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Journal of Chromatography A, 1040 (2004) 115–121
JOURNAL OF CHROMATOGRAPHY A
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Glycolipid class profiling by packed-column subcritical fluid chromatography

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Received 18 February 2004; accepted 29 March 2004

Abstract

The potential of packed-column subcritical fluid chromatography (SubFC) for the separation of lipid classes has been assessed in this study. Three polar stationary phases were checked: silica, diol, and poly(vinyl alcohol). Carbon dioxide (CO_2) with methanol as modifier was used as mobile phase and detection performed by evaporative light scattering detection. The influence of methanol content, temperature, and pressure on the chromatographic behavior of sphingolipids and glycolipids were investigated. A complete separation of lipid classes from a crude wheat lipid extract was achieved using a modifier gradient from 10 to 40% methanol in carbon dioxide. Solute selectivity was improved using coupled silica and diol columns in series. Because the variation of eluotropic strength depending on the fluid density changes, a normalized separation factor product (NSP) was used to select the nature, the number and the order of the columns to reach the optimum glycolipid separation.

Keywords: Class profiling; Subcritical fluid chromatography; Stationary phases, subcritical fluid chromatography; Glycolipids; Lipids

1. Introduction

Lipid class profiling is one of the most widely used chromatographic methods for the characterization of lipid materials. With the use of the evaporative light scattering detector for lipid class profiling [1,2], normal-phase liquid chromatography (LC) has become the technique of choice. Several polar stationary phases have been investigated: silica [3], diol [4], cyano [5], and recently poly(vinyl alcohol) grafted to silica [6,7]. Gradient elution methods are necessary for the separation of a wide range of lipid classes differing in polarity, requiring substantial column reconditioning time especially for unmodified silica stationary phase. On the other hand, lipid class analysis by liquid chromatography often use potentially toxic solvents such as chlorinated solvents.

Subcritical fluid chromatography (SubFC) with packed columns is an interesting alternative to liquid chromatography. Recent reviews [8–10], highlight the applications and recent developments of supercritical and subcritical fluid chromatography. The separation of polar solutes by packed-column supercritical fluid has also been reviewed [11], as has the application of SubFC to carbohydrate derivatives, such as glycolipids [12]. Carbon dioxide (CO_2)-based separations are generally superior to liquid chromatography methods [8]. Column/solvent equilibration is faster and the eluotropic strength of the mobile phase can be adjusted by changing both pressure and temperature. Moreover, an important feature of CO_2-based SubFC is the additional benefit of modifying the eluotropic strength and selectivity by the addition of organic modifier, e.g. from a non-polar solvent such as heptane to a polar modifier like methanol.

Due to the high flow rates used (from 3 to 5 ml min^{-1}) SubFC analyses are generally faster than LC analyses, when selectivity is comparable to LC for a particular application.

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doi:10.1016/j.chroma.2004.03.052

This is facilitated by the higher solute mobile phase diffusion coefficients which permit the use of higher optimum flow rates. In addition, the lower viscosity of supercritical and subcritical fluids leads to lower column pressure drops. Thus, longer columns than normally used in LC can be used to obtain higher plate numbers [13].

Moreover, when scale-up of an analytical chromatographic procedure to a preparative one is a concern, SubFC should received serious consideration. Because of its unique features and of its ability to perform an easy recycling of mobile phase, often composed only of carbon dioxide and of a low amount of lower alcohol such as methanol, SubFC is an attractive method for preparative chromatography [14].

SubFC with packed columns has found many applications in lipid analysis. These applications have mainly addressed the separation of non-polar or slightly polar compounds using reversed-phase columns. Such separations have included triglycerides [15–17], carotenoids [18], ceramide [19], waxes [20], and squalane [21]. Recently a separation with silica packed-column of carbohydrate derivatives, such as glycolipids [22], and stratum corneum lipids such as fatty acids, cholesterol, and ceramides has been described [23].

The aim of this study is to assess the resolution of lipid classes from a crude wheat glycolipid extract containing, in addition to a large amount of neutral lipids, glycosylated sterols, glucosylceramides, and glycoglycerolipids, using packed-column SubFC. Three polar stationary phases were evaluated: unmodified silica, diol bonded silica, and poly(vinyl alcohol) based phase. As the scale-up of the method to a preparative procedure was a further extension of this study, only one modifier is added to carbon dioxide to permit ease of eluent recycling and recovery of the purified compounds.

