ELSEVIER

Journal of Chromatography A, 844 (1999) 307–320

JOURNAL OF CHROMATOGRAPHY A

Separation of *cis*/*trans* isomers of β-carotene by supercritical fluid chromatography

E. Lesellier*, K. Gurdale, A. Tchapla

LETIAM, IUT d'Orsay, Plateau du Moulon, 91400 Orsay, France

Received 13 October 1998; received in revised form 1 March 1999; accepted 10 March 1999

Abstract

The aim of this work was to study and improve the separation of β-carotene *cis*/*trans* isomers in SFC. Five different stationary phases have been tested, and their chromatographic behaviour has been determined. Unfortunately, because of the presence of additional isomers in the sample studied, none of the individual columns were able to isolate the four pure main isomers of β-carotene (9, 13, 15 *cis* and *trans*). The coupling of two different monomeric octadecyl columns was necessary to increase both selectivity and efficiency. The low viscosities of supercritical mobile phases allowed this column association. The influence of temperature, pressure, nature and modifier content were also investigated. Finally, with the optimum chromatographic conditions, six additional isomers have been separated from the four previous ones. One of them has been identified as the 9–9′ di-*cis* β-carotene.

Keywords: Stationary phases, SFC; β-Carotenes

1. Introduction

The increasing interest in the analysis of carotenoid pigments, due to their biological properties, has stimulated development of chromatographic separation methods. As their antioxidant or anticarcinogenic properties are mainly due to the *cis*/*trans* isomerisation of the numerous double bonds, a considerable effort is needed to separate these isomers. Indeed, they only differ by the position (on carbons 7, 11, 13 and 15) or by their *cis* isomerisation number (mono-, di- or tri-*cis*) [1,2].

Two separation techniques were mainly used: high-performance liquid chromatography and sub- or supercritical fluid chromatography. In each case, the influence of the stationary phase seems to play a leading part in the *cis*/*trans* isomer separation, when the role of the mobile phase is more important for the separation of different pigments, like luteine/zeaxanthine or α/β-carotene [1].

Both inorganic polar adsorbents and apolar reversed-phase packings (RP) have been used. In spite of an attractive separation obtained with an alumina column by Vecchi et al. [3], the analysis time of the separation and the restriction resulting from the necessary low range of water in the mobile phase have favoured the utilization of silica and lime adsorbents.

Silica has permitted the separation of some oxygenated carotenoids like acitrecin [4], a second-generation retinoid used as a medicine for skin diseases, or astaxanthin [5], a pigment present in some shellfish species (shrimp, lobster) or fish (salmon).

*Corresponding author.
E-mail address: lesellier@iut_orsay.fr (E. Lesellier)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00364-7

Tsukida et al. [6] were the first to show the separation of 17 compounds produced by thermal isomerisation of β-carotene, among which the 7, 9, 13 and 15 mono-*cis* isomers on lime. Chandler and Schwartz [7] have also used this adsorbent to analyse β-carotene isomers in plants. More recent studies have shown the effect of the addition of *p*-methyl anisole to the hexane–acetone mixture generally employed as an eluent for increasing the peak symmetry [8,9]. With these chromatographic conditions, coelution of 13 mono-*cis* β-carotene and *trans* β-carotene would occur in classical samples (such as carrots and human plasma).

Using a lime column, the separation of a non-cyclic carotene, such as neurosporene, could be improved, by changing the eluent to a hexane–benzene mixture [10]. Unfortunately, lime columns are not commercially available, and require a long time to stabilize the activity of the adsorbent.

The control of the support activity is not so crucial for the bonded stationary phases used in reversed-phase high-performance liquid chromatography.

Octadecyl (C_{18}) chains are necessary to obtain satisfactory separation of *cis*/*trans* isomers, but numerous researchers have also emphasized the importance of the kind of bonded silica (monomeric or polymeric) [11–13] to obtain this separation.

Indeed, *cis* isomerisation leads to a bent spatial configuration of the pigment which is more significant when the isomerisation position is located near the symmetry center of the molecule (15 or 15′ position). Thus, the three-dimensional organization of the C_{18} polyfunctional bonded silica permits a shape selectivity of these isomers. Based on the idea of a shape separation, a C_{30} polyfunctional column has been synthetised, and the results obtained exhibit a greater potential for the separation of linear and bent molecules [14,15].

