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Assessment of the retention properties of poly(vinyl alcohol) stationary phase for lipid class profiling in liquid chromatography

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Abstract

Potentialities of polymerized vinyl alcohol on silica gel were assessed for class separation of simple lipids, sphingolipids, glyceroglycolipids and phospholipids by high-performance liquid chromatography. A screening of pure solvents in binary gradient elution and a chemometric approach was used to define a rugged two segment linear gradient formed from four solvents for total lipid class separation. Triethylamine and formic acid were added in all mobile phase components for acidic phospholipid separation and evaporative light scattering response enhancement. Simple analytical procedures are described for the analysis of complex lipid materials.

Keywords: Stationary phases, LC; Retention behaviour; Gradient elution; Evaporative light scattering detector; Lipids; Poly(vinyl alcohol)

1. Introduction

Detailed lipid class profiling is, in addition to molecular species determination, one of the most widely used lipid characterization method. Since evaporative light scattering detection (ELSD) was introduced for separation of animal lipid classes by Christie [1,2], normal-phase HPLC–ELSD became the method of choice for lipid class analysis.

In addition to simple lipids and phospholipids, plant lipid extracts contain appreciable amounts of glycolipids, such as sterylglycosides, glycosphingolipids, and the glycoglycerolipids mono- and digalactosyldiglycerols.

However, the analysis of a restricted number of lipid classes has mainly been the goal of lipid class separation development, with a particular emphasis on phospholipids. Silica gel remains the most used stationary phase in lipid class analysis, with solvent programs adapted [3,4] from the pioneering work of Christie or either based on hexane/isopropanol/water mobile phases [5–9] or on chloroform/methanol/water [10–12]. The heterogeneous surface of silica gel leads to variable adsorptive properties, irreversible adsorption of solutes and long reconditioning time. Thus, bonded phases appear to be very attractive for lipid class analysis where mobile phase gradients are necessary to cover the whole lipid polarity range from neutral lipids to phospholipids.

Several polar stationary phases alternative to silica

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have been studied for lipid class analysis, such as diol [13–15], cyano [16] and amino grafted silica [17]. A polymerized poly(vinyl alcohol) on silica gel (PVA-Sil®) was recently used for lipid class analysis from potato [16], Arabidopsis [18], marine particulate material [19] and model wine system [20]. The uniform poly(vinyl alcohol) stationary phase is chemically bonded on silica particles. As claimed by the manufacturer, the homogeneous polymer layer bonded on the silica surface allows a constant phase activity with solvents ranging from hexane through water [21]. This characteristic leads to short reconditioning time and absence of irreversible adsorption. Moreover, poor peak shapes often observed with diol phases [22] are not encountered due to the absence of accessible residual silanols. Stationary phase properties and former articles led us to perform an in-depth investigation of plant lipid class analysis with PVA-Sil.

In a first step, a study of polar lipid retention with PVA-Sil and a large range of mobile phases is performed. This screening is designed to describe the potentialities of this stationary phase in lipid analysis and reveal eventual specific properties compared with silica or diol. Retention data obtained with various binary gradients are used for this purpose. An exhaustive range of polar lipids is assessed in order to develop analytical procedures dedicated to specific lipid materials. Besides conventional neutral and polar lipids, we focus on sphingolipids and glycoglycerolipids in order to cover the full range of common plant lipids.

The second step consists of an optimization of an analytical procedure for plant lipid class analysis without prior fractionation. Previously described methods often use complex mobile phases, resulting in poor reproducibility in the case of inter-laboratory studies. In order to develop a robust and simple method, we had limited the design to ternary gradient elution with binary mobile phases.

2. Experimental

2.1. Chemicals

All solvents were HPLC-grade (Fisher Scientific, Elancourt, France). Chloroform stabilized with ~0.75% ethanol was used instead of amylene stabilized chloroform which led to ghost peaks in the elution window of less polar lipids. HCOOH and TEA were, respectively, purchased from Fisher Scientific (Elancourt, France) and from Merck Eurolab (Nogent sur Marne, France). Lipid standards were purchased from Sigma (St. Quentin Fallavier, France).

