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Subcritical fluid chromatography of monosaccharides and polyols using silica and trimethylsilyl columns.

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Abstract

Efficient subcritical fluid chromatography (SubFC) analysis of twelve monosaccharides and polyols on silica and trimethylsilyl (TMS)-bonded silica stationary phases is proposed. Mobile phase composition was studied using CO_2–methanol, CO_2–methanol–water, CO_2–methanol–water–triethylamine in order to obtain high efficiency and resolution. By adjusting the column temperature to 60°C and the flow-rate to 5 ml min^{-1}, a complete separation of eight monosaccharides and polyols is obtained in less than 10 min. Using silica and TMS columns, retentions of carbohydrates and polyols in SubFC are compared with those of some glycolipids. It was found that carbohydrate retention increases when water is added to the eluent, whereas the retention of glycolipids decreases. © 1997 Elsevier Science B.V.

Keywords: Subcritical fluid chromatography; Evaporative light scattering detection; Carbohydrates; Monosaccharides; Polyols

1. Introduction

A wide variety of chromatographic methods are suitable for the analysis of carbohydrates. Nevertheless, no method permits the complete separation of carbohydrates. New analytical methods are important if complementary selectivities are to be obtained and/or if they extend the field of application. The simplest methods are those that do not require derivatization of the solute, such as LC (liquid chromatography), SFC (supercritical fluid chromatography), SubFC (subcritical fluid chromatography) or CE (capillary electrophoresis). The improvement in carbohydrate analysis using derivatization has been discussed recently in a book entitled "Carbohydrate analysis" [1]. CE is a high-resolution analytical technique [2] because high efficiencies can be obtained. It is an attractive method that requires only a small injection volume, however, only a few applications without derivatization of carbohydrates have been published. LC is probably the most common method used [3,4]. Nevertheless, the analysis of carbohydrates can easily be carried out by SubFC with new selectivities [5,6]. The analysis of polar compounds, such as monosaccharides and polyols, requires either the addition of a polar modifier to the carbon dioxide fluid [5,6] or derivatization of the solutes, which increases their solubility in neat carbon dioxide [7]. A high content of polar modifier (10–20%) allows carbohydrates to be analysed without derivatization, if evaporative light scattering detection (ELSD) is used. CO_2–methanol is commonly used as the mobile phase in SubFC. CO_2–methanol is close to a dichloromethane–methanol mixture, in terms of polarity, in comparison with LC. We previously obtained good results in HILIC (hydrophilic interaction chromatography) using dichloromethane–methanol [8–10]. Both CO_2–metha-

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nol and dichloromethane–methanol mobile phases give similar elution orders when silica and various bonded silica phases are used, but the selectivities are different and complementary [11].

Silica-, trimethylsilyl (TMS)- and NH_2-bonded phases have been studied by HILIC [8,9]. Experiments were carried out to study and compare these stationary phases in SubFC and to obtain complementary selectivities for monosaccharides and polyols in comparison with NO_2-, diol- and CN-bonded phases that were used previously [5,6]. Eluents made up of CO_2–methanol, CO_2–methanol–water and CO_2–methanol–water–triethylamine were studied to determine their ability to separate carbohydrates and polyols. It will be shown that the content of water, the presence of a basic additive, such as triethylamine (TEA), and the temperature have a major influence on the capacity factors, selectivity and/or efficiency. Finally, glycolipid behaviour will be discussed in comparison with these carbohydrates.

2. Experimental

2.1. Apparatus

SubFC analyses were conducted with a model SF_3 Gilson apparatus (Villiers Le Bel, France), a Rheodyne (Berkeley, CA, USA) model 7125 injector with a 20-μl sample loop, a CROCO-CIL™ column oven (CIL-Cluzeau, S^{te}-Foy-la-Grande, France). A polar modifier was added to CO_2 using a slave pump (model 302, Gilson). Modifier mixtures were prepared manually.

Detection was performed using an ELSD Sedex 55 Model (Sedere, Alforville, France). The SFC interface of the ELSD was directly connected to the Gilson pressure regulator; the ELSD detector settings were as follows: Photomultiplier, 7; evaporative temperature, 50°C; air pressure, 0.5 bar; nebulizer temperature, 75°C. Data were processed using Shimadzu (Kyoto, Japan) Model CR 5A integrators.

