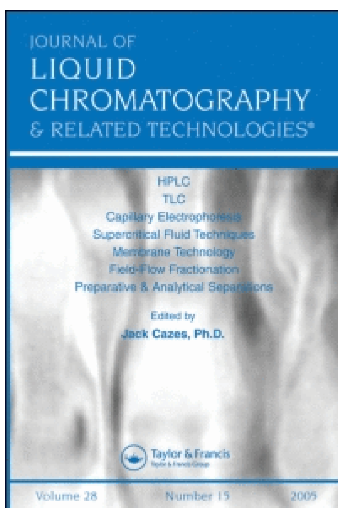
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK




**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Free Bile Acid Analysis by Supercritical Fluid Chromato Graphy and Evaporative Light Scattering Detection

V. Villette[a]; B. Herbreteau[a]; M. Lafosse[a]; M. Dreux[a]
[a] Institut de Chimie Organique et Analytique. URA CNRS 499 Université d'Orléans, Orléans, Cedex, France

**To cite this Article** Villette, V. , Herbreteau, B. , Lafosse, M. and Dreux, M.(1996) 'Free Bile Acid Analysis by Supercritical Fluid Chromato Graphy and Evaporative Light Scattering Detection', Journal of Liquid Chromatography & Related Technologies, 19: 11, 1805 — 1818
**To link to this Article: DOI:** 10.1080/10826079608014007
**URL:** http://dx.doi.org/10.1080/10826079608014007

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 19(11), 1805–1818 (1996)

# FREE BILE ACID ANALYSIS BY SUPERCRITICAL FLUID CHROMATOGRAPHY AND EVAPORATIVE LIGHT SCATTERING DETECTION

V. Villette, B. Herbreteau
M. Lafosse,* M. Dreux

Institut de Chimie Organique et Analytique
URA CNRS 499
Université d'Orléans
B.P. 6759
45067 Orléans Cedex 2, France

Downloaded At: 12:55 24 January 2011

## ABSTRACT

Packed column supercritical fluid chromatography of free bile acids is described. Improvement of the separation of isomeric acids is carried out on diol-bonded silica using methanol or isopropanol as modifier of carbon dioxide. These poor UV absorbing analytes were detected by evaporative light scattering detection showing the potentiality of the SFC-ELSD coupling.

## INTRODUCTION

Bile acids are steroids with 24 carbon atoms biosynthesized from cholesterol and which occur in some biological fluids both in the free and conjugate forms. They include primary bile acids (i.e. cholic and chenodeoxycholic acids) and secondary bile acids by deshydroxylation (i.e. deoxycholic acid and lithocholic acid). The complete simultaneous separation of dihydroxylated acids is complicated by the isomeric forms due to the hydroxyl groups.



Copyright © 1996 by Marcel Dekker, Inc.

Bile acids are present in stools, mainly as free bile acids. They can also be used as therapeutic agents for the dissolution of cholesterol gallstones and in the therapy of bile reflux gastritis.

Usually the determination of free bile acids is based on reverse phase liquid chromatography with buffer due to their pKa value (about 6). Selectivity is highly dependent on the composition of the mobile phase: the nature of the buffer, the ionic strength and the pH value. Thus the limitations of this method are:

- low sensitivity in the detection by UV absorption (amount about 200 nmole).[1]
- gradient elution involves a severe drift of baseline[1] at the low wavelength used (210 nm).

A. Roda[2,3] proposed evaporative light scattering detection (ELSD) in place of UV detection in liquid chromatography. The mobile phase is nebulized by a gas stream and the aerosol droplets are carried through a heated tube to evaporate the mobile phase. The remaining particles of nonvolatile solutes pass through a light beam and the light is scattered and collected by a photomultiplier or photodiode, causing a chromatographic signal which depends on the amount of solute, but not on its nature. The limit of detection of bile acids is about 5 nmole i.e. 30-50 times lower than with UV detection[2] and permits a mass detection. However, the ammonium acetate required to give an ionic strength, needs a sufficiently high temperature to ensure complete vaporization of the mobile phase and to avoid baseline noise . The ELSD used in this work,[2,3] requires a heated temperature of 130°C which is high enough for thermodegradation with thermolabile products.