The following standards were used during method development: triacyl- (TAG), diacyl- (DAG) and monoacylglycerols (MAG), cholesterol (CO), non-hydroxy (CER-NOH) and hydroxy fatty acid (CER-OH) ceramides, monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), bovine brain non-hydroxy (GalCER-NOH) and α-hydroxy (GalCER-OH) fatty acid galactosylceramides, human Gaucher's spleen glucosylceramides (h-GlcCER), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidyglycerol (PG), and the lyso forms of phospholipids (LPC, LPE, LPI, LPG), *N*-acylphosphatidylethanolamine (NAPE), lecithin type II-S. Wheat glycolipids extract was obtained by extracting ''manito'' grade gluten (Eurogerm, Rambouillet, France) with acetone, using a Soxhlet apparatus. To remove glycerolipids and isolate wheat gluten glucosylceramides (w-GlcCER) and steryl glucoside (SG), this glycolipid extract was saponified under mild alkaline conditions with 0.4 *M* KOH in methanol for 2 h at room temperature, followed by a Folch's partition procedure [23].

In some cases, the glycolipid extract was supplemented with this unsaponifiable part in order to increase its sterylglycoside and glycosylceramide content, and is then named as ''unsaponifiable enriched wheat glycolipid extract''.

Samples were dissolved in chloroform prior to analysis, at a concentration near 0.1 mg/ml during method development

2.2. Apparatus

Chromatography was performed on a 150×2-mm column packed with 5 μm YMC PVA-Sil (YMC, Kyoto, Japan) supplied by Interchim (Montluçon, France). A 10×2-mm guard column packed with the same material was used. Detailed elution schemes are given below. Heptane was used instead of *n*-

hexane for safety reasons. Measurements were made with a PU 980 pump (Jasco, Nantes, France) equipped with a Rheodyne model 7125 injection valve with a 5-μl loop (Rheodyne, Cotati, CA, USA). Flow rate was set at 0.5 ml/min for all experiments. Unless described in the text, all gradients were from 100% initial mobile phase to 100% final mobile phase, with a 25-min reconditioning time.

The column dead volume was 0.47 ml. The gradient delay volume was measured with a Kratos Spectroflow 773 detector (ABI, PE, Norwalk, CT, USA) at 265 nm by observing the rise in the baseline while running a mobile phase gradient from 100% methanol to methanol with 0.2% acetone. The gradient delay volume was 1.2 ml.

Detection was performed with a Cunow DDL 11 ELSD (Eurosep, Cergy, France). The drift tube temperature was set at 50°C and air pressure at 1 bar.

The column temperature was set at 35°C with a thermostatically controlled oven (Crococil, Cluzeau, Ste. Foy la Grande, France).

3. Results and discussion

Lipid classes are defined by the polar headgroup nature. Within a single lipid class, different molecular species may occur. For instance, glycerolipid molecular species are related to the fatty acid carbon atoms and double bond number. As a lipid class profiling procedure aims at quantifying lipid class distribution, elution of all molecular species of a defined class in a single and sharp peak will allow the most accurate results. All lipid standards were of natural origin, and from vegetal (soya, wheat) origin when available. No synthetic standards or purified molecular species were studied. These standards have then a complex molecular species distribution, and allow us to underscore an eventual discrimination of molecular species. This choice of natural standards allows the assessment of the separation of complex lipid extracts during the method development.

Of the normal-phase-HPLC lipid class analyses previously reported, only a few were based on systematic development, with most based on successive refinements of an original method. Moreover, these methods were often dedicated to a defined type of lipid material. With diol phase, a chemometric approach was used in order to develop a lipid class analysis [14]. The optimization was performed with five lipid classes, by varying additive content in heptane/isopropanol/water gradient using multivariate methods. Thus, this kind of optimization procedure is only designed to upgrade previously reported solvent systems, and is not adapted to recent stationary phase assessment for which few data are available.

Since, at the start of our study, only four examples of lipid class analysis with PVA-Sil had been reported, we found it necessary to further explore the potentialities of this phase. Gradients used in these studies are derived from HPLC methods developed with other polar stationary phases. In order to highlight the potential specificity of PVA-Sil, we started the study by screening solvents using binary linear gradient. This approach allowed the assessment of the retention properties of plant lipid classes. This screening was then used to define a ternary gradient method dedicated to lipid class analysis of complex materials. Moreover, data from this screening may be useful to design elution procedures for defined and known lipid mixtures.