2.2. Columns

The following columns were used: Zorbax TMS (250×4.6 mm I.D.), Zorbax Sil (150×4.6 mm I.D.), Zorbax NH_2 (150×4.6 mm I.D.), all of which were from Dupont (Wilmington, DE, USA), Lichrospher Diol (125×4 mm I.D.) and Lichrosorb Diol (150×4.6 mm I.D.), which were from Merck (Darmstadt, Germany)

2.3. Reagents

Carbon dioxide was of industrial grade (purity 99.7%) (Air Liquide, Paris, France), methanol (Hipersolv grade, BDH, Poole, UK), TEA (Chromanorm, Paris, France). Water was purchased from Stalabo (Cooperation Pharmaceutique Française, Melun, France). Carbohydrates were purchased from Merck. Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and galactocerebroside type I (Galcer I) were purchased from Sigma (Saint Quentin Fallavier, France). Plant extracts of glycolipids and glycosylsitosterol (GSS) were provided by Parfums Christian Dior (Saint Jean de Braye, France). Solutes were dissolved either in chloroform–methanol (2:1, v/v) or in pure methanol. The solutes were injected separately and injections were made in triplicate.

3. Results and discussion

For these studies, methanol was used as the primary polar modifier. Some investigations using other modifiers (ethanol, butanol, acetonitrile) have been carried out [12], but poor efficiencies were obtained and the elution strength was too low compared with that obtained using methanol. Zorbax NH_2, Lichrospher Diol and Lichrosorb Diol columns were tested and these provided lower selectivities for monosaccharides and polyols than those obtained with Zorbax Sil and Zorbax TMS columns. Zorbax NH_2 provides an acceptable retention time (t_r<10 min) using CO_2–methanol (70:30, v/v) as the eluent, as other stationary phases needed CO_2–methanol (80:20, v/v) as the eluent. In this paper, we will mainly discuss the results obtained on silica and on TMS-bonded silica.

In order to compare the capacity factors, the percentage of modifier and the pressure were set to 20% and 200 bars, respectively. With this high

amount of modifier in the eluent, the critical temperature of the mixture is not reached and analysis can be performed under SubFC conditions [13]. A high methanol content in needed in the CO_2–methanol mixture to elute carbohydrates and polyols with capacity factors from 2 to 10 (Table 1). The methanol acts by increasing their solubility and reducing the interactions of polar groups with the silanols of silica and residual silanols of the TMS packing. Moreover, a similar retention order can be found with TMS and silica columns (Table 1; modifier, 100% methanol), however, an interaction may occur between the ring of the sugar and the TMS group, since the capacity factors are always greater on a TMS stationary phase than on silica.

3.1. Influence of the content of water in the modifier

Variations in selectivity and efficiency were observed when water was added to the modifier for amino acids [14], the phosphine oxide enantiomers [15] and for ranitine and its metabolite [16]. The maximum water content in the modifier depends on the miscibility of water with the CO_2–methanol mixture. Under our conditions, the maximum percentage was 9%, without phase separation of the mobile phase occurring.

3.1.1. Capacity factors and selectivities

Table 1 shows the different capacity factors (k')

