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Advantages of the use of monolithic stationary phases for modelling the retention in sub/supercritical chromatography Application to *cis/trans*-β-carotene separation

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Abstract

The low viscosity of supercritical fluids enables the coupling of columns, which favours both the high efficiency of separation and the ability of tuning the selectivity. However, it increases the inlet pressure then modifies the fluid density, i.e. the eluotropic strength of the mobile phase. In this case, the latter is rather different depending on the number of coupled columns. This fact prevents the calculation of the chromatographic parameters for coupled columns from the results obtained from one. In subcritical conditions, by using silica rod columns, which have bimodal porous structure, the flow resistance parameter is dramatically reduced. Consequently, the addition of monolithic columns induces only slight internal pressure changes and the fluid density does not vary with the column length. In this case, the calculation of retention factor and selectivity based on retention values obtained on each separate column provides accurate results allowing to determine the optimum column length in regard to the studied separation. After a better characterisation of the stationary phase included in the Chromolith column, this paper describes the β-carotene isomers separation obtained by coupling up to six Chromolith columns to an octadecyl bonded particulate one. These compounds were studied because of the difficulty to separate these *cis*/*trans* isomers. No abnormal apparent dead volume change due to fluid density variation was reported, and good correlations between experimental and calculated retention factors and selectivities were observed. The optimum separation requires five highly porous columns coupled to a YMC Pack Pro. Moreover, the use of monolith packing allows to decrease both the retention factor and the analytical time by comparison to previous studies.

Keywords: Monolithic columns; Supercritical fluid chromatography; Column coupling; Carotenes

1. Introduction

The use of supercritical fluids with packed column chromatography offers numerous advantages due to the physico-chemical properties of these fluids [1–3].

Carbon dioxide is currently the most employed fluid, for several reasons: it is inert towards the material, it is not toxic, the critical conditions are experimentally easily attained, and it is miscible to numerous polar modifiers (methanol or acetonitrile (ACN)) and to non polar modifiers (hexane), in proportions varying from 5–40% [4,5]. The supercritical state is obtained for a pressure and a temperature called 'critical'. These pressure and temperature depend on

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the chemical nature of the fluid. For pure carbon dioxide, these values are 7.3 MPa and 31 °C, requiring a counter pressure at the end of the column. Judging by the methylene selectivity (α_{CH2}), the elution strength of the CO_2–methanol and CO_2–acetonitrile mixtures is comparable to that of these two organic solvents when they are used pure in reversed phase high performance liquid chromatography [6]. This last property explains that sub/supercritical chromatography is particularly suitable for the study of hydrophobic compounds, namely: triglycerides, carotenoid pigments, ceramides, or waxes.

Besides, due to the low viscosity of the supercritical fluid, the diffusion coefficients of the compounds in the mobile phase are higher than in liquids. Therefore, the optimum of efficiency is reached for linear speeds that are higher than those observed in liquid chromatography [2,7]. Consequently, the use of flow rates of 3–5 ml min^{-1} allows to reduce the analysis time without altering the quality of the separation.

The low viscosity of supercritical fluids also makes it easier to couple packed columns. Associating columns allows to reach a high number of theoretical plates (200,000 plates per meter with a 1.2 m column) [8]. Coupling columns having stationary phases of different properties, is also possible [9]. This coupling can lead to the fractioning of complex mixtures that would be impossible or difficult to separate with either one or the other stationary phase.

This type of coupling is particularly suitable in supercritical fluid chromatography (SFC) for certain groups of isomers like carotenoid pigments [10] or polycyclic aromatic hydrocarbons (PAHs) [11,12].

However, coupling columns increases the internal pressure, particularly in the first column situated right after the injection valve. The supercritical fluids being compressible, the fluid density of the mobile phase rises [9].

This increase induces a growth of the apparent dead volume of the columns, which means that the amount of pumped fluid necessary to fill in the dead volume increases [13].