Other studies have been achieved by sub/supercritical fluid chromatography (SFC), using octadecyl bonded stationary phases [1,16–22]. The noticeable points of SFC in comparison with HPLC are a consequence of the properties of the sub/supercritical mobile phase, which is often CO_2. A low viscosity, a different solvating power and a high diffusion coefficient enable fast and effective separation. Once more, for octadecyl chains, the kind of bonded silica strongly influences the quality of isomer separation (monomeric or polymeric). A greater selectivity is also obtained with polymeric bonded phases. However, the use of monomeric bonded phases, with a high density (3–4 $\mu mol/m^2$), permits to separate some isomers of β-carotene by SubFC, in the presence of modifiers such as methanol or acetonitrile in the mobile phase. Thus, like in HPLC, monomeric and polymeric phases exhibit different selectivity for the separation of bent forms of the β-carotene isomers in SFC [19,21].

Obviously, because the separation of the *cis*/*trans* isomers of β-carotene is dependent on the type of support used, some studies have achieved the characterization of the reversed stationary phase with these compounds [20–22]. Lesellier and Tchapla have set up a simple chromatographic test based on the subcritical analysis of three carotenoids: zeaxanthine, and *trans* and 13-*cis* β-carotene [21,22]. Concerning the type of bonded silica (mono or polymeric), this test provides results that are correlated with the TbN/BaP selectivity [23].

Thus this work is aimed at the investigation of the possibilities of the separation of β-carotene *cis*/*trans* isomers by sub/supercritical chromatography. For this purpose, different octadecyl bonded phases have been compared, including a polymeric stationary phase grafted with triacontane (C_{30}) chains. After selection of the reversed phase, the mobile phase composition, the temperature and the outlet pressure have been studied.

2. Experimental

2.1. Chemicals

HPLC-grade methanol, acetone, methylene chloride were purchased from SDS (Peypin, France), acetonitrile from Merck (Darmstadt, Germany) and heptane from Carlo Erba (Rodano, Italy). Carbon dioxide (N45-grade, containing <7 ppm water) was purchased from Alphagaz (Bois d'Arcy, France).

All *trans* β-carotene, 9, 13, 15 mono-*cis* β-carotene, 9–9′, 13–15 and 9–15 di-*cis* β-carotene isomers were kindly provided by Hoffman La Roche (Basel, Switzerland).

The isomerisation of β-carotene was carried out in a methylene chloride solution by adding previously

dissolved iodine. Two isomer solutions were obtained. The degree of isomerisation and the isomer quantity are linked to the quantity of iodine added.

2.2. Apparatus

Supercritical fluid chromatography was done using the equipment manufactured by Jasco (Tokyo, Japan). The two pumps (Model 880-PU) were connected to a pulse damper (Sedere, Touzart et Matignon, Les Ulis, France). The head of the pump used for carbon dioxide was cooled to −2°C by a cryostat (Julabo F10c, Seelbach, Germany). The pulse damper was connected to an injection valve (Rheodyne 7125, Cotati, CA, USA) fitted with a 20-μl loop. The column was thermostated in a controlled oven (Crocosil, Cluzeau, Ste. Foy-la-Grande, France), regulated by a cryostat (Haake, D8GH, Karlsruhe, Germany). Detection was performed with a Jasco MD 910 UV-Visible diode array detector built with a high pressure-resistant cell. The eluent was discharged via an automatic back-pressure regulator (Jasco, Model 880-81). Chromatograms were recorded at 450 nm, using an electronic integrator CR6A (Shimadzu, Kyoto, Japan). The flow rate of the mobile phase was 3.0 ml/min.

Spectra were recorded in the UV and visible domains between 195 and 500 nm. One point of measure was done each 2 nm. For the identification of the 9, 13, 15 mono-*cis* and the β-all-*trans* carotene using different columns, the spectra of the standard compounds were collected and compared to the spectra of the isomerized β-carotene solution. The comparison was done using a normalisation between the spectra by the least-squares method.

To assess the peak purity, the comparison of spectra recorded in the increase, the decrease and at the absorbance maximum was done.

Liquid chromatography was performed using a quaternary pump (PU4100, Unicam, Cambridge, UK), an injection valve (Rheodyne 7125, Cotati, USA) and a diode array detector PU 4121, connected to a PC-compatible computer using the PU 6003 software (Unicam, Cambridge, UK).

2.3. Columns

Three columns contained octadecyl chains.