2. Experimental

2.1. Chromatographic system

Chromatographic separations were carried out using equipment manufactured by Jasco (Tokyo, Japan). Two Jasco Model 880-PU pumps were used, one for carbon dioxide and the second for the modifier. Flow rate was set at $3\,ml\,min^{-1}$ for all experiments. The pump head used for pumping the carbon dioxide was cooled to $-2\,^{\circ}C$ by a cryostat (Julabo F10c, Seelbach, Germany, supplied by Touzart et Matignon, Les Ulis, France). After mixing the two solvents (modifier and CO_2), the fluid was introduced in a dynamic mixing chamber PU 4046 (Pye Unicam, Cambridge, UK), connected to a pulsation damper (supplied by Touzart et Matignon). The injection valve was fitted with a 20 μl loop (Model 7125 Rheodyne, Cotati, CA, USA). The column was thermostated in a controlled oven (Crocosil, Cluzeau, Sainte Foy-la-Grande, France) regulated by a cryostat (Haake D8 GH, Karsruhe, Germany). The outlet column pressure was controlled by a regulator Jasco 880-81 (Tokyo, Japan). The outlet regulator tube (internal diameter 0.25 mm) was heated to 80 °C.

Detection was carried out with a light scattering evaporative detector DDL 21 (Eurosep, Cergy-Pontoise, France). Since this detector was set-up after the pressure regulator, no modification was required. The nebulization gas was air, the nebulization pressure 1.5 bar and the nebulization temperature 40 °C. The chromatograms were recorded with an Azur acquisition software (Datalys, Saint Martin d'Heres, France).

2.2. Columns

Three columns were used: silica column Kromasil Si-100, 250 mm × 4.6 mm, i.d., 5 μm (Eka Nobel, Bohus, Sweden), Diol LiCrhrospher 100 5 μm, 250 mm × 4 mm (Merck, Darmstadt, Germany) and poly(vinyl alcohol) PVA-Sil 5 μm, 150 mm × 2 mm (YMC, Kyoto, Japan).

2.3. Chemicals

Carbon dioxide No. 45 grade was purchased form Alphagaz (Bois d'Arcy, France). All solvents were HPLC grade and purchased from Carlo Erba (Milan, Italy).

Lipid standards were purchased from Sigma (St. Quentin Fallavier, France). Wheat glycolipid extract was obtained by extracting "manito" grade wheat gluten (Eurogerm, Rambouillet, France) with acetone using a soxhlet apparatus. In order to increase its sterylglycosides and glucosylceramides content, this wheat lipid extract was, if desired, spiked with its unsaponifiable part, obtained by a saponification under mild alkaline conditions with 0.4 M KOH in methanol followed by a Folch partition procedure [24]. Samples were dissolved in chloroform prior to injection.

3. Results and discussion

A lipid class is defined by the nature of its polar functional group, and among such a defined lipid class, various molecular species can be encountered. For example, five different fatty acids were detected in wheat digalactosyldiacylglycerols [25].

Some discrimination of lipid molecular species may occur during a lipid class analysis by normal-phase liquid chromatography [26]. In order to mimic the structural heterogeneity of natural lipid extracts, which are the targets of this study, lipid standards were selected from natural origin. This approach allows to investigate the separation of lipid molecular species within a defined lipid class since the very first steps of method development. Targeted lipid class structures are shown in Fig. 1 while Table 1 presents the lipid class content of the two test samples.