Besides, the elution strength of the fluid increases too. Consequently, when coupling two particulate columns, this double variation induces a diminution of the total retention factor [6,14]. In this case, the selectivity between two compounds cannot be calculated from the retention factors measured separately on the two supports, and the improvement of the separation is rather based on an empirical approach requiring numerous experiments [15].

The calculation of the chromatographic variables in SFC with a system of coupled columns requires the use of a chromatographic support of high permeability. This would avoid changes in the fluid density in the first column, which is a particulate one.

Lately, monolithic stationary phases have been used in HPLC and capillary electrochromatography (CEC) [16–21]. Developed from a new sol–gel process, these silica rods have a bimodal structure composed of macropores of 2 μm and mesopores of about 13 nm. Due to the presence of large through-pores, the interstitial porosity of monolithic columns is more important than the one of particulate columns. Consequently, the internal pressure of monolithic columns is about five times lower than on a Purospher RP 18e and 20 times lower than on a Capcell Pak C_{18} [16,21] allowing the use of higher flow rates on monolithic columns (up to 9 ml min^{-1}), that dramatically reduces the analysis time [18], without strongly modifying the efficiency in HPLC [16].

On the other hand, the methylene selectivity of the monolithic silica was close to the one of different particulate stationary phases: LiChrospher RP 18 [18], Purospher RP 18e [19,20], Inertsil ODS3 [21], and Capcell Pak C_{18} [16,21], around 1.5/1.6 with acetonitrile–water mobile phases, showing the likeness in the separation mechanism.

The purpose of this paper is to study the possibility of using monolithic columns for coupling in SFC, avoiding fluid density modifications in the first column set in the column chain. In this case, the calculation of k and α versus the monolithic column length could be done, enabling to choose the appropriate column length to achieve a given separation. This calculation methodology is applied to the separation of the *cis*/*trans* isomers of β-carotene.

2. Material and methods

2.1. Reagents

The solvents used were methanol (Carlo Erba, Milan, Italy) and acetonitrile (SDS, Vitry sur Seine, France) from Alphagaz (Bois d'Arcy, France).

All-*trans*-β-carotene was provided by Sigma–Aldrich. *cis*-Isomers (9 mono, 13 mono, and 15 mono) were obtained by addition of diluted iodine in a carotene solution (MeOH–CH_2Cl_2) until the three main mono-*cis* isomers (9, 13, and 15) are in satisfactory amount. After each iodine solution addition, the evaluation of the β-carotene isomerisation was done by analysing the solution with a polymeric bonded silica (Vydac 201 TP 54) with a mobile phase CO_2–MeOH (85:15).

2.2. Apparatus

Chromatographic separations were carried out using equipment manufactured by Jasco (Tokyo, Japan) described elsewhere [5,10,15]. Chromatograms were recorded using the AZUR software (Datalys, France). The chromatographic columns were octadecyl bonded silicas (250 mm × 4.6 mm i.d., 5 μm): Kromasil (TSP-Shandon, Les Ulis, France), YMC Pack Pro C_{18} (supplied by Interchim, Montlucon, France), Restek allure (Evry, France), Vydac 201 TP 54 (Grace Hesperia, USA), and Chromolith RP 18e (Merck, Darmstadt, Germany).

3. Results and discussion

3.1. Characterisation of the monolithic support in SFC

Previous studies carried out in SFC allowed to develop a test aimed at characterising C_{18} bonded silica on the basis of the separation of different carotenoid pigments and their isomers in standard conditions [22–24].

The retention factor of β-carotene gives an estimation of the hydrophobicity of the stationary phase; the selectivity between the 13-*cis* isomer and all-*trans*-β-carotene determines if the phase is monomeric (one bonded octadecyl chain per silanol) or vertically polymerized (more than one bonded chain per silanol), and, for the monomeric phases, the bonding density.

On the Chromolith column, the *cis*/*trans* selectivity is comparable to that observed on Kromasil C_{18} or Purospher RP 18e. We can conclude that the stationary phase is monomeric with high bonding density. The carbon content of 17% and the surface coverage of 3.2 μmol m^{-2} are in good agreement with this hypothesis.