Downloaded At: 12:55 24 January 2011

Supercritical Fluid Chromatography (SFC) using packed column offers the advantage of a simple mobile phase ($CO_2$-organic modifier). The polarity of this mobile phase can be easily modified by varying the modifier content and/or by increasing the column pressure, because density and thus solvent power increases highly with an increase in pressure. This method has been used for bile acids[4] and affords an analysis time faster than that obtained by the reverse phase.[5] In this work,[4] UV detection at low wavelength and the use of methanol lead to a poor sensitivity. Moreover, only five bile acids are separated and the mechanism remains somewhat unclear.

We have proposed a new ELSD in which the temperature of the heated tube is low, thus avoiding a degradation of thermolabile compounds.[6] We also propose coupling this detector with SFC.[7] Hence, this technique appears to be a universal detector for packed column SFC.

The aim of this work is to show that the separation of seven naturally occurring free bile acids can be achieved using packed column SFC. Different parameters are studied to increase the selectivity of the separation and the sensitivity of the detection.

## EXPERIMENTAL

The SFC apparatus consisted of the Series SF3 supercritical fluid chromatographic system (Gilson, Villiers-le-Bel, France): a Model 305 piston pump (main pump) delivers the $CO_2$. Polar modifier was added by a pump Model 302 (slave pump) and both solvents were mixed in a Model 811 C mixer. The head of the Model 305 pump was cooled with a refrigerated bath.

Supercritical or subcritical conditions were maintained by a pressure regulator valve (Model 821) set at the column outlet and before a fused silica capillary, which acts as a restrictor to maintain mobile phase in a critical state before detection. This restrictor is set in the heated specific interface of the ELSD for SFC (Sedex Model 55, Touzart et Matignon, Vitry-sur-Seine, France) which allows both expansion and nebulization of the supercritical mobile phase before its vaporization in the heated tube. Ice formation at the outlet of the restrictor and condensation of the organic modifier in the interface of detector, are avoided thanks to a make-up gas.

The column was thermostated at 50°C in a Croco Cil oven (CILCluzeau, Sainte-Foy la Grande, France). Samples were injected onto the column via a Rheodyne 7125 valve fitted with a 25 µL sample loop. Chromatograms were recorded on a Shimadzu Model CR5A integrator (Touzart et Matignon, Vitry-sur-Seine, France)

Columns: Lichrosorb Diol (5 µm (150x4.6 mm) Interchrom (Interchim, Montluçon, France), Zorbax $NH_2$ and TMS (Dupont, Wilmington, DE, USA), Pecospher HS-3 Silica(Perkin-Elmer, Norwalk, Connecticut, USA). Carbon dioxide was purchased from Air Liquide (Paris, France) and methanol and isopropanol were of HPLC grade from Prolabo (Paris, France).

Bile acids were of analytical grade: lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), hyodeoxycholic acid (HDCA), hyocholic acid (HCA), cholic acid (CA). Fig. 1 illustrates two analytes: a primary acid (CA) and a secondary acid (DCA). Analytes were dissolved in methanol/dichloromethane 50:50 mixture at the concentration of 200 ppm.