Table 1
Capacity factors of monosaccharides, polyols and glycolipids

Column	Zorbax TMS				Zorbax SIL			
Modifier Composition	MeOH 100	MeOH–H_2O 96.0:4.0	MeOH–H_2O 92.0:8.0	MeOH–H_2O–TEA 91.5:8.0:0.5	MeOH 100	MeOH–H_2O 96.0:4.0	MeOH–H_2O 92.0:8.0	MeOH–H_2O–TEA 91.5:8.0:0.5
Deoxyose								
L-Rhamnose	4.1	4.8	7.1	7.9	2.3	3.2	5.1	7.3
Aldopentoses								
D-Ribose	3.7	4.3	6.3	6.9	2.1	2.8	4.6	7
D-Xylose	4.7	5.4	8.1/8.6[a]	9.5	2.5	3.4/3.6[a]	5.6/6.2[a]	8.6
L-Arabinose	4.9	5.7	8.3	9.5	2.5	3.5	5.9	8.6
Ketohexoses								
D-Fructose	6.5	7.2	9.9	12	3.3	4.3	7.5	11.1
L-Sorbose	6.7	7.5	10.7	12.7	3.5	4.8	8.1	11.1
Aldohexoses								
D-Mannose	7.4	8	11.4	13.3	3.7	5.2	8.4	12.5
D-Galactose	8.3	9.2/9.5[a]	12.8/14.3[a]	15.5	4.3	5.9	9.2/10.5[a]	14.3
D-Glucose	8.5	9.2	13.3	15.7	4.3	5.7	9.5	14.8
Polyols								
m.Erythritol	4.2	4.9	7.2	8.1	2.4	3	5.12	7.3
Xylitol	6.6	7.3	9.8	11	3.7	4.6	7.2	11.1
Mannitol	10.6	10.9	13.3	16.6	5.5	6.1	10.2	15.8
Glycolipids[b]								
MGDG	3.6	2.6	1.84	1.5	2.1	1.6	2	2
GSS	4.4	3.4	2.9	2.8	2.7	2	2.5	3
Galcer I	9.3	5.4/5.7[a]	2.9/3.2[a]	2.8/3.2[a]	6	3.4	2.5	3
DGDG	20.3/22[a]	11.5/12.5[a]	5.7/6.3[a]	4.9/5.4[a]	14	6.6/7.5[a]	5.5/6.1[a]	5.9/6.7[a]

The column temperature was 41°C, the flow-rate was 3 ml min^{-1} and the pressure was 200 bars.
The mobile phase consisted of CO_2–modifier (80:20, v/v).
[a]Capacity factors of anomers of carbohydrates or molecular species of glycolipids.
[b]Glycolipid retention times are discussed in Section 3.4.
Abbreviations and formulae are given in Fig. 4.

obtained using silica and TMS columns and with methanol–water as the modifier (water from 0 to 8.0% in the primary modifier). The capacity factors of carbohydrates and polyols increase as the water content increases. Without water, it was noted previously that capacity factors decrease when the methanol content increases in the CO_2–methanol mobile phase. Such behaviour in the presence of water is different for some other polar solutes [14–16] analysed using CO_2–methanol–water eluent. In contrast, similar behaviour was found for carbohydrates analysed using LC with a bare silica column and a mobile phase consisting of dichloromethane–methanol–water [9].

In all likelihood, the retention mechanism is a partition mechanism. An adsorption mechanism cannot be considered because the amount of polar solvent is too high [17]. Using CO_2–methanol as the eluent assumes that the stationary phase swells and that the active site (silanol) is covered [18]. One can surmise that increased solvation of the stationary phase by the hydroorganic modifier or a modification of the surface tension between the CO_2 and the stationary phase could occur [19]. This could explain the variation in carbohydrate retention when water is added to the mobile phase, but more experiments are necessary to validate this explanation.

Table 2 shows the variation in selectivities between some pairs of solutes when the amount of water in the modifier is increased (methanol–water, water from 0 to 8%). Very low variations in carbohydrate selectivities (e.g. fructose–sorbose, mannose–glucose) are observed in the presence of water. However, polyols show a particular behaviour in comparison with carbohydrates. The selectivities between polyols–polyols (e.g. xylitol–mannitol) and/or the corresponding carbohydrate (e.g. mannose–mannitol, xylose–xylitol) decrease, especially on TMS columns. Therefore, it is important to discuss efficiencies, as water induces variations in selectivities and efficiencies.

3.1.2. Efficiency

Efficiency variations are shown in Fig. 1 as a function of the water content in the modifier. The carbohydrates that provide anomer separation are not represented. Efficiencies on TMS columns are higher than on silica. With both columns, the addition of water to the modifier produces higher efficiency. The best efficiencies are obtained with 8.0% water in the modifier, except for glucose (which had its best efficiency when 4.0% water was present). The best example is mannitol, which had a five-to-seven fold increase in theoretical plate number per meter on TMS- or silica columns. This increase in efficiency results in improved resolution. As seen in Table 3, the best resolutions occurred using a high content of water. Experiments were performed in triplicate in

Table 2
Selectivities for some pairs of solutes using Zorbax TMS and Zorbax SIL columns