However, the hydrophobicity of this phase is inferior to that of the Kromasil C_{18} or the Purospher RP 18 stationary phases, which have similar bonding density and surface area (about 300 m^2 g^{-1}).

The porosity of the Chromolith silica is 85–90% versus 60–65% for the particulate silica, which explains that the quantity of silica per unit of volume is lower for the monolithic support. Obviously, this induces a lower retention.

On the other hand, varying the percentage of polar modifier allows to evaluate possible changes in the retention behaviour of a stationary phase. The variation of β-carotene retention factor in function of the percentage of methanol is presented on Fig. 1. The variation of retention is identical on the three types of octadecyl bonded particular stationary phases: low density monomeric bonding (Hypersil ODS), high density monomeric bonding (Kromasil) and polymeric (Vydac 201 TP 54). This retention behaviour has been described elsewhere [5,6,25]. A similar variation of retention is observed with the monolithic support, the minimum being reached at a proportion in methanol of 35%. It seems that changing a particular support for a monolithic support does not affect significantly the retention behaviour studied here, i.e. the properties of the octadecyl bonded silica.

It has been shown in SFC that the three-dimensional structure of the stationary phase depends on the mobile phase adsorption onto the bonded one. Varying the percentage of modifier may then influence the selectivity between compounds having a linear (all-*trans*-β-carotene) or a bent structure (13-*cis*-β-carotene). Considering that the mobile phase adsorption onto the C_{18} bonded particular silica is greater with methanol than with acetonitrile [13], the influence of the amount of methanol on the selectivity of the isomers 13-*cis* and all-*trans* of β-carotene has been studied (Fig. 2).

As could be observed with a particular monomeric stationary phase (Kromasil C_{18}), the increase in the amount of methanol added to the carbon dioxide causes no significant variation of this selectivity on the monolithic support. The adsorption of mobile phase onto the stationary phase remains unchanged, whatever the structure of the silica skeleton.

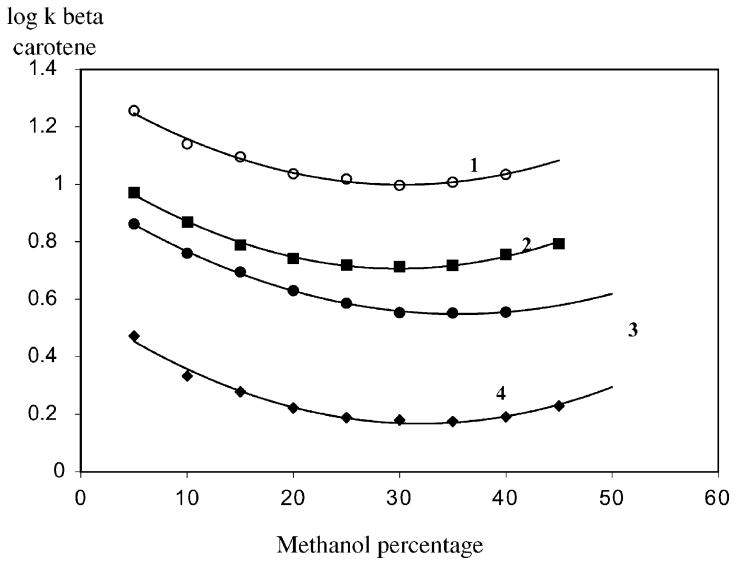

Fig. 1. Variation of the β-carotene log k vs. methanol percentage with different stationary phases. (1): Kromasil C_{18}, (2): Hypersil ODS, (3): Chromolith RP 18e, and (4): Vydac 201 TP 54.

3.2. Coupling and modelling of retention

Actually, in SFC, the separation of isomers often requires the coupling of particulate octadecyl bonded columns of varied properties [10].

Several stationary phases having different retention behaviours have been tested with a mobile phase CO_2–ACN–MeOH (85:13.5:1.5, v/v/v) in order to evaluate the complementarity of these phases with the monolithic one to obtain the separation of the *cis*/*trans* isomers of β-carotene.

