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A single step reversed-phase high performance liquid chromatography separation of polar and non-polar lipids

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ABSTRACT

This paper reports a simple chromatographic system to separate lipids classes as well as their molecular species. By the use of phenyl coated silica as stationary phase in combination with a simple mobile phase consisting of methanol and water, all tested lipid classes elute within 30 min. Furthermore, a method to accurately predict retention times of specific lipid components for this type of chromatography is presented. Common detection systems were used, namely evaporative light scattering detection (ELSD), charged aerosol detection (CAD), electrospray mass spectrometry (ESI-MS), and UV detection.

1. Introduction

The characterization and analysis of natural lipid mixtures needs to deal with the basic situation of lipids being a heterogeneous group of compounds which contain a variety of esterified fatty acids. Lipid classes are distinguished by different polar head groups but within each class molecular species are characterized by the structure and arrangement of the fatty acid (acyl) groups. The separation of intact lipids in mixtures is normally performed stepwise with various chromatographic techniques. There is a need for methodology to get relevant compositional information in a single step. The efforts to reach this goal have in recent years been concentrated to developing the detection and identification by LC–MS, but these methods rarely give an overall picture of the lipid profile of a sample. The authors' view is that the separation of lipids by means of HPLC still needs to be improved, in order to minimize the need for pre-fractionation and work-up steps and to facilitate subsequent identification.

Since many decades, chromatographic separation of lipid classes has been performed on polar stationary phases, mainly silica but also on other phases. The early work was based on thin-layer chromatography, which still is used for this purpose [1]. Normal-phase HPLC has replaced most of the TLC-work, especially after the introduction of suitable detectors such as the evaporative light-scattering detector (ELSD), the charged aerosol detector (CAD), or the mass spectrometer (MS). Beside silica, diol columns and PVA-Sil (polymerized and cross-linked vinylalcohol on silica) have been used extensively. The area is reviewed by Christie and Han [2,3]. The authors of the present paper have recently reported on the use of a cyanopropyl column [4,5].

Lipid separation on reversed-phase HPLC has been devoted to the separation of molecular species within a specific lipid class. In the case of separation of non-polar lipid classes on C18 (or ODS) columns, which by far is the most utilized stationary phase, the separation conditions are sometimes referred to as non-aqueous reversed-phase (NARP), which refers to elution conditions based on solvent mixtures and gradients without any water present. For triglyceride species in vegetable oils acetone–acetonitrile gradients are commonly used [6]. The separation of phospholipid classes usually requires the presence of water in the elution mixture. For example, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecular species respectively were separated on a C18 column using methanol–water–acetonitrile with [7] or without [8] the addition of choline chloride. Reversed phase separation of phospholipids, sphingomyelin and galactolipids has been reviewed by Christie and Han [9].

In contrast to TLC, in which the entire lipid material applied on the starting spot can be displayed for detection after separation, the analysis by column chromatography can only detect compounds that have been transported through the column. Normal-phase

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Table 1
Mobile phase gradient.

t (min)	%Water	%Methanol
0	20	80
2	20	80
20	0	100
30	0	100
31	20	80
40	20	80

To elute cardiolipins, 5 mM of ammonium acetate was added to each eluent.

HPLC may in principle retain very polar components in contrast to reversed-phase HPLC, which therefore should be useful for the analysis of lipid classes. As described above the reversed-phase methods are based on C18 columns, specific for each class, and designed to give best possible separation of molecular species within the class, which in most cases require very long elution times.

The aim of the present work was to find a simple reversed-phase methodology that could be useful for the separation of polar as well as non-polar lipid classes, be compatible with evaporative detectors (light scattering or CAD) but also with UV detectors and electrospray ionization mass spectrometry (ESI-MS).

In order to obtain shorter retention times compared to C18 and C8 columns a phenyl column was chosen. There are few reports in literature on this type of column [10–13] one of which relates to phospholipids [13]. Initial experiments showed that elution with methanol–water could elute triglycerides as well as phospholipids within 30 min. Since this solvent system also fulfilled the criteria of being compatible with the mentioned detection systems, it was selected as the eluent mixture.

The ambition to obtain a complete chromatographic pattern of natural lipid materials in one step will of course result in complex chromatograms. Therefore it would be advantageous to be able to model and predict the retention pattern of components expected to be present in the mixture as an aid for identification. Attempts to predict retention times of lipid components in liquid chromatographic systems have a long history. For sample mixtures consisting of only one class, such as triglycerides, a simplistic model based on the acyl chain lengths and number of double bonds can be utilized to give a reasonable prediction of retention times in reversed phase systems [14]. In such models the elution order can be estimated by considering the partition number (Eq. (1)).

$$PN = CN - 2 * DB \quad (1)$$

where PN is the partition number, CN is the total number of carbons in the acyl chains and DB is the number of double bonds.

However, the effect of the double bonds is dependent of the components in the mobile phase [15,16], and a relationship which in practice corresponds to a replacement of the constant 2 in Eq. (1) for an empirically determined constant has been suggested [17]. Recently a regression model that introduces an extra term for the number of monounsaturated fatty acids in the molecule has been presented [18].

It is obvious that extended prediction models for the retention behavior that takes into account the structure both of the polar head group and the acyl groups would be beneficial for developing and understanding liquid chromatographic separation methods intended for simultaneous analysis of several lipid classes.

2. Experimental

2.1. Chemicals

All solvents were of HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland). Ammonium acetate min 98% was from Merck (Darmstadt, Germany). Lipid class

Table 2
Specific lipid class fragments and parameters.

Lipid class	MS-ion type	MS/MS-ion type[a]	Cone voltage	CC energy[b]
Lysophosphatidylcholines (LPC)	$[M+H]^+$	184	40	30
Lysophosphatidylethanolamines (LPE)	$[M+H]^+$	$[M+H-141]^+$	50	40
Phosphatidylcholines (PC)	$[M+H]^+$	184	40	30
Phosphatidylcholines (PC)[c]	$[M+OAc]^-$	$[FFA-H]^-$	50	40
Phosphatidylethanolamines (PE)	$[M+H]^+$	$[M+H-141]^+$	50	40
Phosphatidylethanolamines (PE)[c]	$[M-H]^-$	$[FFA-H]^-$	50	40
Phosphatidylinositols (PI)	$[M-H]^-$	$[FFA-H]^-$	40	60
Phosphatidylserines (PS)	$[M+H]^+$	$[M+H-185]^+$	40	30
Sphingomyelins (SM)	$[M+H]^+$	184	40	30
Triacylglycerols (TG)	$[M+NH4]^+$	$[M+H-FFA]^+$	40	20

[a] All generated fragments have earlier been reported in [20].
[b] Collision cell energy.
[c] Used to identify the individual fatty acids of a particular species group.

Table 3
Descriptor values for monoolein and dimyristoyl-PE.

	Monoolein		Dimyristoyl-PE	
	No of atoms	Descriptor value	No of atoms	Descriptor value
Molecular weight		356.55		635.86
H	40	11.31	66	10.46
Phosphate P	0	0	1	4.87
sp^2 C with double bond to C	2	6.74	0	0
sp^2 C with double bond to O	1	3.37	2	3.78
sp^3 C	18	60.64	31	58.56
Hydroxyl O	2	8.97	0	0
Acid O	0	0	1	2.52
Ester O	1	4.49	4	10.06
sp^2 O	1	4.49	3	7.55
Ammonium N	0	0	1	2.20
Net charge	0	0	0	0