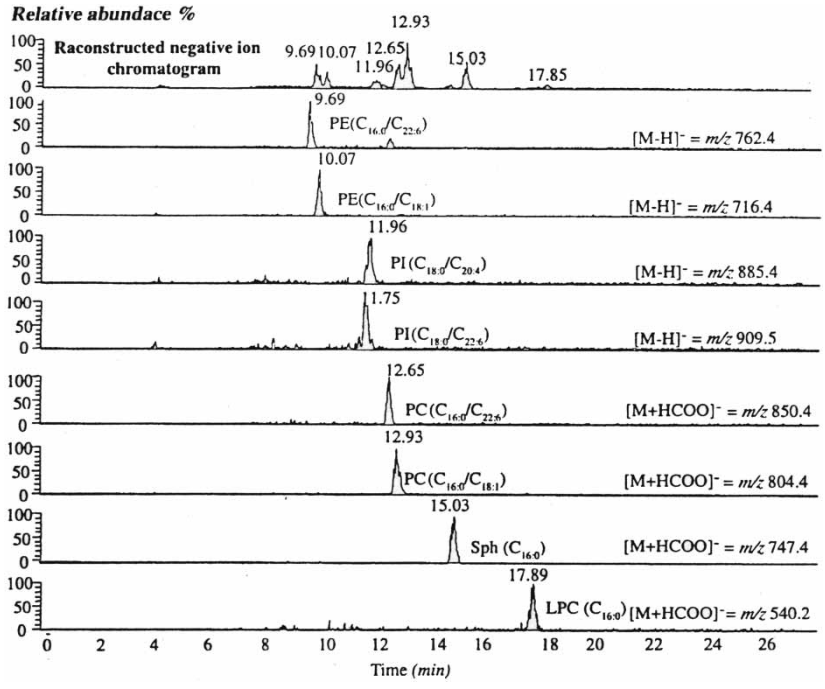

Figure 8. Nagative ion HPLC-ESI-MS analysis of egg phospholipids with the MS operating in scan mode.[24] Column was silica 3 μ, 100 mm $\times$ 4.6 mm. The mobile phase was a gradient of (A) chloroform/methanol/ammonium hydroxide (30%) (80:19.5:0.5) and (B) chloroform/methanol/water/ammonium hydroxide (30%) (60:34:5.5:0.5). The gradient started at 100% of A, decreased to 0% in 10 min, then was held (100% B) for 15 min. For abbreviations, see Figures 1 and 6.

has not been commonly used for the separation of lipid classes. Sas et al.[25] separated lipid classes of phospholipid and lysophospholipid standards on the diol column using a ternary gradient of (A) *n*-hexane, (B) methanol/acetic acid/triethylamine, and (C) acetone/acetic acid/triethylamine as shown in Figure 9. The lipid classes were separated well by this ternary system and ELSD was used for detection.

In a study by Silversand and Haux,[26] two binary gradients, one for polar and one for non-polar (neutral) lipid class standards were applied on a diol column using ELSD detection. For polar lipid classes (Figure 10), the binary gradient of (A) hexane/2-propanol/acetic acid (82:17:1.0), and (B) 2-propanol/water/acetic acid (85:14:1.0) was used with the addition of triethylamine (0.08%) to the solvents. Triethylamine was added to the solvents for HPLC of polar lipids in order to improve peak shape and resolution. Furthermore, triethylamine also improved the reconditioning of the column and made sure that no lipids remained adsorbed in the column

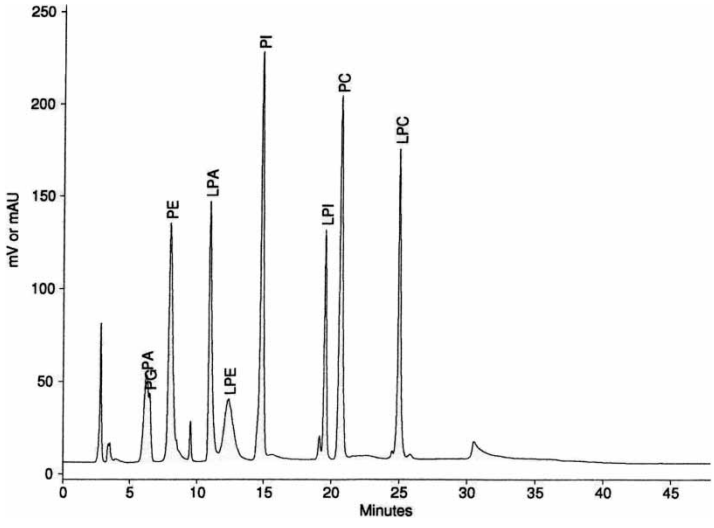

Figure 9. Separation of phospholipid and lysophospholipid standards on a diol column using a ternary gradient of *n*-hexane/methanol/acetone/acetic acid/triethylamine.[25] For abbreviations, see Figures 1, 4 and 6. LPA, lysophosphatidic acid; LPI, lysophosphatidylinositol.