	Modifier	MeOH	MeOH–H_2O	MeOH–H_2O	MeOH–H_2O–TEA
	Composition (v/v)	100	96.0:4.0	92.0:8.0	91.5:8.0:0.5
Fructose–sorbose	Zorbax TMS	1.03	1.04	1.08	1.06
	Zorbax SIL	1.06	1.12	1.08	1
Mannose–glucose	Zorbax TMS	1.15	1.15	1.16	1.18
	Zorbax SIL	1.16	1.10	1.13	1.18
Xylitol–mannitol	Zorbax TMS	1.6	1.49	1.36	1.51
	Zorbax SIL	1.49	1.33	1.42	1.42
Mannose–mannitol	Zorbax TMS	1.43	1.36	1.17	1.25
	Zorbax SIL	1.49	1.17	1.21	1.26
Xylose–xylitol	Zorbax TMS	1.4	1.35	NC[a]	1.16
	Zorbax SIL	1.4	1.35	NC[a]	3.39

Mobile phase consisted of CO_2–modifier (80:20, v/v).
The column temperature was 41°C, the flow-rate was 3 ml min^{-1} and the pressure was 200 bars.
[a]NC=not calculated (anomer resolution).

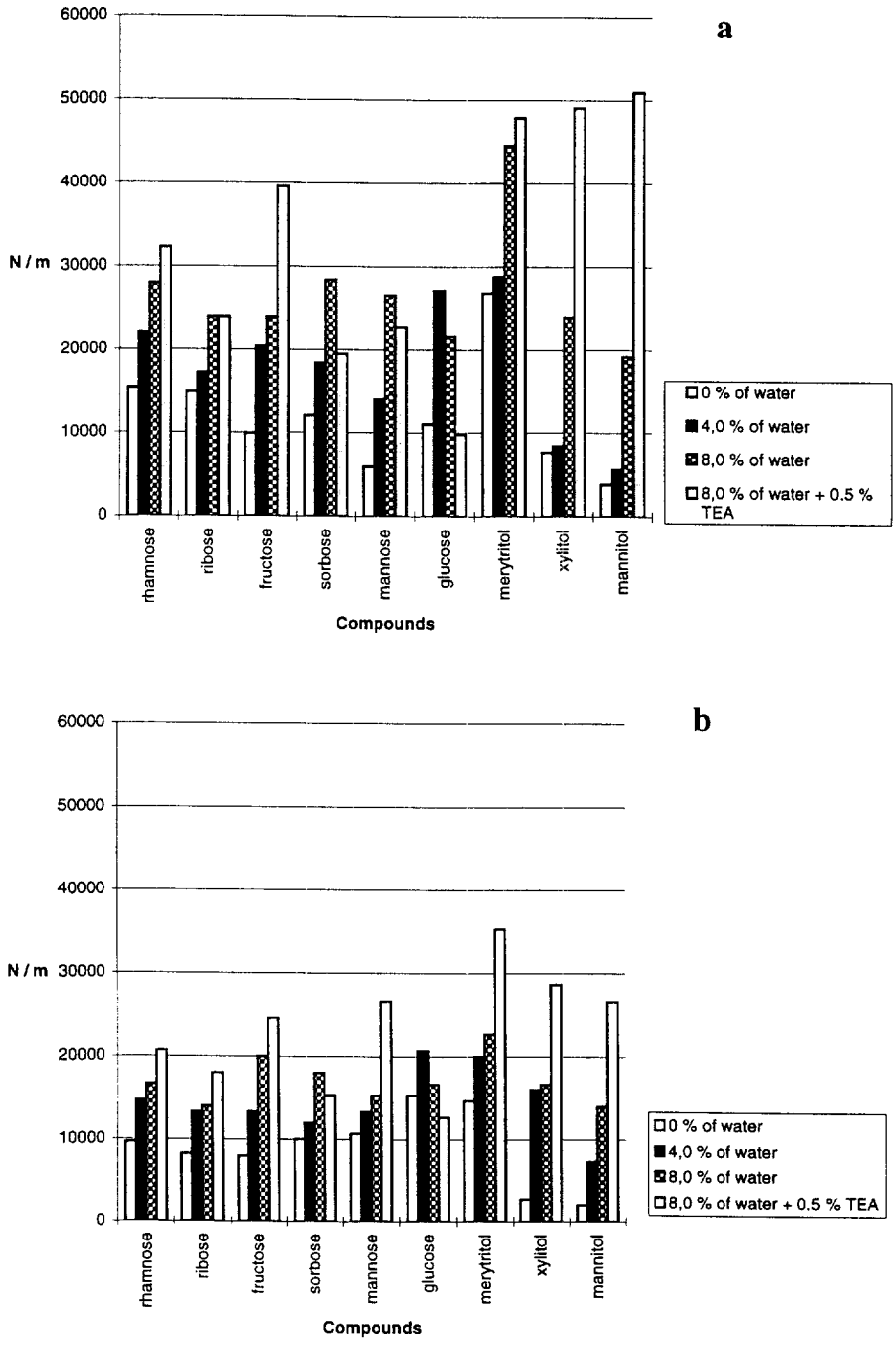

Fig. 1. Variation of efficiency per meter (N/m) as a function of the composition of modifier with a Zorbax TMS column (a) and a Zorbax Sil column (b). Conditions are as given in Table 1.

Table 3
Resolutions for some pairs of solutes using Zorbax TMS and Zorbax Sil columns

	Modifier	MeOH	MeOH–H_2O	MeOH–H_2O	MeOH–H_2O–TEA
	Composition (v/v)	100	96.0:4.0	92.0:8.0	91.5:8.0:0.5