A SubFC separation of DGDG from oat lipids has been reported with a diol stationary phase and CO_2–methanol mobile phase [27]. An efficient SubFC separation of a

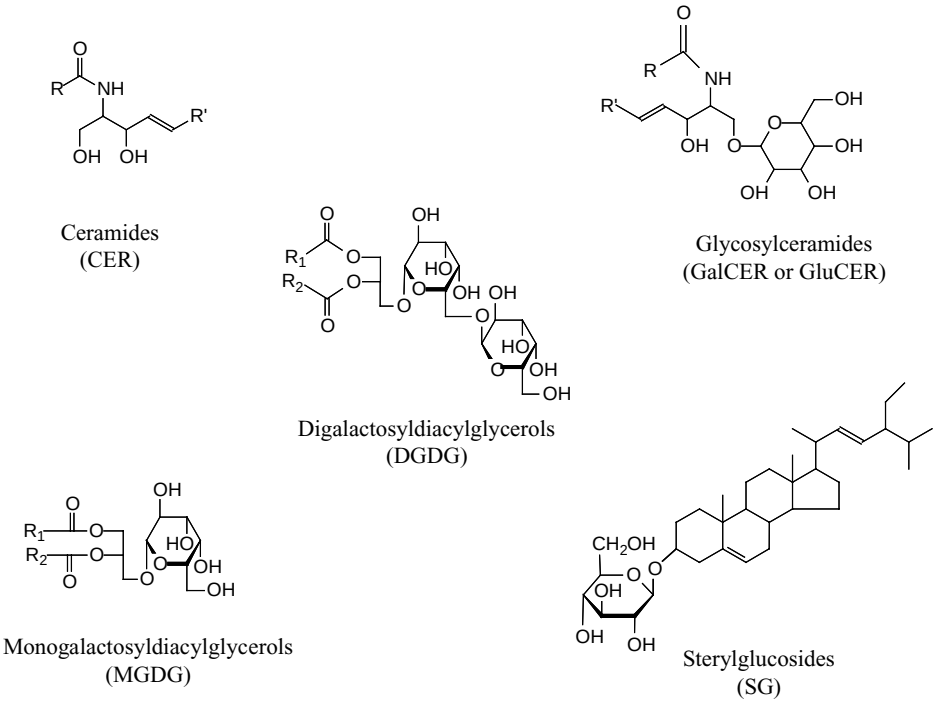

Fig. 1. Structures of lipid classes. Sphingolipid structures are arbitrary presented with a sphingosine base.

glycolipid extract has also been reported using a silica column, and a CO_2–methanol–water mobile phase employing an isocratic elution scheme [22]. However, the above separation addressed a glycolipid extract where no apolar lipids such as triglycerides or sterols were present. Lipid class analysis by liquid chromatography is mainly performed using gradient elution because of the large range of lipid polarity in a natural sample. Therefore, we have considered the option of gradient elution, since our aim is the resolution of a natural lipid extract without prior fractionation.

According to these preliminary considerations, a three-step development strategy was selected.

(1) First, the retention properties of polar stationary phases are established using isocratic elution. Modifier content, pressure, and temperature were investigated with commercial standards and a wheat lipid extract.
(2) These retention data were then considered in selecting between a isocratic or gradient elution for the separation of a crude lipid extract.
(3) The last step was to assess the coupling of columns packed with different stationary phases in order to optimize the separation by selectivity tuning.

3.1. Silica stationary phase

Silica remains the most common polar stationary phase in liquid chromatography. However, limitations such as long reconditioning times with gradient elution have decreased its use in lipid class profiling. Short reconditioning times in SubFC led us to consider this phase for lipid class analysis.

Preliminary investigations led to the choice of methanol as the polar modifier. Use of other modifiers (2-propanol, acetonitrile) resulted in poor efficiencies. We found that about

Table 1
Lipid class content of the standard lipid solution and of the wheat glycolipid extract

Lipids	Abbreviation	Standard solution	Glycolipid extract
Neutral lipids	NL		X
Non hydroxy fatty acid ceramides	CER-NOH	X	
Hydroxy fatty acid ceramides	CER-OH	X	
Monogalactosyldiacylglycerols	MGDG	X	X
Sterylglycosides	SG		X
Bovine brain non hydroxy fatty acid galactosylceramides	GalCER-NOH	X	
Bovine brain α-hydroxy fatty acid galactosylceramides	GalCER-OH	X	
wheat glucosylceramides	GlcCER		X
Digalactosyldiacylglycerols	DGDG	X	X

