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Review

# Supercritical fluid chromatography of carbohydrates

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**Abstract**

Supercritical fluid chromatography (SFC) is a relatively new technique applied to polar solutes such as carbohydrates about 10 years ago. The developments in the SFC of carbohydrates are summarized and a comparison between capillary and packed column SFC is presented. High-efficiency capillary columns are suitable only for derivatized carbohydrates since various packed columns are well adapted for non derivatized mono-, di- and trisaccharides and provide complementary selectivities.

## Contents



## 1. Introduction

A wide variety of chromatographic techniques are necessary to analyse carbohydrates, owing to the numerous problems arising from these compounds: (i) the analytes are small molecules distinguished solely on the basis of differences in stereochemistry or mode of linkage; (ii) they sometimes contain mixtures of oligo- or polysaccharides having a high degree of polymerization (DP) and thus require gradient elution in liquid chromatography (LC) or temperature programming in gas chromatography (GC) in order to obtain a good efficiency of the most retained solutes; (iii) for direct LC analysis a universal detector is needed owing to their lack of a

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chromophore and a refractive index detector is not compatible with gradient elution; (iv) GC analysis requires derivatization to obtain suitable volatile and thermally labile compounds [1], and, in this manner, GC permits the analysis of carbohydrates and glucose oligomers through hexasaccharides (DP = 6) after derivatization [2]. In GC, the universal response of flame ionization detection (FID) is advantageous in most applications. However, analysis of higher molecular mass carbohydrates would require too high temperatures to achieve sufficient volatility, so these conditions involve instability of the analytes.

Supercritical fluid chromatography (SFC) is a relatively new technique compared with LC and GC. It provides greater chromatographic flexibility and employs a mobile phase consisting of a highly compressed gas just above its critical temperature and pressure. This technique has often been used to separate non-polar or low-polarity compounds, as shown in the review of Fowley and Crow [3]. It was first applied to polar materials such as carbohydrates about 10 years ago.

The aim of this review is to summarize the developments in the SFC of carbohydrates during the past decade, and to compare capillary SFC, where columns are similar to bonded-phase GC columns with a smaller inner diameter and a thicker stationary phase film, and SFC using the packed columns used in LC. Recent work from our laboratory using these packed columns is included.

## 2. Supercritical fluid chromatography

Introduced in 1962 by Klesper et al. [4] with supercritical dichlorodifluoromethane, then used with other supercritical fluids such as 2-propanol [5,6] or carbon dioxide [7], SFC was first developed on packed columns as in LC. However, through the 1970s this technique was overtaken by LC. Significant advances were made following the work of Novotny et al. [8] on capillary columns thanks to the development of fused-silica columns in GC.

Carbon dioxide is the preferred solvent for SFC. It has a great number of advantages, in particular its favourable values of critical pressure $P_c$ and temperature $T_c$ ($P_c = 73$ bar, $T_c = 31$°C), low cost and chemical inertness.

When both the pressure and temperature are above these critical values, supercritical fluid possesses properties that are intermediate between gases and liquids:

(i) A supercritical fluid has the advantage of possessing a diffusion coefficient about 100–200 times larger than the coefficient observed in liquid. Since this high diffusivity is an important factor in resistance to mass transfer, optimum velocities of mobile phases are always higher for SFC than for LC. So, for a similar efficiency, separation is about ten times faster in SFC than in LC with packed columns [9].

(ii) The viscosity of a supercritical fluid is about 100 times lower than that of the liquid. This low viscosity involves a much lower pressure drop over the column than in LC, allowing a higher velocity of the mobile phase than in LC.

(iii) The density of a supercritical fluid is similar to that of the liquid and 200–500 times that of the gas. Shorter intermolecular distances and an increase in molecular interactions enhance the solubilizing power relative to GC.

Moreover, the density and so the solvating power can be modified by varying the pressure and temperature. In the region close to and above the critical point (e.g., 40°C) there is a sharp increase in density with only a slight increase in pressure. At higher temperatures (e.g., 100°C), this effect is not so pronounced.

The solubility parameter $\delta$ defines the mobile phase strength of the fluid and increases, as does the density, as a function of the pressure. The parameter of supercritical carbon dioxide varies between that of a perfluoroalkane (for a density close to 0.3 g $ml^{-1}$) and of chloroform (for a density close to 1 g $ml^{-1}$) [9]. Therefore, carbon dioxide has a low polarity and is not a good solvent for polar compounds.

The analysis of polar compounds such as carbohydrates requires either derivatization of solutes to enhance their solubility in pure carbon dioxide, or the addition of a polar solvent

(modifier) to increase the mobile phase strength, modify the selectivity and improve the peak shape by deactivation of active sites on the surface of the stationary phase.

In the second method (use of a modifier), $P_c$ and $T_c$ values of the mixtures vary with the concentration of modifier [10]. Then in some cases, chromatographic analysis is achieved under sub-critical conditions (only either pressure or temperature is above the critical values). Moreover, the addition of small amounts of modifier can be incompatible with some detection systems (e.g., methanol with FID).

## 3. Instrumentation

### 3.1. Apparatus

The schematic diagram of an SFC apparatus is the same as for LC with two exceptions: the pump head is cooled with a circulating refrigerated liquid in order to increase the pump-down efficiency, and at the end of the column a restrictor (capillary tube or valve) is required to create the pressure needed for the supercritical state, because of the low pressure drop of the column. When gradient elution is required, a polar modifier is added via a second pump and the two solvents are mixed in a dynamic mixing chamber.