On the chromatogram obtained with one of these columns (YMC Pack Pro C_{18}), four peaks can be seen, corresponding to the 9, 13 and 15-mono-*cis* isomers

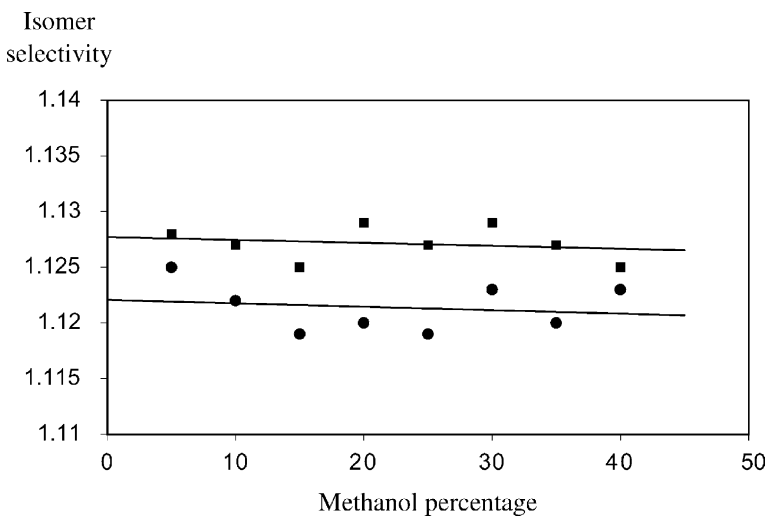

Fig. 2. 13-Mono-*cis*/all-*trans*-β-carotene selectivity vs. the percentage of methanol. Flow rate 3 ml min^{-1}, $T = 25\,^{\circ}C$, upper line: Kromasil C_{18}, and lower line Chromolith RP 18e.

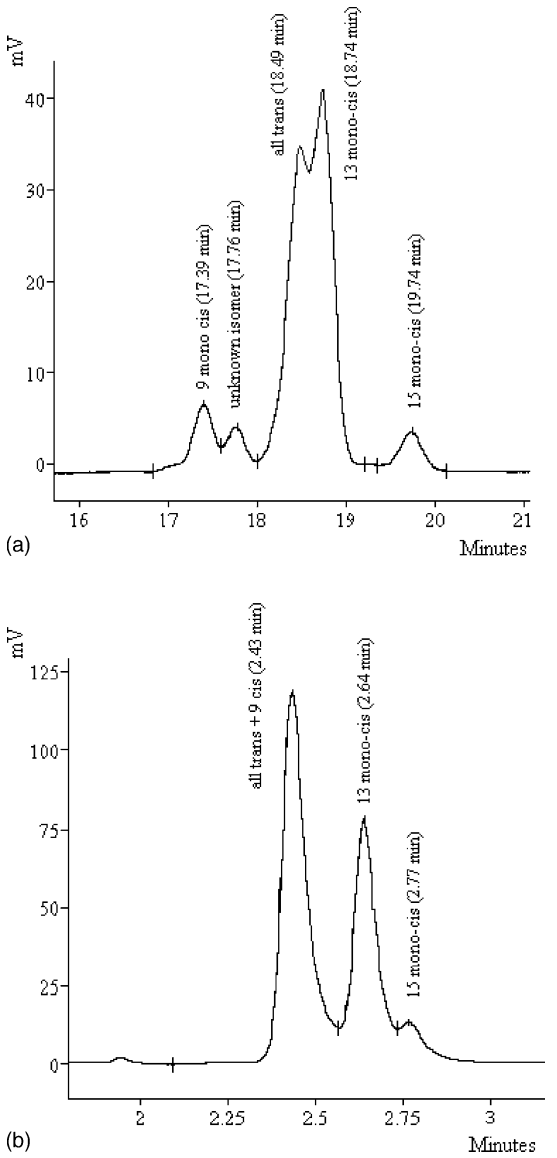

Fig. 3. Chromatograms of a β-carotene isomer mixture in SubFC. Flow rate: 3 ml min^{-1}, $T = 25\,^{\circ}C$, mobile phase: CO_2–MeOH–ACN (90:1:9, v/v/v), stationary phases: (a) YMC Pack Pro C_{18}, and (b) Chromolith RP 18e.

and to the all-*trans*-β-carotene, respectively at 17.39, 18.74, 19.74 and 18.49 min (Fig. 3a). One additional unknown isomer elutes at 17.76 min. However, the all-*trans*-β-carotene and the 13-mono-*cis* isomer are poorly resolved.