Fructose–sorbose	Zorbax TMS	0.36	0.6	1.44	0.88
	Zorbax SIL	0.43	0.91	0.86	0
Mannose–glucose	Zorbax TMS	1.52	2.43	2.44	1.78
	Zorbax SIL	1.36	1.04	1.31	1.59
Xylitol–mannitol	Zorbax TMS	2.61	2.83	4.24	8.98
	Zorbax SIL	1.20	1.75	3.07	4.48
Mannose–mannitol	Zorbax TMS	2.14	2.28	2.3	5.26
	Zorbax SIL	1.20	1.05	1.84	3.11
Xylose–xylitol	Zorbax TMS	2.78	2.62	NC[a]	1.16
	Zorbax SIL	1.20	2.62	NC[a]	3.39

Mobile phase consisted of CO_2–modifier (80:20, v/v).
The column temperature was 41°C, the flow-rate was 3 ml min^{-1} and the pressure was 200 bars.
[a]NC=not calculated (anomer resolution).

the absence of water or with a water content ranging from 4.0 to 8.0% in the modifier and also with a water content of 8.0% or with the water content ranging from 4.0 to 0%, in order to avoid cross influence of swelling of the stationary phase by the different mobile phases. Under these conditions, the two ketohexoses (sorbose and fructose) for example are well resolved using a TMS column. The drawback is the resolution of anomers of some carbohydrates in the presence of water.