### 3.2. Columns

Both capillary and packed columns have been used in SFC. Capillary columns are similar to bonded-phase GC columns [8] with a smaller I.D. (50–100 $\mu$m) and a relatively thicker stationary phase film (0.05–0.2 $\mu$m).

These columns afford high efficiency but the analysis time is often long. Using pure carbon dioxide as the mobile phase, the variation of retention is usually carried out by changing the density through pressure programming at a given temperature. Adding modifier is much less significant in capillary column than in packed column SFC and creates background noise in FID, usually employed with this kind of column. Moreover, the choice of the stationary phase is limited to silicones.

Packed columns are identical with LC columns and allow greater efficiency per unit time. Retention and selectivity can be adjusted by adding modifier and gradient elution is often preferred to pressure programming.

The early commercial instruments used capillary columns while work on packed columns was performed with laboratory-made apparatus. Currently, two manufacturers (Gilson Medical Electronics, Villiers le Bel, France, and Hewlett-Packard, Palo Alto, CA, USA) have developed new commercially available systems for SFC with independent programming of mobile phase pressure, composition and flow-rate (Gilson Series SF3 for packed columns and Hewlett-Packard Model G 1205 for capillary and packed columns).

### 3.3. Detectors

The detectors in SFC have been taken from LC and GC. Absorption detectors (UV–Vis) employed before decompression of the fluid require a high-pressure cell and are not suitable for carbohydrates which are without UV or visible chromophores. Fourier transform infrared spectrometric detection with a high-pressure cell has also been performed [11] to identify derivatization products in the eluate from packed columns with a small diameter (1 mm), but the region (2400–2100 $cm^{-1}$) is masked by the absorbance of supercritical carbon dioxide. For carbohydrates, a universal detector is needed and therefore the FID from GC is widely used with capillary columns after decompression of the fluid. However, FID precludes the use of polar additives necessary to solubilize and elute polar solutes. With packed columns, its use is limited to formic acid and water because the addition of other modifiers generates a high level of noise. Mass spectrometry, which has been shown to provide sensitive, universal and specific detection for capillary SFC [12,13], is usually limited to pure carbon dioxide, like FID.

The evaporative light scattering detector (ELSD), which is a universal detector for LC

[14], and is more practical than the refractive index detector, has been widely used for the LC analysis of carbohydrates [15–18]. Using ELSD in LC, the mobile phase is nebulized by a gas stream and the aerosol produced passes through a heated tube where vaporization of the eluent takes place. The remaining microparticles of the non-volatile analyte are then passed through a light beam. The incident light is scattered by the particles and collected by a photomultiplier, providing the chromatographic signal (Fig. 1).

Some commercial ELSDs have been proposed for LC (ACS, Applied Chromatographic Systems, Macclesfield, Cheshire, UK; Varex, Burtonsville, MD, USA: Sedex, Sedere, Alfortville, France), and a review of the principle, technology and characteristics of these detectors has recently been published [19]. The Sedex Model 55 is available with an interface coupling to SFC. As for FID, a small-diameter fused-silica tube acts as a restrictor to maintain the supercritical state of the fluid. Its design is very important for avoiding analyte precipitation and plugging and the formation of ice at the restrictor outlet, where the cooling effect of decompression is greatest. While the preparation and installation of the restrictor in the FID require great care to minimize spiking in the output signal, the interfaces enabling coupling of ELSD to SFC or LC in the Sedex 55 apparatus are easily interchangeable. For ELSD, make-up gas also enables ice formation and condensation of the organic modifier to be avoided.

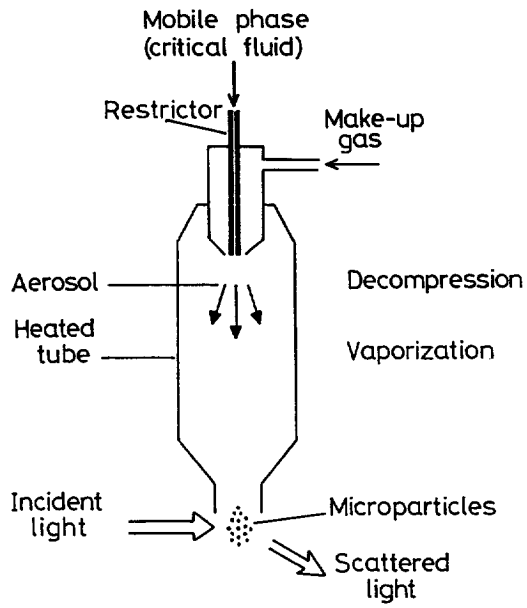


Fig. 1. Schematic diagram of evaporative light scattering detector for SFC.

ELSD is then compatible with SFC as it reveals every solid and liquid analyte in suspension in the gas phase resulting from the decompression of the supercritical fluid and the vaporization of aerosol droplets of modifier. It is also compatible with polar modifiers added to the carbon dioxide, unlike FID. This commercial coupling has been achieved in our laboratory [20,21] and the main factors that influence the detection limit and the reproducibility in SFC have been determined [21]. Various applications in LC and SFC of this coupling have been published [22]. ACS ELSD has also been used in SFC. Its HPLC tube was removed and replaced by a crimped stainless-steel restrictor [23]. A 0.8 mm I.D. tube was used to form the restrictor. However, this method is likely to give low reproducibility between various restrictors of the same type.