On the contrary, as can be seen on the chromatogram of this same mixture on the Chromolith column (Fig. 3b), this separation is nearly perfect, the all-*trans* and the 13-*cis* isomer being well separated (2.43 and 2.64 min), the total analysis time being inferior to 3 min.

The coupling of these two types of bonded silica should enable the association of their separating properties in order to obtain a better total separation. However, as mentioned previously, this coupling must not modify the fluid density in the first column coupled (YMC Pack Pro C_{18}), because a modification of the fluid density induces a change in the eluotropic strength leading to variations of the retention factors and of the selectivity in the first column.

Also, the high permeability of the Chromolith columns is evaluated through the evolution of the dead times (t_0), measured in function of the number of coupled columns (Table 1). The comparison of the experimental and calculated dead times shows a very good concordance between the values, at least up to five Chromolith columns (50 cm) added after the YMC column (25 cm). Because the total apparent dead volume is equal to the sum of the apparent dead volumes of each coupled column, the addition of columns induces no important fluctuation of the fluid density. The calculation of retention factors and of selectivities of coupled systems is done from the values obtained on the supports tested individually, using the following equations:

$$\alpha_{1,2} = \frac{t_{r2}\mathrm{YMC} + nt_{r2}\mathrm{Chromolith} - (t_0\mathrm{YMC} + nt_0\mathrm{Chromolith})}{t_{r1}\mathrm{YMC} + nt_{r1}\mathrm{Chromolith} - (t_0\mathrm{YMC} + nt_0\mathrm{Chromolith})}$$

$$k_{tot} = \frac{t_r\mathrm{YMC} + nt_r\mathrm{Chromolith}}{t_0\mathrm{YMC} + nt_0\mathrm{Chromolith}} - 1$$

where n is the number of Chromolith columns added to the YMC column.

Table 2 gathers the experimental and calculated retention factors of the 15-*cis* isomer, the most retained one, and the experimental and calculated selectivities between the 13-*cis* and all-*trans* isomers of β-carotene, which separation was the most difficult to achieve on the YMC support. As expected, the experimental values are in accordance with the calculated ones, due to the high permeability of the monolithic support. It should be noted that the addition of

Table 1
Experimental and calculated values of t_0 and of all-*trans*-β-carotene t_r vs. nature and number of columns

	YMC	Chromolith	YMC + 1 Chromolith	YMC + 2 Chromolith	YMC + 3 Chromolith	YMC + 4 Chromolith	YMC + 5 Chromolith	YMC + 6 Chromolith
t_0 exp	1.08	0.55	1.65	2.17	2.75	3.28	3.85	4.5
t_0 cal			1.63	2.18	2.73	3.28	3.83	4.38
t_r exp all-*trans*	18.49	2.43	20.64	23.14	25.08	28.30	30.91	33.30
t_r cal all-*trans*			20.92	23.35	25.78	28.31	30.74	33.37

Table 2
Experimental and calculated retention factors of 15-mono-*cis*-carotene and selectivity between 13-mono-*cis*- and all-*trans*-β-carotene

	k_{exp} 15-*cis*	k_{cal} 15-*cis*	α_{exp} 13-*cis*/all-*trans*	α_{cal} 13-*cis*/all-*trans*
1 YMC	17.27		1.014	
1 Chromolith	4.036		1.111	
1 YMC + 1 Chromolith	12.81	12.48	1.026	1.024
1 YMC + 2 Chromolith	10.59	10.61	1.033	1.032
1 YMC + 3 Chromolith	9.27	8.95	1.042	1.041
1 YMC + 4 Chromolith	8.39	8.46	1.049	1.046
1 YMC + 5 Chromolith	7.77	7.84	1.053	1.05
1 YMC + 6 Chromolith	7.30	7.37	1.059	1.055

Chromolith columns brings about a diminution of the retention factor of the 15-*cis* isomer (Fig. 4).