Downloaded At: 12:55 24 January 2011

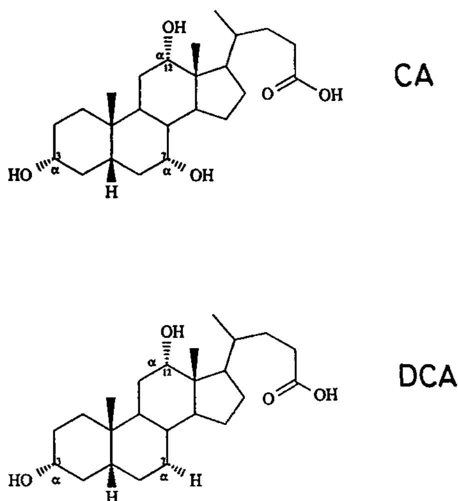



**Figure 1.** Scheme of bile acids : CA = cholic acid, DCA = deoxycholic acid.

Downloaded At: 12:55 24 January 2011

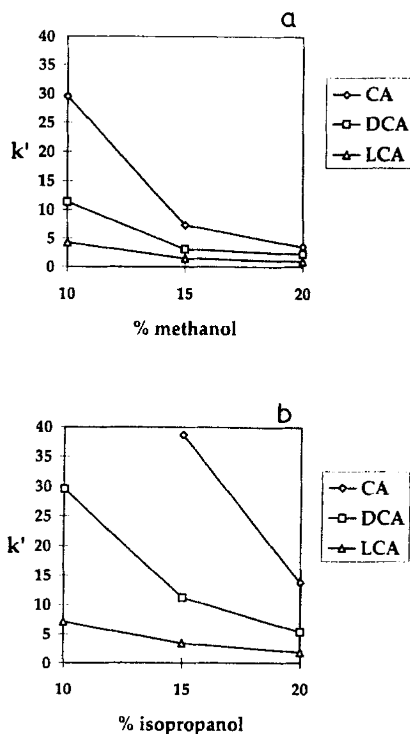
## RESULTS AND DISCUSSION

Several columns (Zorbax $NH_2$, Zorbax TMS, Pecospher Silica and Lichrosorb Diol) were studied with $CO_2$-methanol as mobile phase. Only Lichrosorb Diol affords a good shape of peaks, selectivity and easy use. Therefore, the following study gives results on this stationary phase. Analyses were carried out at the 3 mL/min flow-rate. This value is nearly the optimum value obtained by[8]

$$F_{opt}\,(\text{mL/min}) = 1.25 \times dc^2(\text{mm}^2)/dp\,(\mu\text{m})$$

where dc and dp are respectively the column diameter and the particle diameter of packing and F is the flow-rate.

The optimum value of flow-rate given by this equation is 5.3 mL/min with pure $CO_2$, but since this value depends directly on the diffusion coefficient Dm of solutes in the mobile phase,[8] the 3 mL/min value will be a good compromise since Dm decreases when modifier is added.

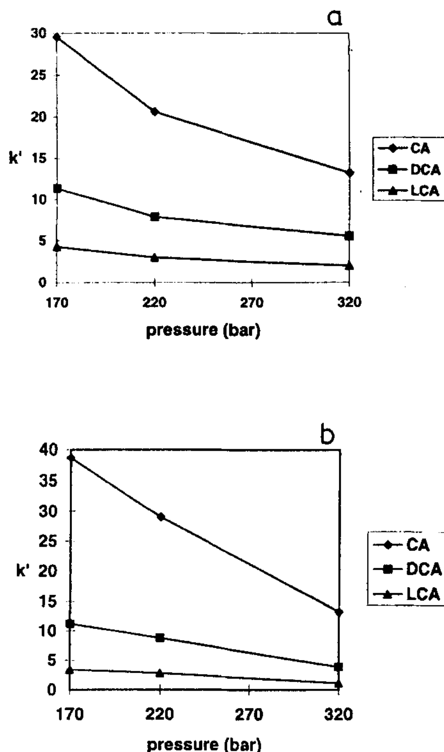



Downloaded At: 12:55 24 January 2011

**Figure 2.** Variation of capacity factors of bile acids with the percentage of modifier in the mobile phase. Column, 150 x 4.6 mm I.D. Lichrosorb Diol, 5 μm; mobile phase, (a) $CO_2$-methanol, (b) $CO_2$-isopropanol; flow-rate, 3 mL/min; inlet pressure, 170 bar; temperature, 50°C; detection, ELSD; solutes CA, DCA, LCA.

## Retention as a Function of the Modifier

Figure 2 illustrates the variation of the bile acid retention versus the modifier content with two organic modifiers: methanol and isopropanol. The capacity factors decrease with increase in the content of methanol or isopropanol, caused by interactions of the polar modifier with diol groups of stationary phase and enhancement of the solubility of analytes in the




Downloaded At: 12:55 24 January 2011

**Figure 3.** Variation of capacity factors of bile acids with the inlet pressure. Column, 150 x 4.6 mm I.D. Lichrosorb Diol, 5 μm; mobile phase, (a) $CO_2$-methanol, (b) $CO_2$-isopropanol; flow-rate, 3 mL/min; temperature, 50°C; detection, ELSD; solutes, CA, DCA, LCA.

mobile phase. In both cases, retention increases in the order monohydroxy (LCA) < dihydroxy (DCA) < trihydroxy (CA). This behaviour is usually observed in a normal phase chromatography.