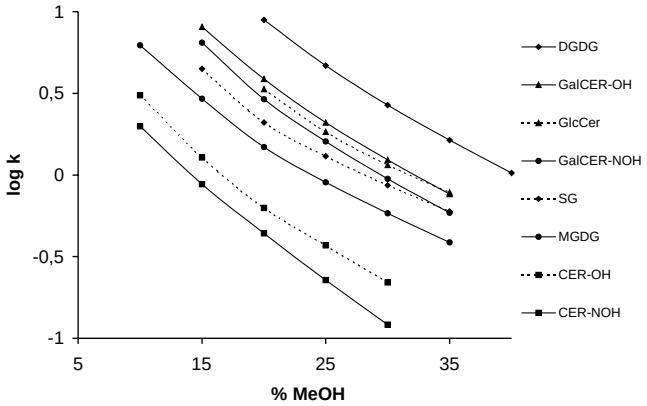

Fig. 2. Variation of $\log k$ vs. the methanol content. Column: silica Kromasil Si-100, $T = 20\,^{\circ}C$, $P = 150$ bar. Abbreviations as in Table 1.

5% (v/v) methanol in carbon dioxide was necessary to ensure the elution of neutral lipids such as triglycerides in the void volume. Lower methanol content led to a later elution of triglycerides as large groups of peaks together with less polar lipids as sphingolipids. This later elution of neutral lipids is a major drawback for the analysis of plant lipid extracts where large amounts of triglycerides are commonly found. This behavior is thought to be related to a too low solubility of neutral lipids in mobile phases with low methanol content.

3.1.1. Influence of methanol content on retention

The variation of retention factors of polar lipids with methanol content, at 20 °C and 150 bar, is presented in Fig. 2. The retention decrease continuously with increasing mobile phase methanol content from 10 to 40% (v/v).

The same behavior was obtained at 40 and 60 °C (data not shown). At these high methanol contents, it can be considered that the silanols are covered with methanol [28], leading to a constant phase activity whatever the modifier content. Consequently, the regular decrease in retention can be related to an increase of polar solute solubility in the mobile phase when increasing the polar modifier content. In former studies on the retention of polar solutes in packed-column supercritical fluid chromatography (SFC), $\log k$ was found to dramatically decrease in a non-linear way with an increase in modifier content [29,30]. The non-linear decrease in retention was explained in a solvatochromic study [30] involving the concept of clustering, resulting in a higher local concentration of modifier around the polar solutes than the modifier content in the bulk of the mobile phase. The influence of modifier content on retention was therein assessed with polar stationary phases and methanol content ranging from 1 to 12% (v/v). With higher methanol content, $\log k$ is herein an almost linear function of modifier content, thus excluding a retention variation governed by such a clustering phenomenon.

Glycolipids were herein found to be eluted in the order commonly observed using liquid chromatography employing a silica column [31]. It is seen from the retention factors in Fig. 2 that the analyses of a complex lipid extract containing all lipid classes from neutral lipids to digalactosyldiacylglycerols through sphingolipids can be achieved with a polar modifier gradient.

3.1.2. Influence of temperature and pressure

The retention was found to continuously decrease with increasing pressure or decreasing temperature. This retention behavior is related to a modification of fluid density. However, within the operating range of parameters of the SubFC experiments, the modification of solute retention produced by a change in fluid composition is larger than those due to density changes at constant composition. Thus, only a modifier gradient allowed the development of a lipid class analysis able to resolve a complex crude lipid extract.

3.1.3. Separation of a natural lipid extract with a polar modifier gradient

A separation of a wheat glycolipid extract by a gradient elution procedure, from 10 to 40% (v/v), methanol is presented in Fig. 3. An important feature is the short reconditioning time of 5 min for this gradient elution scheme, in spite of the large range of methanol content. The four glycolipid classes, sterylglucosides (SG), monogalactosyldiglycerides (MGDG), glucosylceramides (GlcCER), and digalactosyldiglycerides (DGDG) were resolved and eluted as sharp peaks. Only GlcCER were eluted as a double peak. Wheat glucosylceramide has three major ceramides [32], d18:2 sphingoid base with h16:0 α-hydroxy fatty acid, d18:1 with h16:0 and d18:2 with h20:0. The discrimination of wheat glucosylceramides molecular species is thought to be related to the degree of unsaturation of the ceramide backbone rather than on fatty acid chain length.