Because the retention factor obtained on the Chromolith column is four times lower than that obtained on the YMC column, the addition of the first two Chromolith columns reduces rapidly the total retention factor. Then, between the third and the fourth Chromolith, that is to say when the contribution of the Chromolith columns to the retention is proportionally equal to that of the YMC, the retention decrease becomes less important.

Variations of selectivity between the different couples of neighbouring isomers when increasing the length of Chromolith column are linear (Fig. 5).

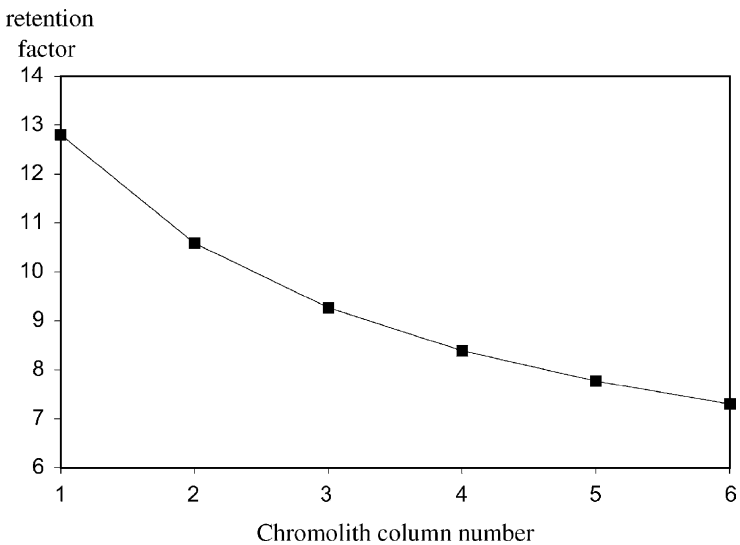

Fig. 4. Variation of the retention factor of the 15-mono-*cis*-β-carotene vs. the Chromolith column number. Analytical conditions (see Fig. 3).

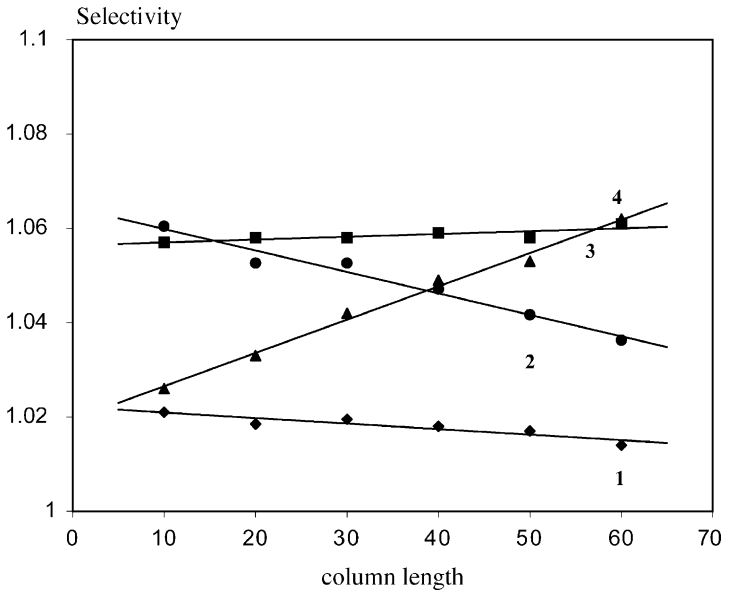

Fig. 5. Variation of the selectivity of different compound couples of β-carotene isomers vs. column length. (1): unknown isomer/9-mono-*cis*, (2): 9-mono-*cis*/all-*trans*, (3): 15-mono-*cis*/13-mono-*cis*, and (4): 13-mono-*cis*/all-*trans*. Analytical conditions: see Fig. 3.