When we compare both modifier effects, a similar elution strength can be noted for a 10% methanol content and 15% isopropanol content, which illustrates the higher elution strength of methanol and confirms the proposed mechanism .

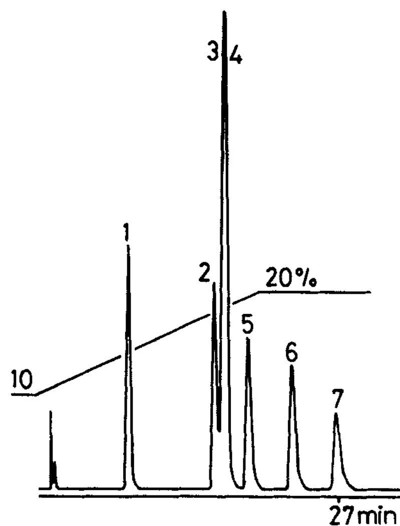



**Figure 4.** SFC chromatogram of a bile acid mixture. Column, 150 x 4.6 mm I.D. Lichrosorb Diol, 5 µm; mobile phase $CO_2$ (A) - isopropanol (B), gradient elution from A/B (90:10) to A/B (80:20) in 20 min.; flow-rate: 3mL/min; pressure 170 bar; column temperature : 45°C; solutes : 1=LCA, 2=DCA, 3=CDCA, 4=UDCA, 5=HDCA, 6=HCA, 7=CA.

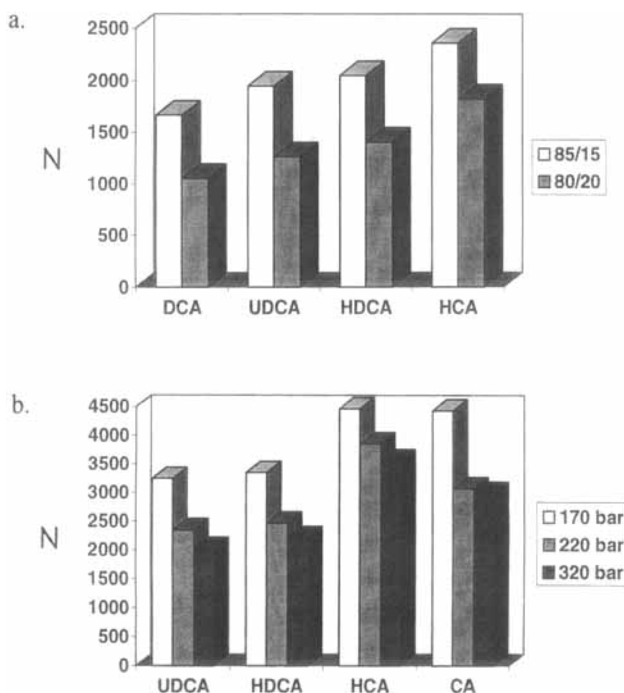
Downloaded At: 12:55 24 January 2011

## Table 1

### Selectivity as a Function of the Modifier

| % Modifier | DCA/LCA | CDCA/DCA | UDCA/CDCA | HDCA/UDCA | HCA/HDCA | CA/HCA |
|---|---|---|---|---|---|---|
| 10 | 2.65 | 1.09 | 1.27 | 1.04 | 1.67 | 1.07 |
| 15 | 2.10 | 1.09 | 1.24 | 1.00 | 1.62 | 1.07 |
| **% Isopropanol** | | | | | | |
| 15 | 3.30 | 1.13 | 1.00 | 1.34 | 1.63 | 1.40 |
| 20 | 2.94 | 1.00 | 1.00 | 1.21 | 1.52 | 1.38 |

Pressure: 170 bar.




Downloaded At: 12:55 24 January 2011

**Figure 5.** Influence of experimental conditions on column efficiency. Conditions as Fig. 2 except mobile phase and pressure. (a) mobile phase, $CO_2$- isopropanol (80:20 or 85:15, v/v); (b) mobile phase, $CO_2$-methanol (90:10), inlet presssure, 170, 220 or 320 bar.

## Retention as a Function of Pressure

As shown in Fig. 3, the retention of bile acids decreases, as pressure increases, by enhancing the density and the eluting power of the mobile phase. Comparison of both Fig. 2 and 3 shows that the influence of polar modifier on the retention on SFC packed column is higher than that of the pressure.