3.1. Screening of pure solvents with binary gradient elution

Commercial standards were used during this development. Two weak solvents were considered: chloroform and heptane. Strong solvents were chosen in various Snyder's selectivity groups [24]: acetone, methanol, 2-propanol and acetonitrile. Retention data are reported in Table 1. Triacylglycerols, diacylglycerols and cholesterol were eluted in the solvent front with chloroform as weak solvent. Methanol, 2-propanol and acetone as strong solvent gave the same elution order for sphingolipid and glycoglycerolipid standards. Glycoglycerolipid, sphingolipid and zwitterionic phospholipid classes were resolved with a chloroform–methanol gradient. The eluotropic strength of isopropanol was too weak to elute the most polar phospholipids such as phosphatidylcholine.

With heptane as weak solvent, acetone and 2-propanol gave the same classical elution order for

Table 1
Retention times of lipid standards (min)

Weak solvent	Chloroform			Heptane	
Strong solvent	Acetone	Methanol	Isopropanol	Acetone	Isopropanol
TAG	0.94	0.94	0.94	1.73	1.75
CO	0.94	0.94	0.94	4.90	3.93
DAG[a]	0.94	0.94	0.94	4.96–5.27	3.93–4.12
MAG	1.63	1.61	1.61	7.67	5.81
CER-NOH	1.67	1.68	1.69	8.29	5.01
CER-OH	5.60	4.02	4.13	10.03	5.49
MGDG	6.74	4.31	4.75	14.39	8.38
GalCER-NOH	12.36	5.29	6.33	17.62	9.46
GalCER-OH	15.73	5.69	6.99	19.77	10.15
DGDG	20.08	6.37	7.83	24.53	13.65
PE		7.63	13.20		17.99
PC		9.01			
LPE		9.35			
LPC		14.81			

Pure solvent used in binary linear gradient from 100% weak solvent to 100% strong solvent, 4% slope. Column dead time: 0.94 min. Abbreviations as in experimental Section 2.1.

[a] Mixture of 1,3 and 1,2 diacylglycerols isomers.

sphingolipids and glycoglycerolipids. Heptane–acetonitrile gradient did not yield any satisfactory results, as only less polar lipids were eluted as very broad and tailed peaks.

Chromatograms obtained with heptane–acetone gradient are presented in Fig. 1. This binary solvent program was found efficient for sphingolipid and glycolipid separation, as lipid class retention time windows are very large, allowing a straight identification of lipid classes in complex lipid material.

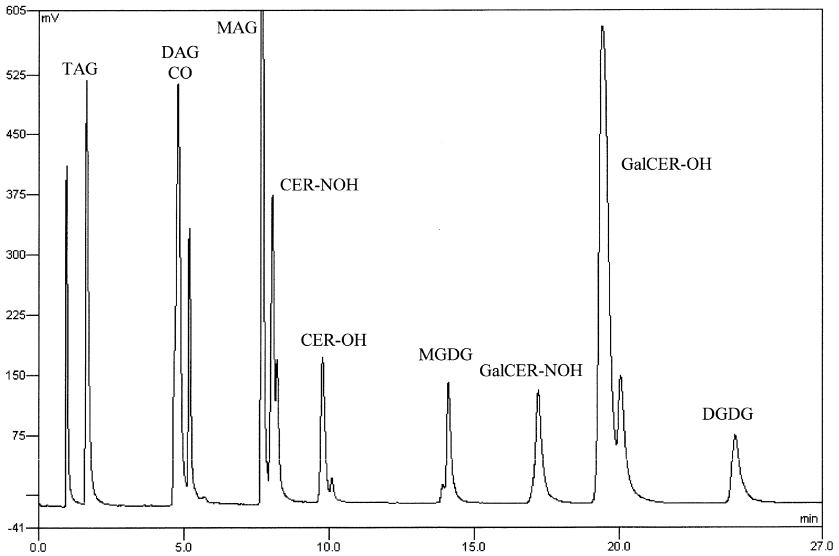

Fig. 1. Separation of commercial standards. Binary linear gradient from 100% heptane to 100% acetone, slope 4%/min. CER-OH, hydroxy ceramides; CER-NOH, non-hydroxy ceramides; CO, cholesterol; DAG, diacylglycerols; DGDG, digalactosyldiacylglycerols. GalCER-OH, hydroxy galactosylceramides; GalCER-NOH, non-hydroxy galactosylceramides; MGDG, monogalactosyldiacylglycerols; TAG, triacylglycerols.