(1) Monomeric bonded silica (Hypersil ODS; 150×4.6 mm I.D.; pore size, 120 Å; surface area, 170 m^2/g; carbon content, 9–10%, Shandon, Runcorn, UK);

(2) Heavily loaded monomeric bonded silica (Ultrabase UB 225; 250×4.6 mm I.D. and 150×4.6 I.D.; pore size, 100 Å; surface area, 340 m^2/g; carbon content, 19%; bonded density, 3.4 μmol/m^2, Shandon). It should be noted that Ultrabase silica is another name for Kromasil silica (Eka Nobel, Bohus, Sweden). Thus, the results obtained in this paper with Ultrabase can be reproduced with Kromasil column.

(3) Polymeric bonded silica (Vydac 201 TP 54; 250×4,6 I.D.; pore size: 300 A°; surface area: 90 m^2/g; carbon content: 8%, The Hesperia group, Santa Clara CA, USA).

A polymeric triacontane (C_{30}) column (YMC; 250×4.6 mm I.D., Wilmington NC, USA), and a silica coated by a polymer bonded with octadecyl chains (γbond C_{18}; 250×4.6 mm; ES Industrie, Berlin, USA) completes the supports.

3. Results and discussion

Preliminary HPLC analysis was performed prior to SFC, using the C_{30} column and the optimum conditions for the β-carotene *cis*/*trans* isomer separation described by Emenhiser et al. [24].

This experiment was done both to identify the main mono-*cis* isomers and to assess the purity of the chromatographic peak. The chromatogram obtained displays the separation of the 9, 13, 15 mono-*cis* β-carotene and all-*trans* β-carotene isomers (Fig. 1). With the high isomerised solution we used, a poor resolution occurs for the 13 and 15 mono-*cis* β-carotene, because one unknown compound is co-eluted both with the 13 and the 15 *cis* β-carotene, whereas another is coeluted with the 13 *cis* β-carotene. These unknown compounds could be two different di-*cis* β-carotene isomers, as the injection of standards has showed a partial coelution of the 13–15 di-*cis* and the beginning of the 13 mono-*cis* peak, and of the 9–15 di-*cis* and the end of the 13 mono-*cis* peak.

These results confirm that with these analytical

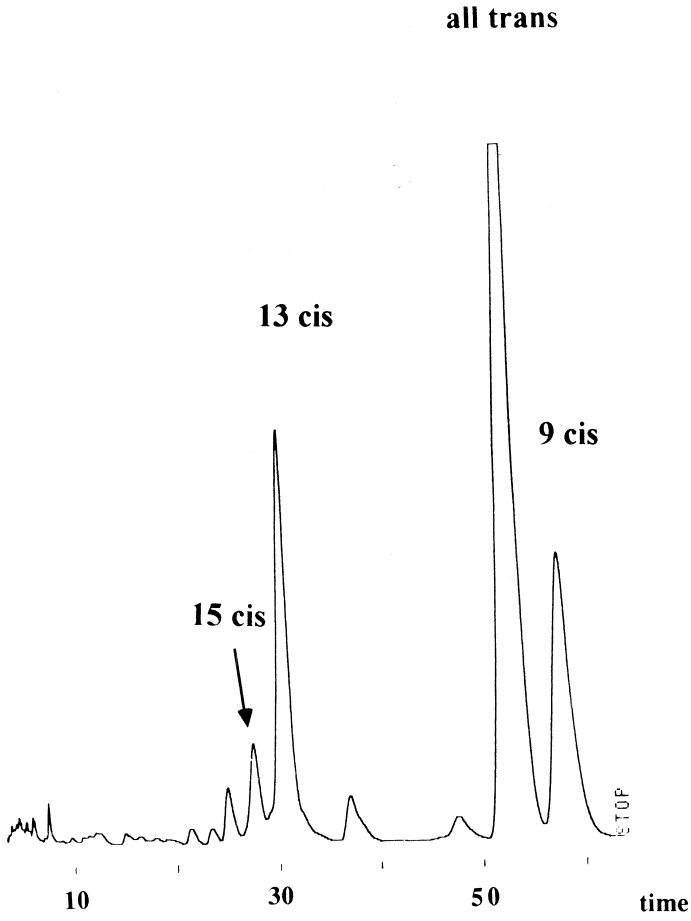

Fig. 1. Chromatogram of isomerized β-carotene separated by HPLC with a C_{30} polymeric bonded silica. Column: YMC 30; mobile phase, methanol–MTBE (89:11, v/v); flow-rate, 1.0 ml/min; detection, 450 nm.

conditions, it seemed difficult to properly quantify these isomers with the C_{30} support by HPLC.