As an illustration of the effect of modifier or pressure on retention, Fig. 4 shows the possibility of using a gradient of modifier without baseline drift. This is a major advantage of the use of ELSD over UV detection at low wavelength which requires a high quality grade for such gradient elution.

**Table 2**

**Selectivity as a Function of Pressure**

| Pressure (Bar) | DCA/LCA | CDCA/DCA | UDCA/CDCA | HDCA/UDCA | HCA/HDCA | CA/HCA |
|---|---|---|---|---|---|---|
| **$CO_2$-Methanol 90:10** | | | | | | |
| 170 | 2.65 | 1.10 | 1.27 | 1.04 | 1.67 | 1.07 |
| 220 | 2.63 | 1.10 | 1.23 | 1.05 | 1.67 | 1.09 |
| 320 | 2.74 | 1.08 | 1.19 | 1.06 | 1.59 | 1.09 |
| **$CO_2$-Isopropanol 85:15** | | | | | | |
| 170 | 3.30 | 1.13 | 1.00 | 1.34 | 1.63 | 1.40 |
| 220 | 3.14 | 1.12 | 1.00 | 1.32 | 1.61 | 1.38 |
| 320 | 3.53 | 1.12 | 1.00 | 1.34 | 1.62 | 1.41 |

Downloaded At: 12:55 24 January 2011

## Selectivity as a Function of the Modifier

With an average efficiency of 2000 theoretical plates and capacity factors from 5 to 30, selectivity values from 1.12 to 1.10, are respectively required for a resolution of one unit. Table 1 gives the selectivities of consecutive solutes obtained using various mobile phases. It appears that the selectivity varies very weakly with the modifier content, but in contrast, it depends on the nature of the modifier.

For example, the selectivity of the couple of dihydroxyl isomers HDCA/UDCA is low (1.00-1.04) with methanol modifier, whatever the modifier content, since high selectivities are obtained (1.21-1.34) with isopropanol. In contrast, the selectivity of the two dihydroxyl isomers UDCA/CDCA is better (1.24-1.27) with methanol, than with isopropanol. The same drastic result is noted for the trihydroxyl isomers CA/HCA couple: selectivity is 1.07 with methanol and about 1.39 with isopropanol.

Resolution and sensitivity can be improved by comparing of efficiency as a function of the modifier content. As the temperature of the column was 50°C, the addition of modifier means the mobile phase is under subcritical conditions; consequently the diffusion coefficients and the efficiency are reduced in

comparison with supercritical conditions. Fig. 5a illustrates the influence of an increase of modifier content at constant pressure: since the viscosity of the mobile phase increases, the efficiency is lowered.

### Selectivity as a Function of Pressure

With $CO_2$-methanol (90:10) at 3 mL/min, a 320 bar pressure permits an analysis time of 15 min which is increased up to 30 min with 170 bar, with no changes in the separation. In fact, the selectivity is not influenced by the pressure variation as shown in Table 2. An increase in pressure at constant modifier content, increases the viscosity of mobile phase and simultaneously decreases the diffusion coefficient of solutes, lowering the efficiency as shown in Fig. 5b.

In conclusion the pressure and modifier content will be used to regulate the analysis time and the nature of organic modifier to enhance the separation parameters.

Downloaded At: 12:55 24 January 2011

### Influence of the Addition of Water in Organic Modifier

With methanol or isopropanol used as modifier, the complete resolution of the seven bile acids is not obtained. For this reason, the effect of water in polar modifier was also examined. Increasing the amount of water in alcohol results in a slow decrease in retention. However, Table 3 shows that the addition of a small amount of water in methanol as modifier increases only weakly the selectivity of UDCA/CDCA couple, whatever the pressure.

With isopropanol as modifier, the addition of water similarly increases the selectivity of UDCA/CDCA couple but, decreases drastically the selectivity of CA/HCA couple. The $CO_2$-modifier (85:15) mobile phase with 5% water in isopropanol, allows a separation of nearly all compounds, but the resolution of UDCA/CDCA couple is poor (0.67) and the analysis time is long (34min).