3.2. Bonded polar stationary phases

A diol phase was first studied. As with silica, the retention relationship continuously decreases with an increase in

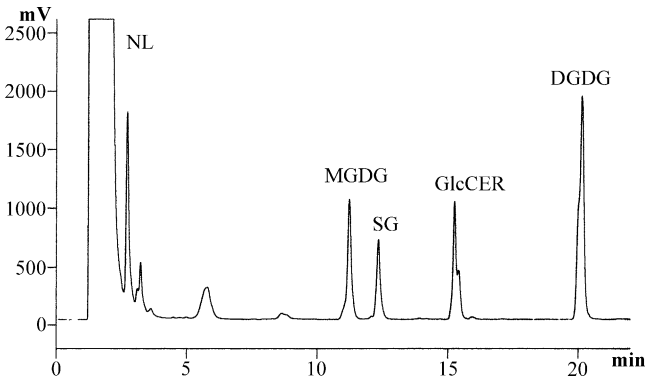

Fig. 3. Gradient separation with a silica column of a wheat glycolipid extract, spiked with its unsaponifiable matter. $T = 40\,^{\circ}C$; $P = 100$ bar. Linear gradient from 10% methanol during 5 min to 40% methanol at a 1.5% per minute slope, reconditioning time 5 min.

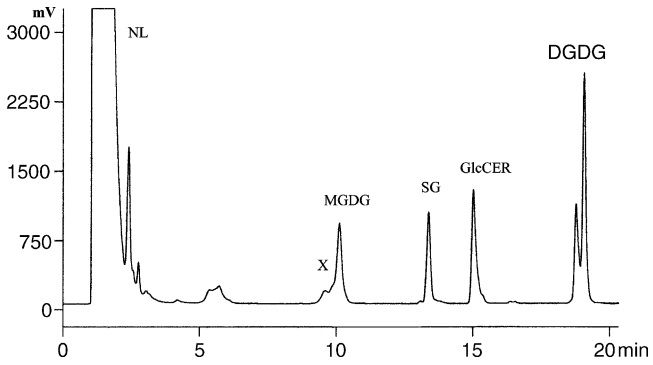

Fig. 4. Gradient separation with a diol column of a wheat glycolipid extract, spiked with its unsaponifiable matter. $T = 40\,^{\circ}C$, $P = 100$ bar. Linear gradient from 10% methanol during 5 min to 40% methanol at a 1.5% per minute slope, reconditioning time 5 min.

methanol content from 10 to 25% (v/v) methanol content. Compared with silica, the selectivity was different for glycolipids. If SG was closely eluted near MGDG with silica, SG and GlcCER are closely eluted at an intermediate retention time between MGDG and DGDG on diol phase.

A gradient elution was performed to analyze the wheat glycolipid extract, as presented in Fig. 4. An unknown solute (X) is eluted in front of the MGDG peak during the analysis of the wheat lipid extract. The analysis with this gradient on diol stationary phase of wheat MGDG collected with a silica column (experimental conditions as in Fig. 4) led to the detection of a single MGDG peak. Thus, the peak named X did not result from the resolution of MGDG molecular species.

An important feature is the enhanced separation of DGDG molecular species with a diol phase, as seen from Fig. 4, where identical gradient conditions led to the elution of DGDG as a single peak with silica. The elution of DGDG as two resolved peaks was previously reported [33] with diol phase and a CO_2–methanol–triethylamine mobile phase. However, the enhancement of molecular species resolution with the diol column compared with silica phase in SuBFC is not linked to a difference of fluid density in the diol and silica columns, as the internal pressure was close for these two columns under these conditions. An effect of the stationary phase is thus suspected. However, on both stationary phases, this separation of molecular species was herein found to increase with increasing temperature and decreasing pressure.

The applications of poly(vinyl alcohol) stationary phase (PVA-Sil) in lipid class profiling were recently reported [6,7], and led us to assess this promising stationary phase in SFC. The methanol content was first investigated at 150 bar and 40 °C. About 5% (v/v) methanol was also necessary to ensure the elution of a polar lipids such as triglycerides in the void volume. However, the addition of 5% v/v methanol led to the elution of the less polar glycolipid MGDG near the void volume, as PVA-Sil is less retentive than silica or diol.