The most important variation is obtained for the couple 13-*cis*/all-*trans*-β-carotene, which was poorly separated on the unique YMC Pack Pro C_{18} column. The addition of Chromolith columns increases this selectivity, which was the aim of this coupling. The selectivity between the isomers 15 and 13 is constant as it was nearly identical on the two types of stationary phases (1.056 on YMC and 1.062 on Chromolith). In the same manner, the selectivity between the 9-*cis* isomer and the unknown compound is nearly constant, while that between the 9-*cis* isomer and the all-*trans*-β-carotene reduces more rapidly.

These last two variations lead to a progressive diminution of the separation of the first three compounds, on the contrary to the 13-*cis* and all-*trans* isomers.

This reduction forces us to use a final length of Chromolith column of 50 cm to avoid the superposition of the first compounds. Fig. 6 shows the chromatogram obtained with one YMC column and five Chromolith ones. The total analysis time is only multiplied by two, although the total length of stationary phase is three times bigger (75 cm versus 25 cm). The improved separation between 13-*cis* and all-*trans*-β-carotene lets a minor peak appear between

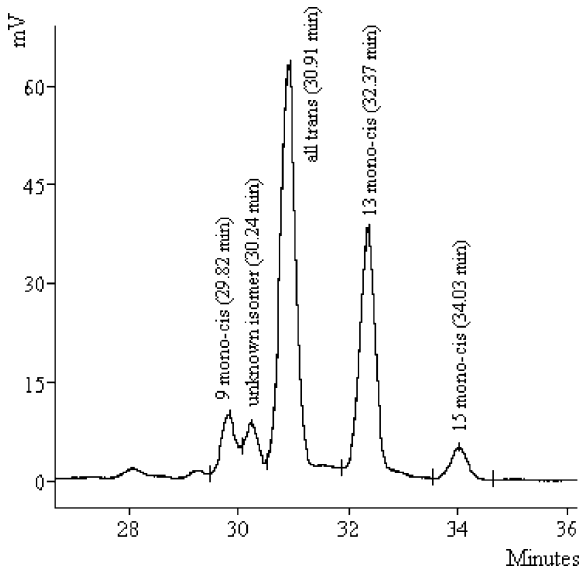

Fig. 6. Chromatogram of a β-carotene isomer mixture in SubFC by using six coupled columns (total column length equal to 75 cm). Analytical conditions: see Fig. 3, stationary phase: one particulate YMC Pack Pro C_{18} column (250 mm × 4.6 mm) followed by five monolithic Chromolith columns: (100 mm × 4.6 mm each).

these compounds. This could be the 9,15-di-*cis* isomer or the 13,15-di-*cis* isomer. Compared to the first separation (Fig. 3a), this coupling allows the separation of two more isomers of β-carotene. The separation profile obtained is complementary to a previous one obtained by coupling four particulate columns [10].

However, the analytical retention time is reduced to 35 min with the coupling including the five Chromolith columns against 50 min with the previous one.

4. Conclusion

The use of monolithic columns in subcritical fluid chromatography in standard conditions (10% of modifier, 25 °C, 15 MPa at the end of the column, and flow rate: 3 ml min^{-1}) avoids an increase of the fluid density in the chromatographic system.

Thus, in the particulate column, neither the apparent dead volume of the column, nor the elution strength of the fluid is modified by the addition of 50 cm of high permeability stationary phase. In these conditions, the calculation of the chromatographic variables k and α can be carried out to model the influence of the column length on the evolution of the separations. The rational choice of the column length to be used to obtain a given separation is then possible, based on this model. The difficulty to separate the isomers of β-carotene allowed to illustrate the precision of this modelling by comparison of the experimental and calculated values. The Chromolith support, being less retentive than many particular supports having neighbouring qualities of separation, can be coupled in important length without increasing the analysis time too much.

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