Resolution of molecular species occurred only for some sphingolipid classes. Hydroxylated glycosphingolipids are eluted as a double or shouldered peak. Non-hydroxy and hydroxy fatty acid ceramides are eluted as a shouldered peak with heptane–acetone gradient. Other lipid classes, among them digalactosyldiacylglycerols (DGDG), were always eluted as a single and sharp peak, unlike a previous report describing PVA-Sil stationary phase, where discrimination of glyceroglycolipid molecular species was reported [18].

3.2. Choice of injection solvent

Heptane-based mobile phase was necessary in order to separate less polar lipids from neutral lipids. However, polar lipids have poor solubility in such solvent mixtures. Injection solvent composition has then a critical influence on separation. Total lipid extraction and purification is often performed with chloroform/methanol mixture. When documented, this solvent mixture was often the injection solvent in lipid class analysis, as injection solvent should be able to dissolve sample. For instance, previously reported studies claimed that when using a silica column, and a heptane–isopropanol–water gradient, the digalactosyldiacylglycerol peak was very broad or sometimes not present when samples were dissolved in pure chloroform [7]. These authors showed that lipid samples should be dissolved in chloroform–methanol (2:1) prior to analysis, as DGDG was found not to be as soluble in hexane–chloroform (1:1) or in pure chloroform.

Since PVA-Sil appeared to be less retentive than silica, the presence of small amounts of methanol, or other polar solvents in the sample, led to peaks broadening and dramatic retention time shift of less polar lipids such as ceramides. It is well known that using an injection solvent significantly stronger than the mobile phase causes the sample to be smeared across a significant part of the column length, resulting in misshapen and abnormally wide peaks.

At the concentrations used with our method, DGDG and polar lipids appear to be sufficiently soluble in pure chloroform, and thus lipid extract should be vacuum dried and then dissolved in pure chloroform prior to injection.

3.3. Addition of ionic species

If former gradients allowed a satisfactory separation of lipids from TAG to zwitterionic phospholipids, acidic phospholipids phosphatidylinositol, phosphatidylglycerol, phosphatidylserine were not resolved and eluted as very broad peaks.

Previously reported analysis of acidic phospholipids used the addition of small amounts of ionic species as modifiers to the mobile phases [18]. We found that the addition of 0.1% (v/v) of triethylamine (TEA) and stoichiometric formic acid (HCOOH) gave satisfactory resolution of acidic phospholipids with former binary gradients. Binary gradient screening was then again performed with addition of 0.1% stoichiometric TEA and HCOOH mixture. To ensure additive dissolution in heptane, addition of 2% isopropanol was necessary. Addition of TEA/HCOOH to all solvents with the aid of 2% isopropanol in case of heptane induced no loss of resolution. Retention times were reduced, with a better peak shape for zwitterionic phospholipids.

Moreover, we found that the addition of TEA and HCOOH significantly enhanced solute ELSD response. This phenomenon had already been reported for ceramide HPLC analysis, using non-aqueous mobile phases with C_{18} or porous graphitic carbon phases [25], and was generalized to other lipid classes and chromatographic techniques [26].

We were interested by an inversion in elution order observed for acidic phospholipids and glycolipids with the addition of TEA/HCOOH as described in the retention data detailed in Table 2. Chloroform–2-propanol mobile phases led to elution of phosphatidylglycerol (PG) near monogalactosyldiacylglycerols (MGDG), and of phosphatidylinositol (PI) and lysophosphatidylglycerol (LPG) closely after DGDG. Heptane/isopropanol gradient exhibited the same behavior as chloroform/isopropanol gradient, apart from the retention of PG, which are eluted between hydroxy galactosylceramides (GalCER-OH) and DGDG. Due to the high viscosity of isopropanol leading to high column pressure, more polar phospholipids were not chromatographed. Chloroform–methanol mobile phases led to a coelution of LPG with GALCER-OH, while PI eluted before DGDG.