## 4. Capillary SFC of carbohydrates

The first analyses of carbohydrates using SFC were performed on capillary columns [24]. Most carbohydrates do not have sufficient solubility in carbon dioxide, and a polar modifier, which could increase their solubility, prohibits the use of FID, thus limiting the direct analysis of these compounds.

Derivatization of low-molecular-mass samples has been successful for GC. Trimethylsilylation, peracetylation or permethylation are usually used for the derivatization of carbohydrates; comparable methods [24–28] have been utilized to enhance analyte solubility in low-polarity carbon dioxide. Chester and co-workers [13,24] showed that trimethylsilyl derivatives of maltooligosaccharides with a degree of polymerization up to DP 20 can be eluted on methyl-

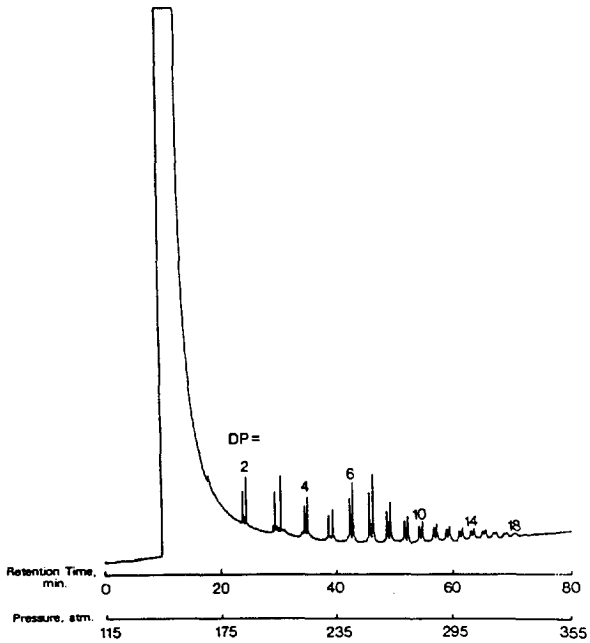


Fig. 2. Capillary SFC–FID separation of silylated oligo- and polysaccharides in a corn syrup. The numbers represent the degree of polymerization, that is, the number of glucose units. (Reproduced from Ref. [24], with permission of Hüthig.)

polysiloxane phases bonded on fused silica (Fig. 2).

For derivatized carbohydrates, capillary SFC is clearly superior to GC in molecular mass range because in GC much larger carbohydrates require too high temperatures of the columns, limiting the stability of analytes. Chester and Innis [24] noted that the upper molecular mass limit of silylated carbohydrates analysed by SFC–FID is imposed principally by the spiking on detection with condensation of solute rather than by mobile phase elution strength limits. A variety of designs of restrictors have been proposed (linear, frit, integral, tapered) to avoid precipitation and plugging when the cooling effect of decompression is greatest. Thermal stability is not necessary, because separation has already taken place [25], which is why the detector is maintained at a high temperature (390°C) [27].

Analyses by capillary SFC are often carried out above 100°C because in this temperature range the variation of density with the pressure is quasi-linear and linear pressure programming is used. With this method, separation of derivatized oligosaccharides requires a long time compared with their direct LC analysis using gradient elution and ELSD [22].

Derivatization of carbohydrates before chromatographic analysis can involve complicated chromatograms owing to the formation of $\alpha$- and $\beta$-anomers and one carbohydrate usually gives two peaks provided that they can be resolved in the column used [29]. To avoid this anomerization, in GC methoximation (MO derivatives) before silylation or acetylation is preferred. Shirota et al. [30] compared these two methods and showed the chromatograms of TMS derivatives of glucopyranose oligomers with FID and MO-TMS derivatives with a nitrogen-sensitive detector (Fig. 3). Marking the analyte with nitrogen improves the sensitivity of detection and this selective detector enables one to obtain a picogram detection limit whereas for FID it is of the order of a few nanograms.

Glycosphingolipids are amphoteric glycolipids of a fatty acid and one or more carbohydrates. Capillary SFC has been preferred to LC for mass spectrometry interfacing [27]. Samples were permethylated [27] and analysed using a pro-

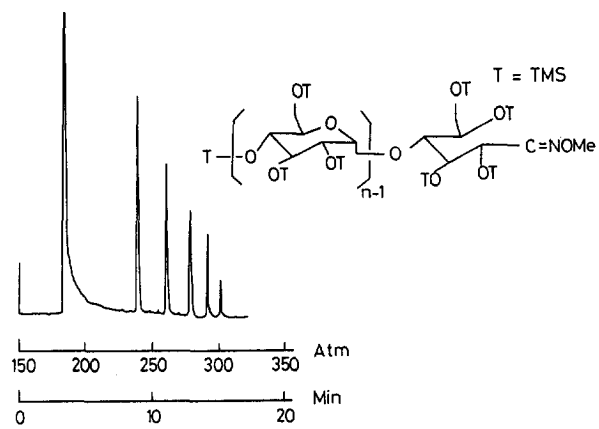


Fig. 3. Chromatogram of derivatized standard glucopyranose oligomer methoxime–trimethylsilyl derivatives with nitrogen-sensitive detector. (Reproduced from Ref. [30], with permission of MicroSeparations.)

grammed increase in density. Using natural extracts, the derivatization leads to complex chromatograms, which limits the identification and requires direct interfacing of SFC to a mass spectrometer [27]. Pinkston et al. [12] also found that capillary SFC–MS is well suited to confirm the identity of the TMS derivative of inositol triphosphate.