3.2. Influence of TEA

Using CO_2–methanol–water as the eluent, anomer

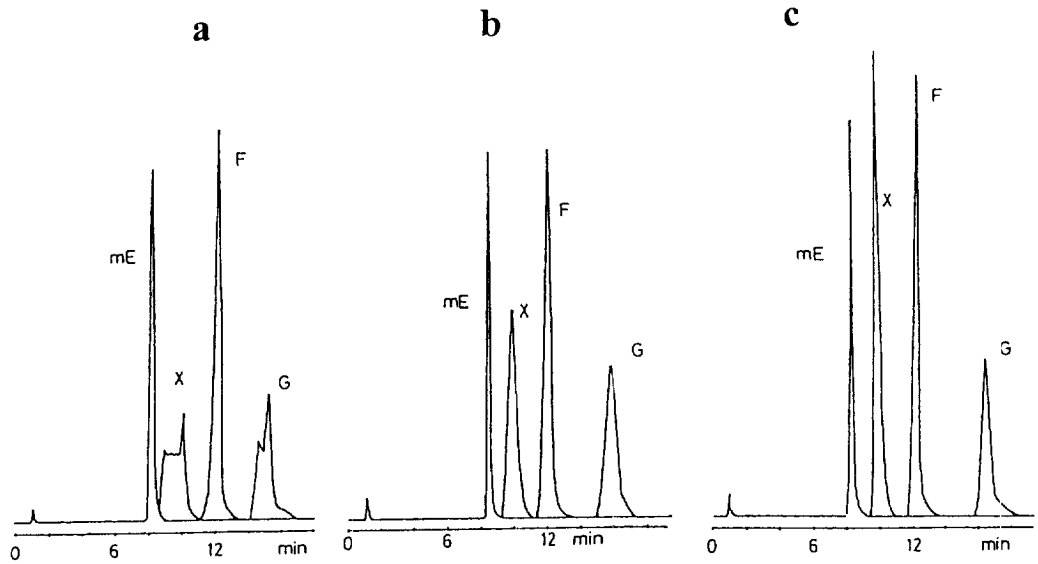

Fig. 2. Chromatograms of monosaccharides and polyols on a Zorbax TMS column (250×4.6 mm I.D.). Eluent: CO_2–modifier (80:20, v/v). Modifier: MeOH–H_2O–TEA (91.5:8.0:0.5, v/v/v). Flow-rate, 3 ml min^{-1}; pressure, 200 bars; temperature (a) 20°C, (b) 40°C and (c) 60°C. Solutes: m.Erythritol (mE); D-xylose (X); D-fructose (F); D-glucose (G).

separation can occur for xylose and galactose (Table 1). The use of TEA to increase the mutarotation rate was effective in LC [8,20,21] since anomer separation did not occur when TEA was added to the eluent. In SubFC, the addition of TEA can also be used successfully (Table 1). The addition of a small amount of TEA to the modifier (0.5%) slightly increases the retention (Table 1). The effect of TEA is probably complex since (i) interactions with the solutes and/or (ii) a strong interaction with the silanols and the residual silanols can occur. With TEA, the two aldopentoses (xylose and arabinose) and the two ketohexoses (sorbose and fructose) were not well separated. Therefore, the selectivity between some solutes can be improved (for a comparison of methanol–water and methanol–water–TEA as the eluent, see Table 3). Concerning the polyols, efficiencies are improved by a factor of two–thirteen when TEA is added to the eluent (modifier, methanol to methanol–water–TEA; Fig. 1). With regard to the monosaccharides, the variations are not uniform. With the TMS column (Fig. 1a), the efficiencies increase except for sorbose, mannose and glucose. With the silica column (Fig. 1b), the efficiencies improved for the majority of compounds, with the exception of glucose and sorbose. Regarding resolutions (Table 3), results vary widely. Therefore, much higher resolution can be obtained using TEA (e.g. xylitol–mannitol) on a TMS column.

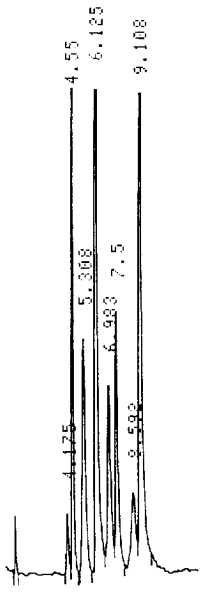

Fig. 3. Chromatograms of eight monosaccharides and polyols on a Zorbax TMS column (250×4.6 mm I.D.). Eluent: CO_2–modifier (80:20, v/v). Modifier: MeOH–H_2O–TEA (91.5:8.0:0.5, v/v/v). Flow-rate, 5 ml min^{-1}; pressure, 200 bars; temperature, 60°C. Solutes: D-ribose, m.Erythritol, D-xylose, xylitol, L-sorbose, D-mannose, D-glucose and mannitol.

3.3. Influence of temperature and flow-rate

The influence of temperature on retention in SFC is well known. Experiments show that the retention times of carbohydrates increase with temperature. An increase in temperature does not bring about a change in selectivities, but improves efficiencies. The