A chloroform to methanol binary gradient was

Table 2
Retention times of lipid standards (min)

Weak solvent	Chloroform		Heptane/isopropanol 98/2 (v/v)	
Strong solvent	Methanol	Isopropanol	Acetone	Isopropanol
CER-NOH	1.07	1.16	7.275	4.13
CER-OH	2.23	2.21	9.49	4.99
MGDG	2.69	2.73	13.74	7.36
PG[a]	3.95	4.55		11.35
GalCER-NOH	4.65	5.35	16.275	8.35
GalCER-OH	5.11	6.54	18.38	8.89
LPG[a]	5.15	7.57		15.38
PI[a]	5.8	8.25		
DGDG	6.03	7.55	23.39	12.15
PE	7.17			
PC	8.25			
LPE	8.51			
LPC	11.59			

Binary linear gradient from 100% weak solvent to 100% strong solvent, slope 4%/min. All mobile phases contained 0.1% (v/v) stoichiometric TEA/HCOOH. Column dead time: 0.94 min.

[a] Acidic phospholipids.

found to be suitable for class analysis of polar lipids, ranging from hydroxylated ceramides to lysophosphatidylcholine. Only highly acidic phosphatidylserine gave a large peak ahead of phosphatidylethanolamine, which was not symmetrical, and still presents abnormal tailing. However, retention time windows were not large enough to allow lipid class identification without ambiguities from a complex lipid extract. Moreover, some lipid classes were not resolved such as LPG and GalCER-OH. Thus, a gradient from pure chloroform to methanol should be used only for phospholipids containing materials with a limited range of lipid classes. However, this fast and simple binary solvent program could be of great value if used in combination with prior fractionation, such as recently developed solid-phase extraction procedures [18].

3.4. Ternary gradient development

As none of these binary gradients gave adequate separation for a sample containing all tested lipid classes from triacylglycerols to phospholipids, binary mobile phases were assessed. Heptane based mobile phase was necessary for separation of less polar lipids. Since we observed different selectivity with chloroform/isopropanol and heptane/isopropanol phases, it seemed that the investigation of gradient starting from heptane to chloroform/isopropanol mixtures could allow an adequate selectivity tuning.

This first gradient step from heptane to a chloroform/isopropanol mobile phase was optimized to achieve the separation of lipid classes with polarity ranging from triacylglycerols to lysophosphatidylglycerol. A test mixture containing sphingolipids, glycoglycerolipids, PG and LPG, was analyzed with a gradient elution program starting from heptane/isopropanol 98/2 to various compositions of chloroform/isopropanol mixtures. All mobile phases contained 0.1% (v/v) equimolar TEA/HCOOH. To evaluate the class separation, time-corrected calibrated normalized resolution product (TC-CNRP) was used as optimization criterion [27].

Calibrated normalized resolution product r^* is obtained from Eq. (1):

$$r^* = \prod_{i=0}^{n} \left(Rs_{(i,i+1)} / \overline{Rs} \right), \quad (1)$$

where n is the number of peak, Rs the peak resolution, $\overline{Rs}$ the mean of peak resolutions, and $i=0$ corresponds to the column dead time.

Time correction factor t_{ne} was obtained from Eq. (2):

$$t_{ne} = t_{max} / Rs_{min}^2 \quad (2)$$

where t_{max} is the retention time of the last eluted solute, and Rs_{min} the minimum peak resolution.

TC-CNRP is calculated from Eq. (3):

$$TC\text{-}CNRP = \sqrt[n]{r^*}/t_{ne} \quad (3)$$

TC-CNRP allows a simultaneous optimization of solvent strength and selectivity, as its aim is to achieve a chromatogram in which all peaks appear at constant resolution intervals while accounting for the analysis time.

As seen in Fig. 2, two maximums of this criterion were found with the test mixture. Final mobile phase composition corresponding to these maximums was further assessed with a unsaponifiable enriched wheat glycolipid extract, containing neutral lipids, monogalactosyldiacylglycerols, steryl glucoside (SG), glucosylceramides and DGDG, in order to assess the separation of a natural extract containing lipid classes which were not used during the optimization step as SG and glucosylceramides. With a 20/80 (v/v) chloroform/isopropanol mobile phase, SG were too closely eluted after MGDG. Moreover, a high proportion of isopropanol associated with the last mobile phase composition resulted in a late elution of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) as broad and asymmetrical peaks. The best results for resolution of SG and MGDG were obtained with 65/35 (v/v) chloroform/isopropanol phase, and only PE is eluted as a broad peak at the end of this gradient. Thus, this mobile phase composition was considered as the optimum. Fig. 3 displays the analysis of commercial standards using this mobile phase composition.