To overcome this behavior, the addition of a second modifier could be assessed. However, this investigation is beyond the scope of this work where only a single modifier was considered, because of a forthcoming scale-up of the analytical procedure. As previously performed in LC [7], an exhaustive screening of modifier properties should precede the use of PVA-Sil for lipid class analysis in SubFC, as its unique retention properties hinder the direct transfer of a method as developed with silica or diol.

3.3. Selectivity tuning by serial column coupling

The chromatographic behaviors of diol and silica were herein found to be complementary in terms of selectivity for lipid classes and DGDG molecular species separation. The low viscosity of supercritical fluid allows the use of higher column length than in LC, which results in the possibility of selectivity tuning with the coupling of different stationary phases. A serial coupling of silica and diol columns was thus investigated to tune the separation selectivity.

Up to three columns were coupled, and the separation was assessed with the gradient elution procedure described in Figs. 3 and 4. The effect of the number and nature of coupled columns on the retention factors of wheat glycolipid classes is presented in Fig. 5. With increasing column length, the solute retention factors decrease. As supercritical fluids are compressible, a longitudinal pressure gradient occurs along the column. The pressure is regulated at the column outlet, thus the fluid density increases with increasing column length leading to an increase of its eluotropic strength. Therefore, with tandem columns and isocratic elution, the density variation leads to a lower residence time of solutes in the first column than in the second column.

When a mobile phase composition gradient is performed, another major feature leads to a decrease of retention with the serial coupling of columns. If the same solvent program is used with each coupling conditions, a modulation of eluotropic strength occurs depending on the position of

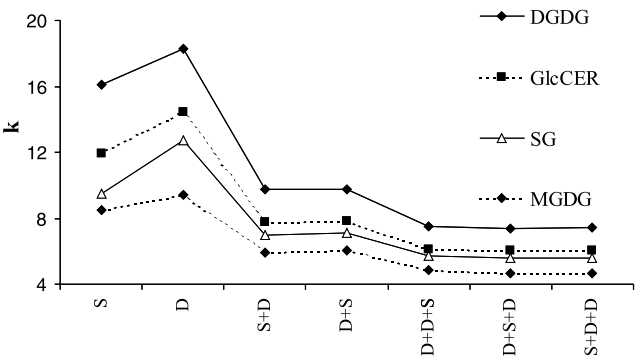

Fig. 5. Serial coupling of silica and diol columns. Variation of the retention factor k with the nature and position of columns. S for a silica column, D for a diol column. S + D means the coupling of a silica column followed by a diol column. Separation was assessed with analysis of the wheat glycolipid extract with the linear gradient elution scheme described in Fig. 4, $P = 100$ bar.

columns [34]. This feature is well understood with the comparison of a single column with a tandem set-up. With a modifier gradient, the methanol content when a solute enters the second column is different compared to the composition when it enters the first column. Consequently, the eluotropic strength in the second column will then be higher than in the first column due to this higher polar modifier content, leading to a lower residence time of solutes in the second column.

It was also demonstrated that the apparent dead volume, measured by the elution time of an unrefined solute, varies with the mobile phase density [35]. With one column in isocratic elution, this previous report showed that the measured dead volume varies with the modifier content in the mobile phase. This variation was explained by a variation of fluid density, leading to a variation of the mobile phase volume filling the column void volume, and by a modification of stationary phase volume due to the adsorption of modifier onto the stationary phase. If the stationary phase volume variation is negligible, the apparent void volume increases with an increase of modifier content and fluid density. In the case of column coupling and in the absence of stationary phase volume variation, the fluid density increases with increasing column length. The apparent void volume then also increases, and can be considered as an additional cause of variation of retention factors in the case of the modifier gradient.

The retention variation observed for a column coupling in SFC thus results from multivariate and interdependent parameters. It is worth pointing out that the order of coupled columns has herein only a minor effect on the retention (Fig. 5). This feature is certainly linked to the nearly identical retention capacity of silica and diol columns.