Thus, we have developed analyses by supercritical fluid chromatography (SFC) to investigate the ability of SFC to separate the four β-carotene isomers (9, 13, 15 mono-*cis* and *trans*) and additional isomers.

3.1. Influence of the temperature using different bonded silicas

As it was shown that the separation of *cis*/*trans* isomers of β-carotene was strongly dependent on the temperature and on the octadecyl stationary phase, we first studied the temperature effect using five different reversed stationary phases.

The chromatograms, recorded under the same analytical conditions, show many changes of the elution profile on the different columns. Sometimes, coelutions or inversions in the retention order of the solutes were observed (Fig. 2).

3.1.1. Octadecyl bonded silicas

At room temperature, for the four octadecyl columns (Hypersil, Ultrabase, Vydac and γ-bond C_{18}), the elution order for the *cis* isomers is the same (9 mono-*cis* then 13 mono-*cis* and 15 mono-*cis* β-carotene) for each column, but the position of the all-*trans* form versus the mono-*cis* isomers is modified. These separation are generally encountered using mono- or polymeric bonded silicas [21,22].

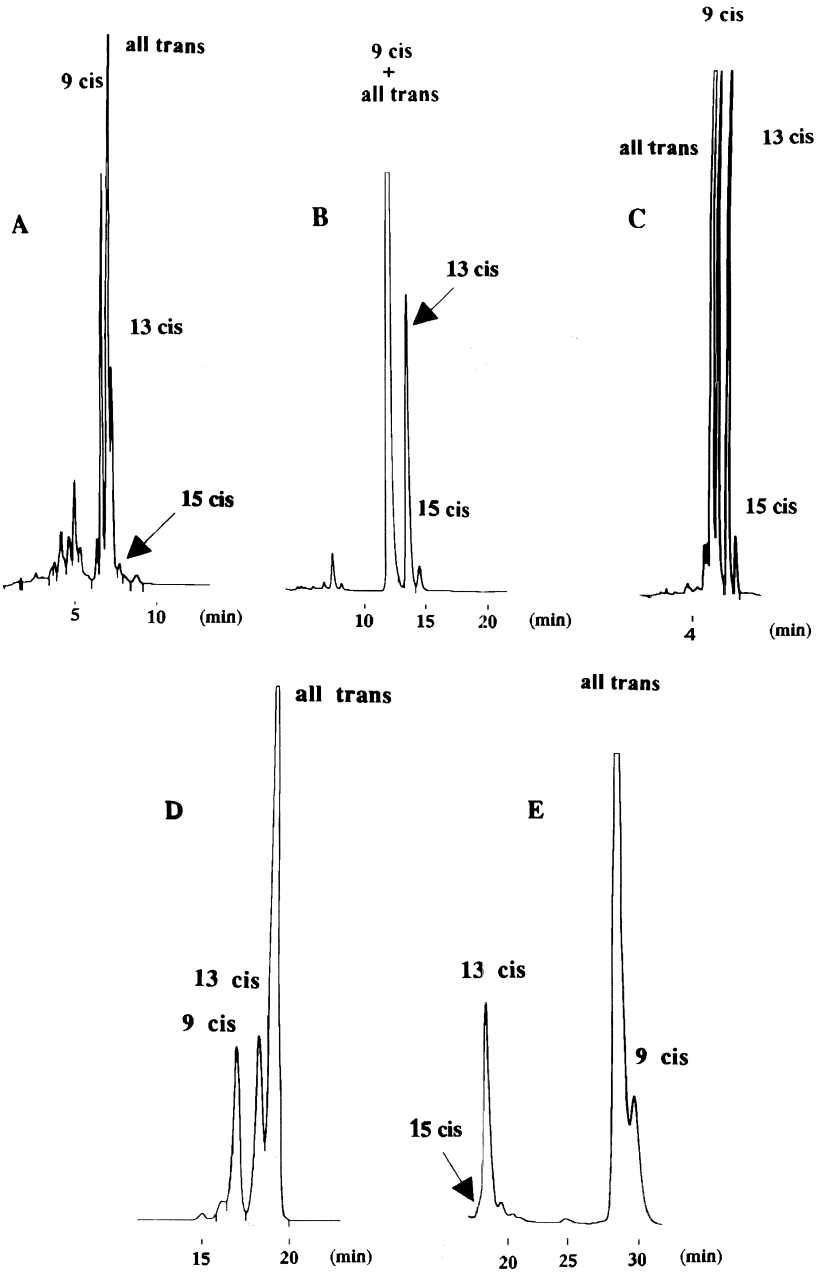

Fig. 2. Subcritical chromatography of isomerized β-carotene. Mobile phase: CO_2–methanol (85:15, v/v); outlet pressure, 15 MPa; temperature, 25°C; flow-rate, 3.0 ml/min; detection, 450 nm. Columns: (A) Hypersil ODS; (B) Ultrabase UB 225; (C) Vydac 201 TP 54; (D) Gammabond C_{18}; (E) YMC 30.