after each analysis. For the non-polar lipid class standards (Figure 11), the binary gradient of (A) hexane/acetic acid (99:1.0), and (B) hexane/2-propanol/acetic acid (84:15:1) was used. These HPLC systems allowed the baseline separations of lipid classes as shown in Figures 10 and 11, except

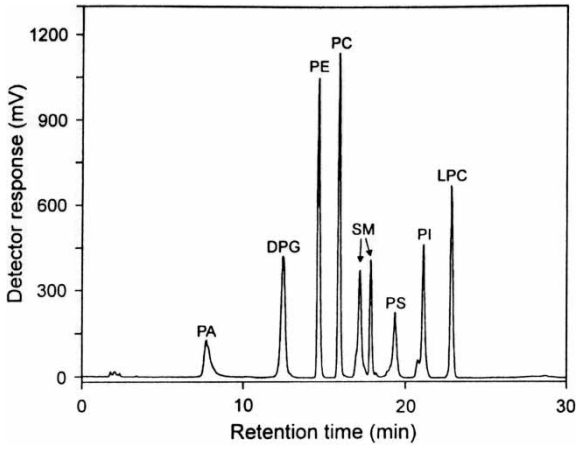

Figure 10. Separation of phospholipid standards on a diol column using a binary gradient of *n*-hexane/2-propanol/waer/acetic acid/triethylamine.[26] For abbreviations, see Figures 1, 4 and 6.

cholesterol ester and wax ester in Figure 11. Earlier, Balazs et al.[27] used a similar binary gradient to separate polar lipid classes (phospholipid) on a diol column. This HPLC system[27] was an International Lecithin and Phospholipid Society method. In a later paper by Neron et al.[28] a similar binary gradient was used to separate phospholipids including NAPE (*N*-acylphosphatidylethanolamine) and NALPE (*N*-acyllysophosphatidylethano lamine).

Twelve non-polar lipid class standards were separated recently by Schaefer et al.[29] on a diol column using a binary gradient of (A) isooctane, and (B) MTBE (*tert*-butyl methyl ether)/acetic acid (99.9:0.1) as shown in Figure 12. The system allowed baseline separation of these non-polar lipids. Since the ELSD response depends on the chain length and the degree of unsaturation, the quantification of a lipid class with unknown composition is only semi-quantitative.

LC-MS was used on a narrow bore diol column for the separation of some phospholipid standards with the binary gradient of (A) chloroform and (B) 25% ammonia in methanol with 0.2% formic acid, pH 5.3.[30] The molecular species of the phospholipids could be identified and quantified by electrospray mass spectrometry. Later, the binary gradient was modified to (A) chloroform and (B) ammonia buffered methanol, pH 5.3 with 0.1% formic acid and 0.05% triethylamine.[31] Chromatographic baseline separation was obtained among PG (phosphatidylglycerol), PC, PE, LPC, PI, and PS (phosphatidylserine).

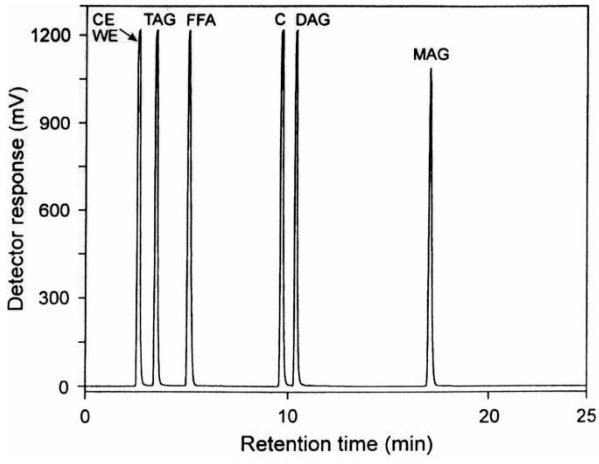

Figure 11. Separation of non-polar (neutral) lipid standards on a diol column using a binary gradient of *n*-hexane/2-propanol/acetic acid.[26] Abbreviations, CE, cholesterol ester; WE, wax ester; TAG, triacylglycerol; FFA, free fatty acid; C, cholesterol; DAG, diacylglycerol; MAG, monoacylglycerol.