It is worthy of note that of the acidic phospholipids, phosphatidylglycerol (PG) is eluted before DGDG, and lysophosphatidylglycerol (LPG) and phosphatidylinositol (PI) after DGDG. No elution of neutral phospholipids occurs with this solvent system. With other mobile phases developed with PVA-

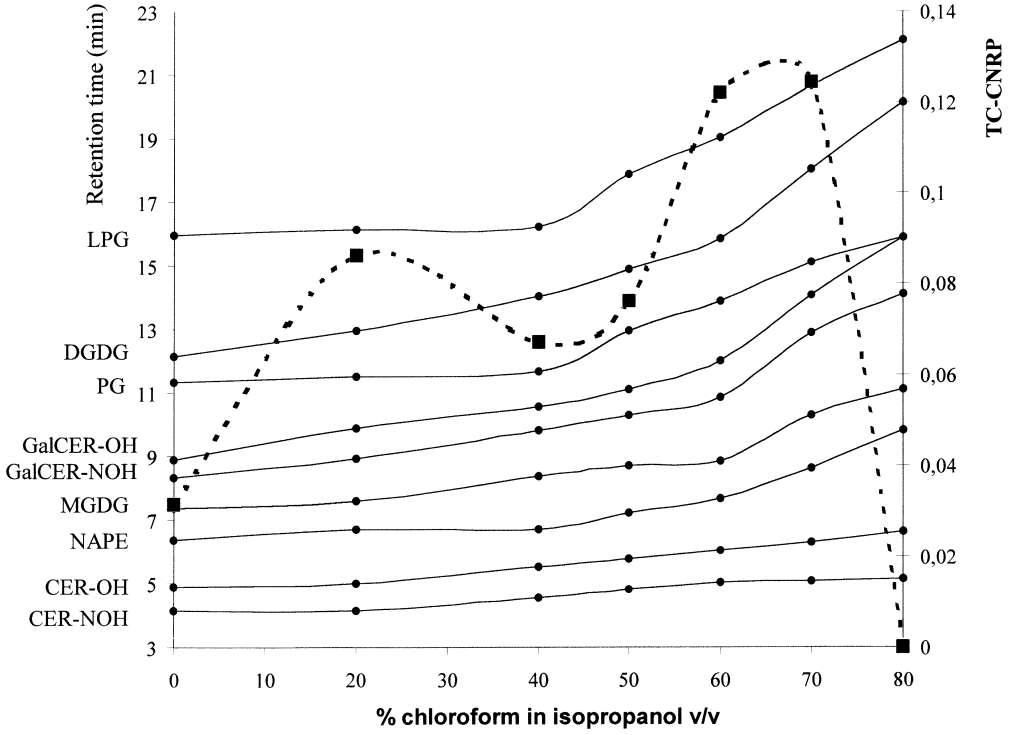

Fig. 2. Optimization of the chloroform/isopropanol mobile phase composition. Binary linear gradient from 100% heptane/isopropanol 98/2 (v/v) to 100% chloroform/isopropanol phase, 4%/min slope. All mobile phases contained 0.1% (v/v) equimolar TEA/HCOOH. Dashed line for TC-CNRP. Thin line for standard retention times. LPG, lysophosphatidylglycerol; NAPE, *N*-acylphosphatidylethanolamine; PG, phosphatidylglycerol. Other abbreviations as in Fig. 1.