## 5. Packed column SFC of carbohydrates

As in capillary SFC, precolumn derivatization of carbohydrates is possible to increase their solubility in pure carbon dioxide and the separation of maltodextrin derivatized with trifluoroacetic anhydride has been reported using FID and a CN packed column [31]. An interesting solventless injection system was described and removal of excess anhydride in an on-line precolumn eliminated sample work-up steps.

The advantages of packed column SFC for carbohydrate analysis as compared with capillary SFC are as follows: analysis is faster and often without a gradient and so is less time consuming, and selectivity can be varied by varying the nature and composition of the mobile phase (although carbon dioxide–methanol mixtures enable a number of problems to be solved) while keeping the stationary phase the same. There is a wider choice of stationary phases than in capillary SFC; ELSD as a universal detection method is compatible with polar modifiers, in contrast to FID, and this modifier–carbon dioxide mixture allows the elution of raw carbohydrates and avoids the derivatization which often involves complex chromatograms.

Polar modifiers enhance the solubility of polar carbohydrates, which is necessary for an acceptable elution time. Hence a binary mobile phase in combination with packed columns and ELSD allows the direct analysis of carbohydrates [20].

In LC, silica-based packings for carbohydrate analysis are usually aminopropyl-bonded silica and occasionally diol-bonded silica, which avoids the disadvantages of the former [32]. On the other hand, this alternative method offers poor selectivity for monosaccharides. Octadecyl-bonded silica has been applied in the LC of oligosaccharides using gradient elution [22] but without resolution of monosaccharides.

In SFC, a wider variety of silica-bonded packings has been applied with carbon dioxide–methanol as the mobile phase and ELSD. Stationary phases as apolar as ODS and TMS [33] and as polar as CN, diol or $NO_2$ [34] have been successfully applied to the separation of monosaccharides.

Determination of carbohydrates in tobacco [33] has been performed on apolar bonded silicas (Zorbax ODS and Zorbax TMS) with a retention mechanism close to "normal phase" (Table 1). The separation between glucose, fructose and sucrose is much better than in LC with the same apolar phases. The detection limit with ELSD is similar to that obtained when ELSD is coupled to LC (about 10 ng injected). This value depends strongly on the quality of the outlet of the restrictor and the decompression temperature of the carbon dioxide.

Table 1
Determination of carbohydrates in three tobacco extracts (triplicate runs)

| Run No. | Tobacco 1 | | | Tobacco 2 | | | Tobacco 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | F(%) | G(%) | S(%) | F(%) | G(%) | S(%) | F(%) | G(%) | S(%) |
| 1 | 6.2 | 7.4 | 11.9 | 6.8 | 9.2 | 15 | 5.6 | 3.7 | 0 |
| 2 | 6.3 | 7.2 | 12.4 | 6.9 | 8.1 | 16 | 5.7 | 3.4 | 0 |
| 3 | 6.2 | 7.2 | 11.6 | 6.6 | 8.4 | 15.6 | 5.6 | 3.7 | 0 |

Column: Zorbax TMS, 7 $\mu$m (250 × 4.6 mm I.D.). Eluent: $CO_2$–methanol (5.4:0.8). Detection: ELSD. Pressure: 220 bar. Temperature: 40°C. Solutes: F = fructose; G = glucose; S = sucrose; internal standard: esculin. (Reproduced from Ref. [33], with permission of Institut National Polytechnique de Lorraine.)

In contrast to the results obtained in LC with acetonitrile–water mobile phases, diol-bonded silicas afford high selectivity for monosaccharides, as shown in Fig. 4a. Other silicas bonded with groups such as CN and $NO_2$ have also permitted excellent resolution of carbohydrates, as shown in Fig. 4b and Table 2.

Useful separations can be obtained on SFC systems in comparison with LC systems. For example, mannose–glucose and glucose–mannitol–sorbitol are poorly resolved using aminopropylsilica and acetonitrile–water as LC mobile phase, since these carbohydrates are well resolved in a short time on diol- and/or $NO_2$-bonded silica with a carbon dioxide–methanol system (Fig. 4a and b).

Moreover, different and complementary selectivities can be obtained using SFC on different stationary phases. For example, μBondapak CN permits the separation of galactose–mannose but not galactose–mannitol couple (Table 2). In contrast, Zorbax CN does not allow the separation of the first pair but provides good selectivity for the second. SFC on bonded silica gels seems to be influenced by the nature of the bonded moiety, the nature of silica and/or the linkage mode of the stationary phase.

SFC separation of raw carbohydrates on bare silica has not yet been reported. A modifier such as methanol–water–pyridine–triethylamine, previously used for SFC of non-derivatized amino acids [35], provides good selectivity and rapid resolution on bare silica columns, as shown in Fig. 4c. Studies on such systems are in progress in our laboratory.

Di- or trisaccharides are also easily eluted with an appropriate percentage of polar modifier. Moreover, gradient elution can be achieved without baseline drift and allows the analysis of different mono-, di- and trisaccharides in the same run (Fig. 4d). As gradient elution is possible with ELSD, identical efficiency was obtained for raffinose and fructose. A fused-silica capillary tube (160 × 0.75 mm I.D.) was used as a restrictor instead of the conventional nebulizer for LC.