### Complete Resolution of Seven Bile Acids

Neither of the two modifiers allows complete separation of the seven bile acids. Results given in Table 1 show the complementarity of $CO_2$-methanol and $CO_2$-isopropanol in selectivity between dihydroxyl isomers DCA, UDCA, CDCA and HDCA.

**Table 3**

**Influence of Water Content on the Selectivity**

**$CO_2$/Modifier (90:10) 320 Bar**
**Modifier: Methanol-Water Mixture**

| % Water in MeOH | DCA/LCA | CDCA/DCA | UDCA/CDCA | HDCA/UDCA | HCA/HDCA | CA/HCA |
|---|---|---|---|---|---|---|
| 0 | 2.74 | 1.08 | 1.19 | 1.06 | 1.59 | 1.09 |
| 2 | 2.41 | 1.07 | 1.25 | 1.06 | 1.55 | 1.06 |
| 5 | 2.46 | 1.07 | 1.26 | 1.06 | 1.58 | 1.06 |

**$CO_2$/Modifier (90:10) 170 Bar**
**Modifier: Methanol-Water Mixture**

| % Water in MeOH | DCA/LCA | CDCA/DCA | UDCA/CDCA | HDCA/UDCA | HCA/HDCA | CA/HCA |
|---|---|---|---|---|---|---|
| 0 | 2.65 | 1.10 | 1.27 | 1.04 | 1.67 | 1.07 |
| 2 | 2.65 | 1.09 | 1.39 | 1.00 | 1.66 | 1.00 |
| 5 | 2.67 | 1.09 | 1.38 | 1.00 | 1.60 | 1.03 |

**$CO_2$/Modifier (85:15) 170 Bar**
**Modifier: Isopropanol-Water Mixture**

| % Water in Isopropanol | DCA/LCA | CDCA/DCA | UDCA/CDCA | HDCA/UDCA | HCA/HDCA | CA/HCA |
|---|---|---|---|---|---|---|
| 0 | 3.30 | 1.13 | 1.00 | 1.34 | 1.63 | 1.40 |
| 5 | 3.42 | 1.11 | 1.07 | 1.34 | 1.68 | 1.17 |
| 8 | 3.21 | 1.12 | 1.10 | 1.42 | 1.94 | 1.00 |

**$CO_2$/Modifier (80/20) 170 Bar**
**Modifier: Isopropanol-Water Mixture**

| % Water in Isopropanol | DCA/LCA | CDCA/DCA | UDCA/CDCA | HDCA/UDCA | HCA/HDCA | CA/HCA |
|---|---|---|---|---|---|---|
| 0 | 2.93 | 1.00 | 1.00 | 1.21 | 1.52 | 1.38 |
| 2 | 2.57 | 1.12 | 1.00 | 1.20 | 1.52 | 1.28 |
| 5 | 2.44 | 1.08 | 1.07 | 1.23 | 1.53 | 1.15 |
| 8 | 2.58 | 1.10 | 1.09 | 1.29 | 1.52 | 1.07 |

That is why a mixture of both modifiers was tried to separate these four isomeric solutes. Figure 6 shows the difficult separation of CDCA/DCA and HDCA/UDCA with methanol as modifier (Fig. 6a), the difficult separation of UDCA/CDCA with isopropanol as modifier (Fig. 6b) and the complete separation by mixing both modifiers (Fig. 6c).

Downloaded At: 12:55 24 January 2011

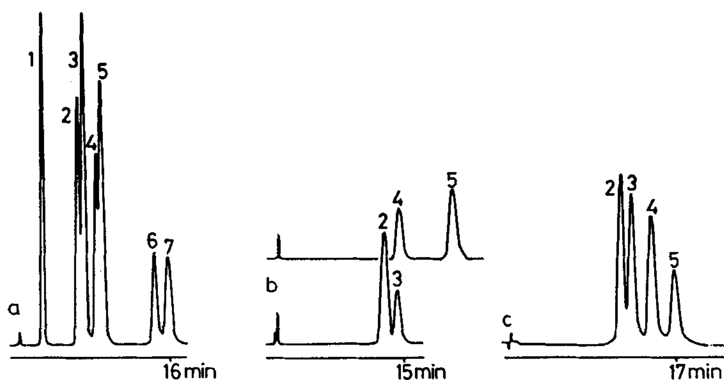



**Figure 6.** SFC of bile acids. Conditions as in Fig. 2 except (a) $CO_2$-methanol (90:10, v/v); inlet pressure, 320 bar; (b) $CO_2$-isopropanol (85:15, v/v); inlet pressure, 170 bar; (c) $CO_2$-methanol-isopropanol (90:5:5); inlet pressure : 320 bar, solutes : see Fig. 4.