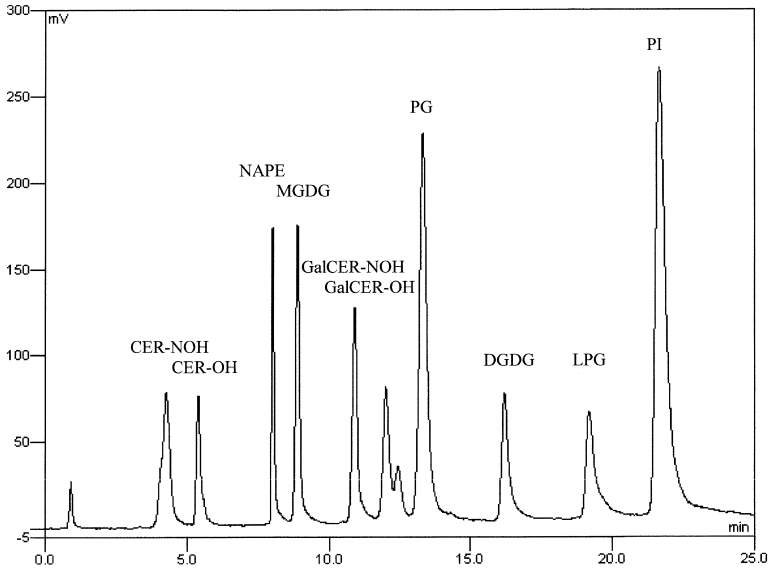

Fig. 3. Analysis of lipid standards with optimized conditions. Binary linear gradient from 100% heptane/isopropanol 98/2 (v/v) to 100% chloroform/isopropanol 65/35, gradient slope 4%/min. Abbreviations as in Fig. 2.

Sil [16,19], acidic phospholipids are eluted near neutral phospholipids. As an example, PG and PI are eluted between PE and PC with these solvent systems. Thus, this binary gradient is adapted for the separation of an extract containing neutral lipids, sphingolipids, neutral glyceroglycolipids, glycosylated sterols and acidic phospholipids. Only the neutral phospholipids and phosphatidylserine are retained too much to be eluted with this simple binary gradient. We found previously that the elution of phospholipids was achieved with a chloroform to methanol gradient. This optimized chloroform/isopropanol mobile phase miscible both with hydrocarbons and methanol is then well suited to mediate the transfer from the heptane/isopropanol starting phase to pure methanol.

3.5. Separation of all lipid classes

The last development step was then to develop separation of all lipid classes, which means extension of the previous binary gradient for the separation of neutral phospholipids. Methanol was selected as the final elution solvent, as it results in the elution of neutral phospholipids. As PE eluted with high proportions of 65/35 (v/v) chloroform/isopropanol, the content of chloroform/isopropanol mobile phase before addition of methanol was optimized. As the optimal composition of the chloroform/isopropanol phase was defined with a 4%/min gradient slope, this slope was fixed and we found that the first gradient step should be stopped with 88% chloroform/isopropanol phase to ensure that the elution of PE does not occur before methanol is added.

Then the slope of the gradient from heptane/chloroform/isopropanol to methanol was simply optimized by assessing the resolution of phospholipid classes with slopes from 0.7 to 4%/min, and set at a 2%/min for optimal separation. Methanol content was stopped at 40% content to ensure the elution of the most polar phospholipid lysophosphatidylcholine.

Table 3 presents the optimal solvent program for total lipid class analysis. Separation of two natural lipid extracts is shown in Figs. 4 and 5. Retention times of lipid standards are displayed in Table 4. All target lipid classes were resolved, with retention time windows separated enough to avoid lipid class overlap associated with eventual discrimination of molecular species. The acquisition time was 40 min, followed by a 30-min reconditioning program. This procedure is adapted for the separation in a single

Table 3
Optimized solvent program for the separation of all lipid classes

Time (min)	Heptane/isopropanol (%) 98/2 (v/v)	Chloroform/isopropanol (%) 65/35 (v/v)	Methanol (%)
0	100	0	0
22	12	88	0
42	0	60	40
44	0	100	0
47	0	100	0
48	100	0	0
68	100	0	0

All mobile phases contained 0.1% (v/v) stoichiometric TEA/HCOOH.

run of a complex lipid extract which contains all tested lipid classes. Only the acidic phospholipid phosphatidylserine (PS) still elutes as a broad peak, which is a limitation of the method. However, no appreciable amount of PS is present in our natural samples.

Elution order obtained with this gradient was quite different than orders obtained with solvent systems previously developed with silica, diol or PVA-Sil. Separations are obtained without the need for water for the elution of phospholipids contrary to previous described methods using a silica or diol or PVA-Sil column. Thus, PVA-Sil seems to be an interesting substitute as the absence of water allows shorter reconditioning time and a simpler recovery of purified lipid classes for further characterization.