Generally, with such a restrictor, varying the viscosity of the mobile phase during gradient elution [22] provides a pressure gradient. When the temperature is about 40°C, this pressure variation can provide a large increase in density. Hence the polarity of mobile phase varies with both effects of an increase in the amount of modifier and an increase in density in the supercritical fluid mixture. For retention to be influenced solely by the effect of the modifier during a gradient elution, the pressure must be kept constant. With an automated regulation valve (Gilson Series SF3 apparatus) before an available restrictor (30 × 0.05 mm I.D. or 30 × 0.075 mm I.D.), gradient elution can be obtained under constant pressure.

The nature of the injection solvent is also very important. Carbohydrates must be dissolved in methanol or chloroform–methanol to avoid deformation of the shape of peaks.

## 6. Packed column SFC of carbohydrate derivatives

As SFC is claimed to be well adapted to the analysis of non-polar solutes, SFC of carbohydrate derivatives less polar than carbohydrates, such as alkylglycosides, glucosinolates or glycolipids, should be also successful.

O-Alkylglycosides are biological detergents used as membrane solubilizers and it is very important to determine their purity. Different O-alkylglucopyranosides and maltosides are commercially available and various O-alkylgalactopyranosides and xylopyranosides have been synthesized in our laboratory. They were analysed by SFC on polar columns with carbon dioxide–methanol mixtures as mobile phase [36]. Table 3 gives the retention times of these compounds. Selectivity is governed by the nature of the carbohydrate moiety and not by alkyl chain length. Table 3 also shows that the retention time of an alkylglycoside is lower than that of the corresponding carbohydrate (e.g., octylxylopyranoside and xylopyranose; octylglucopyranoside and glucopyranose; octylgalactopyranoside and galactopyranose). Hence monitoring of residual carbohydrate content in O-alkylglycosides can be carried out in a short time