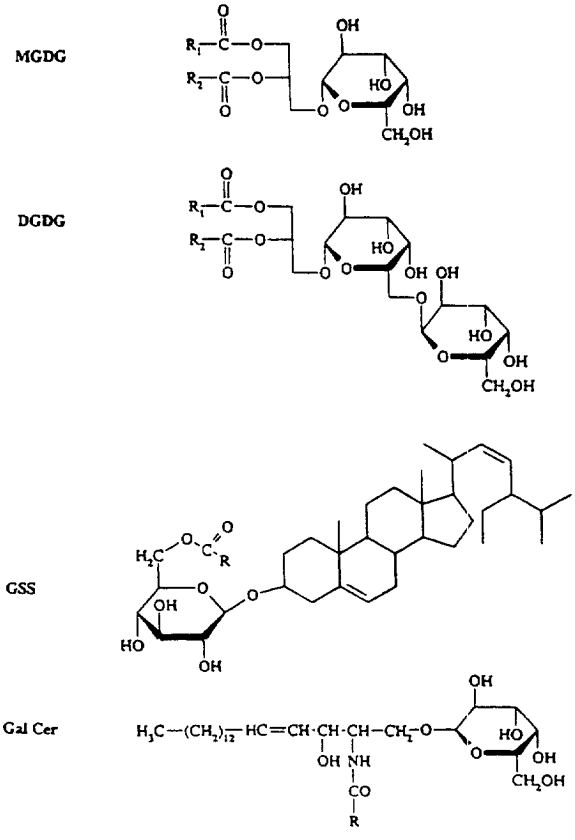

Fig. 4. Structure of glycolipids. MGDG=monogalactosyldiacylglycerol; DGDG=digalactosyldiacylglycerol; GSS=glucosylsitosterol and Gal Cer=galactocerebrosides. R, R1, R2=alkyl chains.

best efficiency is obtained at 40°C for the polyols and at 60–90°C for the monosaccharides (Fig. 2).

Temperature is known to increase the mutarotation of carbohydrates in a water eluent. However, in an organic solvent (e.g., dichloromethane–methanol), the mutarotation rate remains very slow [22]. As seen in Fig. 2, higher temperatures (60°C) and the addition of TEA can both be used successfully to suppress the anomer separation of carbohydrates in SubFC.

With regard to efficiencies as a function of linear velocity (u), the minimum in the H vs. u plot (H=theoretical plate height) in SFC is shifted towards higher linear velocities in comparison with LC [23]. From these results, one should be able to shorten the analysis time by a factor of 1.7 using higher mobile phase flow-rates, with no loss of column efficiency or resolution (Fig. 3). A similar observation has been made concerning the separation of phenylthiohydantoin (PTH) amino acids [24] and other solutes [25].

3.4. Comparison of the behaviour of sugars and glycolipids

Glycolipids are polar neutral lipids consisting of a carbohydrate moiety and one or more acyl or apolar groups. The most important class in plant tissues are MGDG and DGDG. The four glycolipids and the abbreviations used for the studies are given in Fig. 4. Carbohydrates can be present in glycolipid fraction extracts. In a previous paper [26], we described a method that allowed glycolipids, phospholipids and carbohydrates to be analysed in a single run using SubFC without any derivatization. However, no optimization of the eluent composition was undertaken. This has been carried out in this work in order to obtain the best resolution of four glycolipids that could be found in a plant extract. The capacity factors of the four glycolipids are reported in Table 1. SubFC analysis shows some double peaks (DGDG, Galcer I) probably due to variation in the chain length (mainly from C_{16} and C_{18} in plant tissue