The aim of this selectivity tuning was to obtain a chromatogram where peaks tend to appear at constant time intervals. The separations were thus assessed by the calculation of the normalized separation factor product (NSP), as in Eq. (1):

$$\mathrm{NSP} = \prod_{i=1}^{n-1} \left(\frac{\alpha_{(i,\, i+1)}}{\bar{\alpha}} \right) \tag{1}$$

where n is the number of peak (here $n = 4$), α the separation factor, and $\bar{\alpha}$ the mean of separation factors. The highest value of NSP corresponds to a chromatogram where the peaks are distributed having the closest separation factor.

The variation of glycolipid separation factors and of NSP is presented in Fig. 6. An important feature is the minor effect of column order on the peak distribution, as expected from the minor effect of column order on retention factors. The weight of each stationary phase on the separation factor distribution is thus dependent on the number of column for each phase, rather than on the position of column. The maximal values of NSP occurred with the coupling of two columns, one silica and one diol. One of the corresponding separations is presented in Fig. 7. The solutes X and MGDG are resolved, and the peaks are distributed

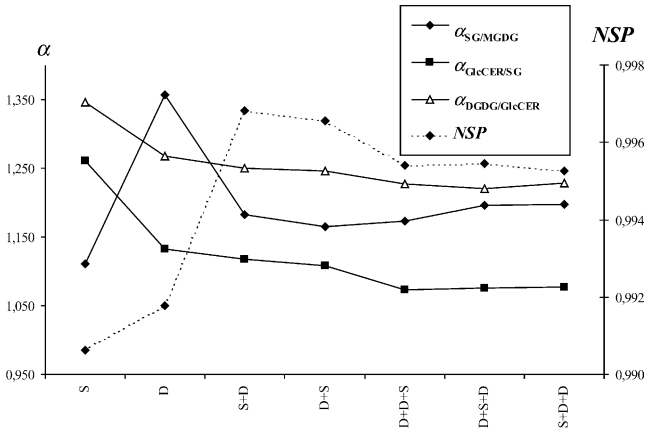

Fig. 6. Variation of separation factor and of the normalized selectivity product (NSP) with the nature and position of columns. Abbreviations and experimental conditions as in Fig. 7.

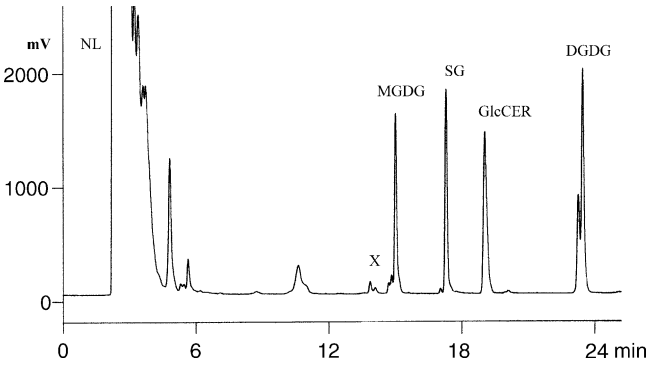

Fig. 7. Optimized separation of a wheat glycolipid extract, spiked with its unsaponifiable matter. One silica column was followed by one diol column. $T = 40\,^{\circ}\mathrm{C}$, $P = 100$ bar. Elution gradient from 10% methanol during 5 min to 40% methanol at a 1.5% per minute slope, reconditioning time 5 min.

in a more homogenous way than with either silica or diol columns.

This result confirms that SubFC offers a possibility of selectivity tuning by combination of columns packed with different stationary phases [34,36,37].

4. Conclusion

Packed-column supercritical fluid chromatography with CO_2 and MeOH as mobile phase is a recommended alternative to liquid chromatography for lipid class analysis. Without prior fractionation, glycolipid classes from a complex natural lipid extract were resolved with silica or diol stationary phases and a methanol gradient in carbon dioxide. Despites the large range of lipid and mobile phase polarity, a 5 min reconditioning time only was necessary. A fluid density and stationary phase dependent separation of DGDG molecular species was noticed. Due to the easy recovery of purified solutes in SubFC, this lipid class profiling method may be used as preparative method for further analysis of

lipid molecular species. A separation optimization by serial coupling of silica and diol columns has confirmed the potentialities of SubFC compared with LC for tuning of solute separation.

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