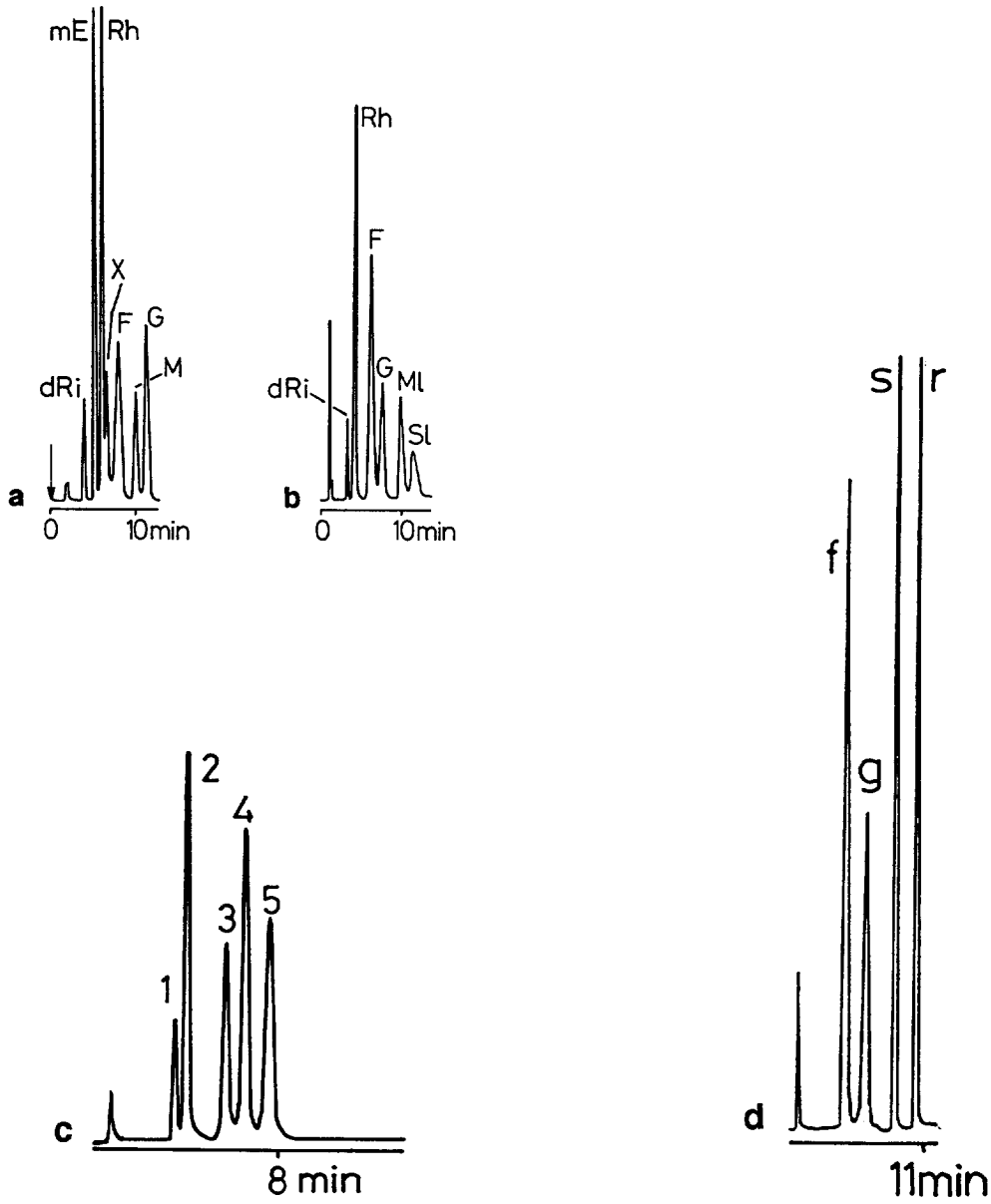


Fig. 4. Separation of carbohydrates by SFC using polar bonded and bare silica and evaporative light scattering detection. Temperature, 40°C. (a) LiChrospher Diol column. Eluent: $CO_2$–methanol (84.5:15.5, w/w), 1.77 ml $min^{-1}$, 3500 p.s.i. Solutes: dRi = 2-deoxyribose; mE = *meso*-erythritol; Rh = rhamnose; X = xylose; F = fructose; M = mannose; G = glucose. (Reproduced from Ref. [34], with permission of Elsevier Science.) (b) RSil $NO_2$ column. Eluent: $CO_2$–methanol (87:13, w/w), 3.8 ml $min^{-1}$, 3500 p.s.i. Solutes: as in (a); Ml = mannitol; Sl = sorbitol. (Reproduced from Ref. [34], with permission of Elsevier Science.) (c) Zorbax Sil column. Eluent: $CO_2$–modifier (83:17); modifier = methanol–water–pyridine–triethylamine (91.95:4:4:0.05, v/v); 3.5 ml $min^{-1}$; 180 bar. Solutes: 1 = ribose; 2 = rhamnose; 3 = fructose; 4 = mannose; 5 = glucose. (d) LiChrosorb CN column. Eluent: $CO_2$–methanol (A–B), gradient 94% during 5 min then 94% A to 82% A in 4 min; 2.42 ml $min^{-1}$ during 5 min then 2.42 ml $min^{-1}$ to 2.84 ml $min^{-1}$ in 4 min. Initial pressure: 3000 p.s.i. Solutes: as in (a); s = sucrose; r = raffinose. (Reproduced from Ref. [22] with permission of Hüthig.)

Table 2
Retention times (min) of monosaccharides and polyols on polar bonded silicas in SFC at 40°C with $CO_2$–methanol mixtures (reproduced from Ref. [34] with permission of Elsevier Science)

| Compound | Column No.[a] | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| 2-Deoxy-D-ribose | 2.4 | 2.1 | 1.1 | 3.9 | 3.3 |
| L-Rhamnose | 3.6 | 3.2 | 4.5 | 5.8 | 4.1 |
| D-Ribose | 3.8 | 3.4 | 4.8 | 5.6 | 4.7 |
| *meso*-Erythritol | 3.9 | 3.5 | 3.6 | 5.0 | 4.6 |
| L-Arabinose | 4.4 | 3.7 | 3.5 | 6.2 | 5.0 |
| D-Xylose | 4.5 | 4.0 | 4.5 | 6.4 | 4.6 |
| D-Fructose | 6.0 | 5.2 | 3.7 | 7.6 | 6.5 |
| L-Sorbose | 6.2 | 5.2 | 3.7 | 9.0 | 6.2 |
| Xylitol | 7.0 | 5.1 | 8.7 | 7.3 | 7.6 |
| D-Galactose | 8.0 | 7.5 | 7.0 | – | 8.0 |
| D-Mannose | 8.0 | 4.5 | 6.7 | 9.4 | 7.0 |
| D-Glucose | 8.5 | 7.8 | 6.5 | 10.6 | 8.0 |
| *meso*-Inositol | 13.0 | 12.0 | 9.8 | 19.0 | – |
| D-Mannitol | 14.0 | 8.0 | 15.0 | 10.8 | 10.2 |
| D-Sorbitol | – | – | – | 11.0 | 11.7 |

[a] 1 = Zorbax CN (150 × 4.6 mm I.D.), $CO_2$–methanol (93.5:6.5, w/w), 4.35 ml $min^{-1}$, 3700 p.s.i.; 2 = μBondapak CN (150 × 3.9 mm I.D.), $CO_2$–methanol (95.9:4.1, w/w), 3.37 ml $min^{-1}$, 3900 p.s.i.; 3 = LiChrosorb CN (150 × 4.6 mm I.D.), $CO_2$–methanol (96.4:3.6, w/w), 3.35 ml $min^{-1}$, 3900 p.s.i.; 4 = LiChrospher Diol (250 × 4.6 mm I.D.), $CO_2$–methanol (83.7:16.3, w/w), 1.79 ml $min^{-1}$, 3900 p.s.i.; 5 = RSil $NO_2$ (250 × 4.6 mm I.D.), $CO_2$–methanol (87.0:13.0, w/w), 3.8 ml $min^{-1}$, 3500 p.s.i.

Table 3
Retention times of glycosides and O-alkyl glycosides on polar packed columns in SFC at 40°C (reproduced from Ref. [36] with permission of Elsevier Science)

| Compounds | Retention time (min) | |
|---|---|---|
| | Column 1[a] | Column 2[a] |
| Octyl β-D-xylopyranoside | 3.6 | 2.0 |
| Hexyl β-D-glucopyranoside | 10.0 | 3.5 |
| Octyl β-D-glucopyranoside | 10.9 | 3.6 |
| Decyl β-D-glucopyranoside | – | 3.8 |
| Dodecyl β-D-glucopyranoside | 10.7 | 3.9 |
| Octyl β-D-galactopyranoside | 10.9 | 4.3 |
| Methyl α,β-D-glucopyranoside | 14.8 | 4.45 and 4.73 |
| Methyl α-D-glucopyranoside | 14.8 | 4.73 |
| Decyl β-D-maltoside | >30 | 21.2 |
| Dodecyl α,β-D-maltoside | >30 | 22.5 |
| α,β-D-Xylopyranose | – | 4.5 |
| α,β-D-Galactopyranose | >30 | 8.0 |
| α-D-Glucopyranose | >30 | 8.7 |

[a] 1 = Lichrospher Diol (125 × 4 mm I.D.), $CO_2$–methanol (93.75:6.25, w/w), 3.9 ml $min^{-1}$, 145 bar; 2 = Zorbax CN (150 × 4.6 mm I.D.), $CO_2$–methanol (93.0:7.0, w/w), 3.5 ml $min^{-1}$, 210 bar.