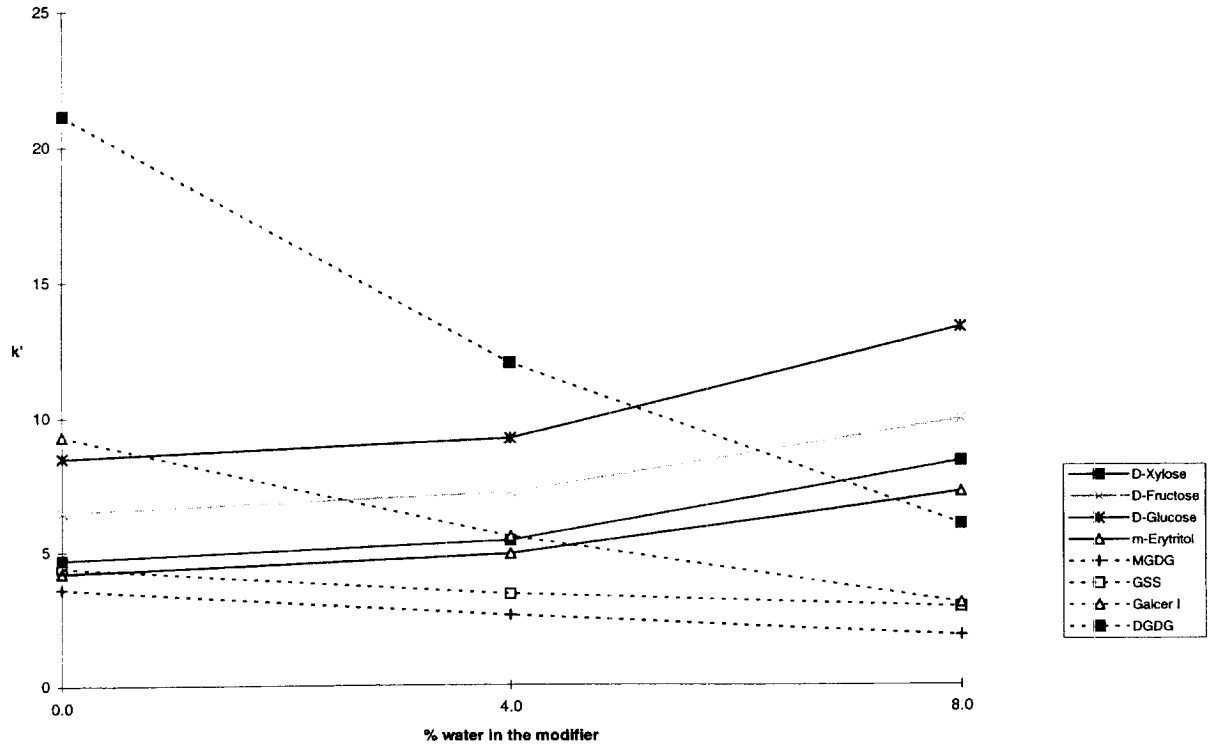

Fig. 5. Comparison of capacity factors (k') between monosaccharides/polyols and glycolipids on a Zorbax TMS column (250×4.6 mm I.D.) as a function of water content in the eluent. Conditions as in Table 1.

glycolipids [27]). The retention of glycolipids decreases when the water content in the eluent increases. Therefore, the behaviour of glycolipids is opposite to that of the carbohydrates. Elution order and selectivities vary with the eluent used (Fig. 5). DGDG and Galcer I can be eluted after polyols and monosaccharides with only methanol as the modifier. Glycolipids can be eluted before polyols and carbohydrates with methanol–water as the modifier (Fig. 5).

Using 8.0% water in the modifier, GSS and Galcer I are not well separated. The addition of TEA does not improve the selectivity (Table 1) and it decreases the retention of glycolipids.

The different behaviours of glycolipids and carbohydrates as a function of water added to the eluent permits us to easily differentiate between carbohydrate and glycolipid peaks. Two experiments with different percentages of water in the modifier are necessary. The peak for which the retention time increases when the water content increases can be identified as a carbohydrate peak. The peak for which the retention time decreases can be identified as a glycolipid peak. Fig. 6 shows a plant extract analysis. A small amount of galactose has been detected.

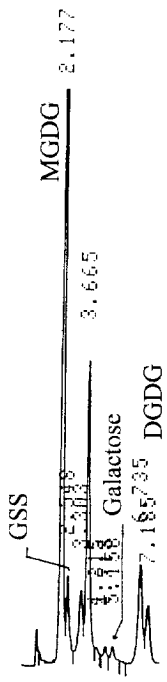

Fig. 6. Analysis of a glycolipid plant extract. Column, Zorbax SIL (150×4.6 mm I.D.). Eluent, CO_2–modifier (80:20, v/v). Modifier: MeOH–H_2O (96.0:4.0, v/v). Flow-rate, 3 ml min^{-1}. Temperature, 41°C. Pressure, 200 bars. See Fig. 4 for abbreviations.

4. Conclusion

In SubFC, silica and TMS-bonded silica are both good choices as a stationary phase in order to analyse underivatized carbohydrates and polyols. Moreover, higher efficiencies and resolution are obtained if water and TEA are added to the methanol as the polar modifier. The separation mechanism seems to be similar on silica and TMS-bonded silica columns. Additional experiments are necessary to explain more precisely the retention mechanism and to explain why there is increased retention of monosaccharides and polyols when water is added in the eluent. Glycolipids, which are carbohydrate derivatives, are of interest because their behaviour is reversed in the presence of water in the eluent. The analytical method proposed for carbohydrates is simple. SubFC is not difficult to run in comparison with LC and routine SubFC analysis of polar compounds should be developed in analytical laboratories.

Acknowledgements

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