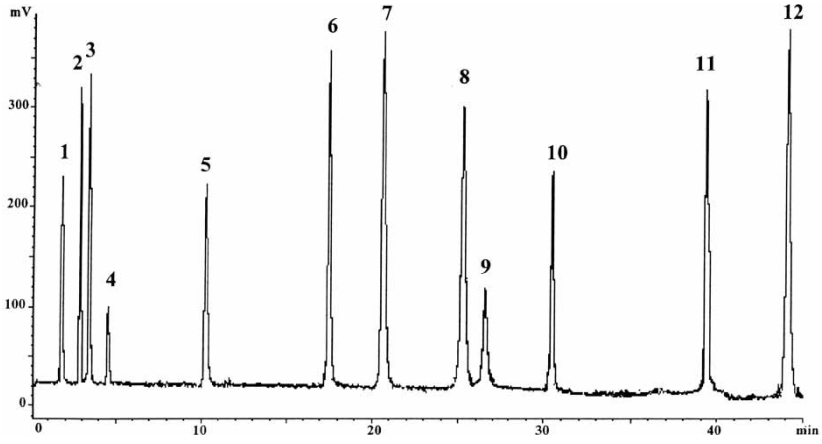

Figure 12. Separation of non-polar lipid classes on a diol column using a binary gradient of isooctane/MTBE(*tert*-butyl methyl ether)/acetic acid.[29] Lipid classes were as follows: (1) PAR (paraffin, liquid), (2) WE (*n*-hexyldecyl palmitate), (3) FAME (stearic acid methyl ester), (5) TAG (glycerol tripalmitate), (6) FOH (hexadecyl alcohol), (7) FFA (stearic acid), (8) cholesterol, (9) 1,3-DAG (glycerol-1,3-dipalmitate), (10) 1,2-DAG (glycerol-1,2-dipalmitate), (11) MAG (glycerol monopalmitate) and (12) FAA (erucylamide).

OTHER NORMAL-PHASE COLUMN HPLC

While the use of silica and diol columns predominate in the separation of lipid classes, other HPLC columns have also been used. Earlier, Christie et al. used a cyano column to separate polar lipid classes using a ternary gradient[32] and non-polar lipid classes using a binary gradient.[33] Mawatari and Murakami[34] used an amino column and isocratic elution to separate phospholipids using a UV (210 nm) detector and phospholipid hydroperoxides using a UV (234 nm) detector. Nordback et al.[35] separated lipid classes including monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), and sulphoquinovosyldiacylglycerols (SQDG), by a polyvinyl alcohol bonded stationary phase (PVA-sil) column using a ternary gradient system. PVA-sil has separation properties similar to that of silica. Rabinovich-Guilatt et al.[36] separated phospholipids and lysophospholipids on PVA-sil column using a binary gradient from pure chloroform to methanol/water (94:6).

CONCLUSION

Acyl lipid classes, both polar and non-polar, can be separated by normal-phase HPLC with binary gradient. Lipid class standards may be used for co-chromatography for tentative identification. However, different molecular

species of a lipid class might be eluted at slightly different times as shown in Figure 8. When the polarities of the fatty acid are very much different, e.g., between normal fatty acid and hydroxy fatty acid, the elution times of the different molecular species could vary significantly as shown in Figure 4. ELSD together with its calibration curves can be used for quantitation of lipid classes in natural samples. The addition of a small amount of acid or base to the solvents may improve peak shape and resolution. Similarly, we have previously added a small amount of ammonium hydroxide as a silanol suppressing agent to the solvents to improve the peak shape and resolution of the molecular species of PC[37] and PE[38] in reversed-phase C_8 HPLC. Using UV (205 nm) detection together with a flow scintillation analyzer can be applied to a metabolism study with radiolabelled compounds. This review showed the HPLC chromatograms separating different acyl lipid classes on different columns and different gradients. The HPLC systems may be used as presented or modified to better suit particular applications.

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