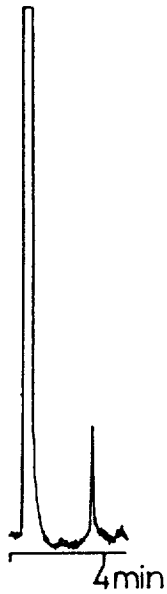


Fig. 5. Chromatogram of octyl-β-D-glucopyranoside (amount 100 ng) on a Zorbax CN column (150 × 4.6 mm I.D.). Eluent: $CO_2$–methanol (93.75:6.25, w/w), 3.9 ml $min^{-1}$, 145 bar. (Reproduced from Ref. [36], with permission of Elsevier Science.)

on Zorbax CN column. Fig. 5 shows that the detection limit of O-alkylglycoside is about 30 ng and it is similar for carbohydrates.

Glucosinolates are found in various plant materials and cause physiological effects in animal such as loss of appetite and goitrigenic effects [37]. SFC analysis can be carried out [36] on the desulphoglucosinolate form, the structure of which is shown in Fig. 6. Compounds A, B and C, not yet found in plants, were synthesized to be used as internal standards in the analysis of natural glucosinolates such as structure D (Fig. 6). ELSD affords the advantage of a mass response compared with UV detection, which depends on aryl substituents. Table 4 shows a retention of desulphoglucosinolates higher than that of glucose, good selectivity relative to the aromatic moiety and a significant difference between the behaviour of both polar columns.

Table 4
Retention times of desulphoglucosinolates on polar packed columns in SFC at 40°C (reproduced from Ref. [36] with permission of Elsevier Science)

| Compound | Retention time (min) | |
|---|---|---|
| | Column 1[a] | Column 2[a] |
| A | 10.7 | 6.2 |
| B | 15.9 | 12.3 |
| C | 14.6 | 7.4 |
| D | 15.7 | 7.6 |
| D-Glucose | 8.1 | 7.1 |

[a] 1 = LiChrospher Diol (125 × 4 mm I.D.), $CO_2$–methanol (90:10, w/w), 4.1 ml $min^{-1}$, 180 bar; 2 = RSil $NO_2$ (250 × 4.6 mm I.D.), $CO_2$–methanol (89.3:10.7, w/w), 4.6 ml $min^{-1}$, 245 bar.

Studies of the analysis of glycolipids are in progress in our laboratory. Fast isocratic analysis can be performed on bare silica or TMS-bonded silica (Fig. 7), since LC analysis requires gradient elution [38] and capillary SFC requires derivatization [27]. As with alkylglycosides [36], carbohydrates are eluted under the same conditions as carbohydrate derivatives, and the simultaneous determination of these two classes of compounds is then possible in a single run. Fig. 7a and b show that a similar selectivity can be obtained on bare silica with a suitable modifier and on apolar TMS-bonded silica with a binary methanol–carbon dioxide eluent.

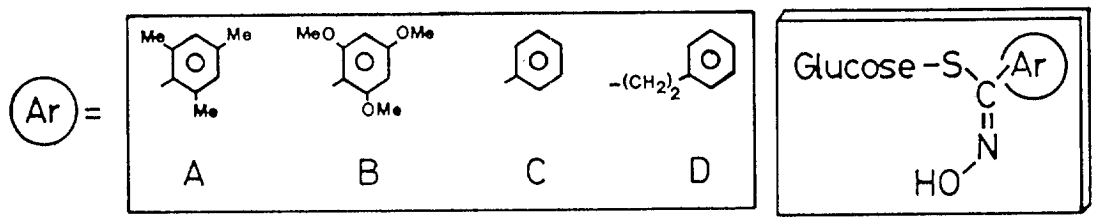


Fig. 6. Structures of desulphoglucosinolates.