Downloaded At: 12:55 24 January 2011

## Quantitative Determination of Free Bile Acid in Pharmaceutical Preparation

As determined for liquid phases, the ELSD response in SFC is nonlinear. Using logarithmic coordinates, the response exhibits a linear variation as a function of the concentration C in mg/L. For UDCA as analyte, the response is Log A = 1.70 Log C + 5.36 (r=0.999) with $CO_2$-methanol (90:10) where A is the peak area. The slope value is similar to that reported for pyrene response.[7]

This value, higher than 1, is interesting for trace component analysis[9] because both detection limit and resolution are increased. With this mobile phase the detection is 800 pmoles for a signal-to-noise ratio S/N 9.6. It becomes 200 pmoles for a similar ratio, using a $CO_2$-modifier (80:20) with a water content of 5% in isopropanol as modifier.

UDCA has been determined in pharmaceutical preparation. The content of the preparation is dissolved in methanol then filtered and injected into the chromatographic system. This rapid, easy and simple method affords a good recovery compared to the value given by the manufacturer. Moreover, owing to the good sensitivity of this quasi universal detection, Fig. 7 shows the absence in the pharmaceutical preparation of other bile acids (over 0.2 - 0.5 % relatively to UDCA) in particular LCA and DCA which are commonly present in raw material.

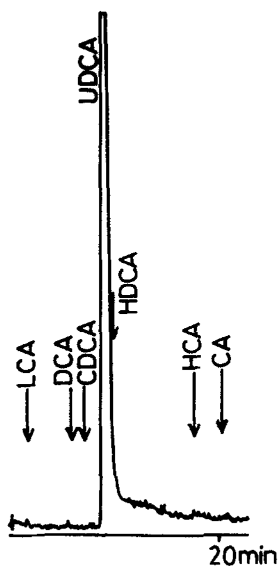



**Figure 7.** SFC chromatogram of a UDCA in pharmaceutical preparation. Column: Lichrosorb Diol, 150 x 4.6 mm; Mobile phase : $CO_2$-polar modifier (90:10), polar modifier : methanol-isopropanol (50:50); flow-rate : 5 mL/min; Inlet pressure : 245 bar, outlet pressure : 220 bar; column temperature : 60°C, nebulizing temperature : 50°C. UDCA concentration in methanol (2000 mg/l).

Downloaded At: 12:55 24 January 2011

This chromatographic method is more sensitive (about a thousand times) than UV detection and could be applied subsequently to biological samples such as stools.

## REFERENCES

1. J. H. Nichols, R. D. Ellefson, J. Hermansen, K. Schifferdecker, M. F. Burritt, J. Liq. Chromatogr., **16(3)**, 681 (1993).

2. A. Roda, C. Cerre, P. Simoni, C. Polimeni, C. Vaccari, A. Pistillo, J. Lipid Res., **33(9)**, 1393 (1992).

3. A. Roda, R. Gatti, V. Cavrini, C. Cerre, P. Simoni, J. Pharm. Biomed. Anal., **11(8)**, 751 (1993).

4. S. Scalia, D. E. Games, J. Pharm. Sci., **82(1)**, 44 (1993).

5. S. Scalia, J. Chromatogr. Biomed. Applications, **431**, 259 (1988).

6. M. Dreux, M. Lafosse, Analusis, **20**, 587 (1992).

7. P. Carraud, D. Thiebaut, M. Caude, R. Rosset, M. Lafosse, M. Dreux, J. Chromatogr., **25**, 395 (1987).

8. F. Verillon, D. Heems, B. Pichon, K. Coleman, J. C. Robert, American Laboratory, June 1992.

9. L. E. Oppenheimer, T. H. Mourey, J. Chromatogr., **323**, 297 (1985).

Received October 6, 1995
Accepted October 30, 1995
Manuscript 4010

Downloaded At: 12:55 24 January 2011