With optimized gradient conditions, retention times of wheat glucocerebrosides isolated from wheat gluten after mild alkaline treatment were different from those of bovine brain galactocerebrosides or human Gaucher's disease glucocerebrosides. Wheat glucosylceramide eluted as a double peak. Wheat glucosylceramide has three major ceramides [28], d18:2 sphingoid base with h16:0 α-hydroxy fatty acid, d18:1 with h16:0 and d18:2 with h20:0 α-hydroxy fatty acid. No molecular species discrimination was observed for other lipid classes where fatty acid distribution is the only cause of microheterogeneity. Thus wheat glucosylceramides molecular species discrimination seems to be related to sphingoid bases rather than to fatty acid chain length. This absence of discrimination based on acyl

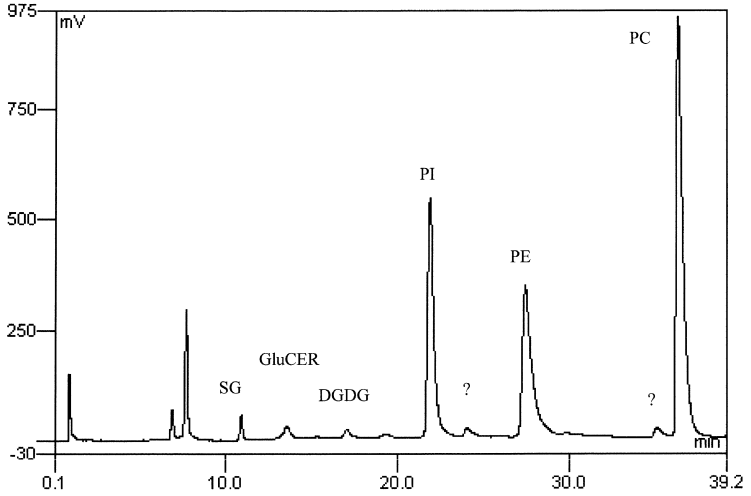

Fig. 4. Analysis of lecithin II-S with the total lipid class analysis procedure. (?) indicates unknown compounds.

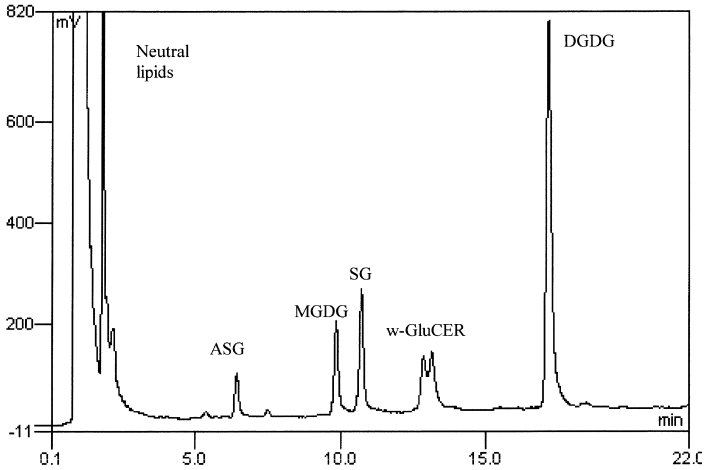

Fig. 5. Analysis of the ''unsaponifiable enriched wheat glycolipid extract'' with the total lipid class analysis procedure. ASG, acylated sterylglycosides; DGDG, digalactosyldiacylglycerols; SG, sterylglycosides; w-GluCER, wheat glucosylceramides.

Table 4
Retention time of lipid standards analysed with the optimized solvent program described in Table 3

Lipid class	Retention time (min)
TAG	0.95
CO	1.92
	2.39
DAG[a]	1.92
	2.39
CER-NOH	4.72
MAG	5.50
CER-OH	6.05
NAPE	7.08
MGDG	9.52
SG	10.95
GalCER-NOH	12.47
GalCER-OH	13.66
PG	14.10
DGDG	17.27
PI	22.23
LPG	19.25
PS	24.05[b]
PE	27.04
LPI	28.33
LPE	35.27
PC	36.41
LPC	39.75

[a] Mixture of 1,3 and 1,2 diacylglycerol isomers.
[b] PS eluted as a large peak.

moieties allows a straight identification of lipid classes from natural materials where high micro-heterogeneity is often encountered.

4. Conclusion

A rational screening of solvent properties for lipid class analysis with PVA-Sil was performed. This preliminary screening highlighted specific properties of PVA-Sil. Simple solvent systems were developed for the separation of lipid materials of reduced complexity. Analysis of all lipid classes was optimized with a ternary gradient using binary mobile phases without water and under rugged conditions.

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