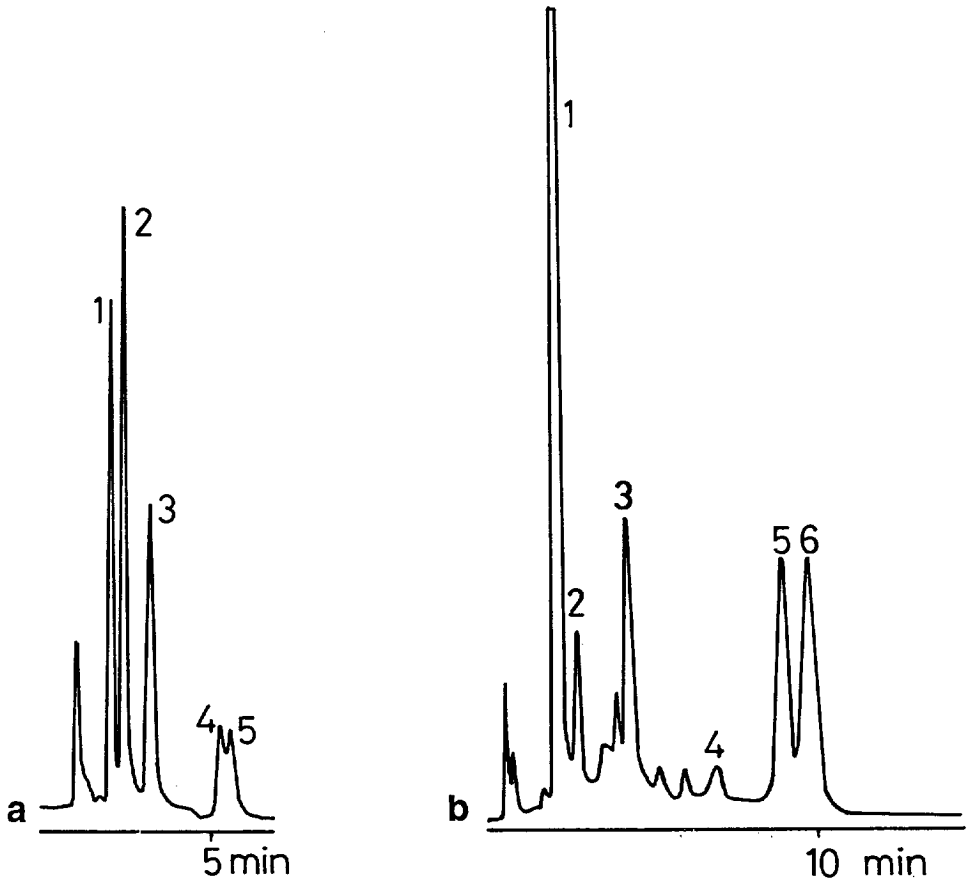


Fig. 7. Chromatograms of glycolipids and carbohydrates in packed column SFC at 40°C. In collaboration with Parfums Christian Dior. (a) Zorbax TMS column (250 × 4.6 mm I.D.). Eluent: $CO_2$–methanol (75:25, v/v), 3.5 ml $min^{-1}$, 180 bar. Solutes (glycolipid standards): 1 = monogalactosyldiacylglycerol; 2 = glucosylsitosterol; 3 = galactocerebroside type I; 4 and 5 = digalactosyldiacylglycerol. (b) Zorbax Sil column (150 × 4.6 mm I.D.). Eluent: $CO_2$–modifier (83:17, v/v); modifier = methanol–water–pyridine–triethylamine (91.95:4:4:0.05, v/v); 3.5 ml $min^{-1}$; 180 bar. Solutes (vegetable extract): 1 = monogalactosyldiacylglycerol; 2 = glucosylsitosterol; 3 = unknown; 4 = galactose; 5 and 6 = digalactosyldiacylglycerol.

## 7. Comparison of the chromatographic behaviour of carbohydrates in packed column SFC and LC using chemometric methods

In order to compare the separation capacities of SFC and LC systems, SFC results should be compared with those obtained using an organic LC mobile phase (e.g., dichloromethane–methanol [39,40], the polarity of which is close to that of carbon dioxide–methanol mixtures. Using such eluents, the separation mechanism of polar solutes such as monosaccharides and polyols on very polar and very apolar stationary phases is not well understood and some results are especially surprising.

SFC and LC retentions of twelve monosaccharides and polyols using twelve or twenty SFC and LC systems [41,42] were analysed using chemometric methods. A hierarchical ascendent classification method (HAC) and principal component analysis (PCA) were used. HAC of the solutes in the space of the chromatographic systems shows that the classification is directly related to the number of hydroxyl groups with

only an abnormal position of the ketohexoses which are closer to the pentoses than to the aldohexoses. HAC of the systems in the space of the solute gives various information about the similarities of systems, but the main result is the non-segregation of liquid and supercritical fluid systems.

The results of PCA were more precise. PCA of the retention of the solutes in the space of the systems gives a main axis contributing 88% to the retention. The compounds are clustered along this axis by the number of hydroxyl groups and this pure mathematical system is close to the Zorbax CN stationary phase and methanol–carbon dioxide mobile phase. The position of the products on this axis was correlated with their solvent-accessible surface computed by a molecular mechanics approach. A good correlation was found between the area of the polar part of this surface and the coordinate on the factorial axis, clearly showing that the main interactions are related to this part of the solutes. The PCA of the systems on the space of solutes gives one main axis (93%). This shows the homogeneity of the different systems.

## 8. Conclusion

SFC analysis of carbohydrates is not yet a widely used method, but it presents major advantages. Both capillary and packed columns, adapted from GC and LC, respectively, can be used. Capillary columns provide high efficiency and high sensitivity, especially with thermionic detection, and can be coupled to mass spectrometry. However, a derivatization step is necessary before analysis. Capillary SFC has been applied especially to oligosaccharide analysis.

Packed columns are well adapted for non-derivatized mono-, di- and trisaccharides, adding modifier to the carbon dioxide and using ELSD (nanogram level). The great selectivity obtained on different packed columns is a major advantage since the carbohydrates have a large number of isomers which are not separated in a particular LC system.

The combination of LC and SFC makes it possible to solve separation problems with this kind of solute. Progress in the interpretation of retention mechanisms, in addition to its theoretical aspects, will permit the analyst to choose the best system for a specific separation.

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