Considering the three pure octadecyl bonded silicas, the 9 mono-*cis* is eluted before the *trans* β-carotene on the Hypersil ODS column, presents the same retention time on the UB 225 column and is more retained than the all-*trans* β-carotene on the Vydac column. Following this column evolution, the

15 mono-*cis*/13 mono-*cis* selectivity is also increased.

These results show that the increase in the phase density between the monomeric phases (changing from the Hypersil to the UB 225) leads to the increase in the relative retention of the mono-*cis* isomers (based on the all-*trans* retention).

The same behavior is observed when the nature of the stationary phase is charged from monomeric to polymeric phases (passing from UB 225 to Vydac 201 TP 54), suggesting that the discrimination of the β-carotene isomers increases as a function of the tightness of alkyl bonded chains.

It has been reported that the higher the graft density of octadecyl-bonded supports, the more difficult the penetration of both rigid and high-molecular mass compounds into the bonded phase is, i.e. when an isotropic phase is replaced by an anisotropic (ordered) one [25]. However, as some of the non-linear molecules (13 and 15 mono-*cis* isomers) are more retained than the linear compounds, this retention order cannot be explained by the slot model developed by Sander and Wise for the PAH [23].

Fig. 3 shows the modifications of the selectivity of the *cis*/*trans* isomers of β-carotene selectivity due to temperature changes. Because the *trans* β-carotene is take as reference, its selectivity value is equal to 1 whatever the column and the temperature. For the isomers eluting later than the *trans* form, this value is greater than 1, and for the ones eluting earlier, it is lower than 1.

For the three octadecyl-bonded silicas studied, the temperature increase leads to a decrease of the retention time. The shift is greater for the *cis* isomers than for the all-*trans* one.

These results are in agreement with previous reports that the modification of the retention of *cis*/*trans* β-carotene isomers that modification occurs when the temperature changes [11,16,20]. Overall isomer resolution decreases with increasing temperature.

At high temperature, the penetration of all isomers into the stationary phase is believed to be easier, but that of the linear all-*trans* isomer is greater than that of the non-linear mono-*cis* isomers. Thus, the relative retention of mono-*cis* isomers and their separation decrease. Because the ability of the compound to penetrate into the stationary phase depends on the rigidity of the bonded chains, showing that the rigidity of the bonded alkyl chains can modify the shape selectivity for *cis*/*trans* β-carotene isomers. Thus, when the column temperature increases, this bonded chain rigidity decreases, which allows the penetration of the compounds inside the stationary phase [26,27].

On the contrary, for a lower temperature, the penetration of carotenes into the bonded phase is restricted, which permits a shape discrimination with a more external contact [19,21].

3.1.2. Triacontyl bonded silica

The retention order of the β-carotene isomers on the C_{30} polymeric bonded silica is identical in SFC and in HPLC, and, as reported by Emenhiser et al. [24], it is identical to the one obtained on calcium hydroxide in HPLC [8,9] (Fig. 2).

Using this triacontane phase, increasing temperature in SFC (until 50°C) leads to a drastic change in the retention, because, as for C_{18} supports, the 9 mono-*cis* isomer becomes less retained than the all-*trans*. However, the retention order between the bent compounds (13 and 15 mono-*cis*) and the most linear ones (9 mono-*cis* and all-*trans*) appears to be unaffected by the temperature modification.

To conclude on the influence of the type of stationary phase, it can be emphasized that the separation orders described in SFC at room temperature for the four previous apolar stationary phases are similar to those observed in NARP liquid chromatography. This can strengthen the idea that these separation mechanisms are the same when the mobile phase is composed of usual solvents or when it is mainly composed of CO_2. On the other hand, an increase in temperature allows a fluctuating penetration of carotene, and reduces the separation capacity between the different isomers. This conclusion concerning the influence of the temperature on compound penetration is opposite to that proposed by Pursh et al. [28]. They described a penetration mechanism at lower temperature for the same kind of results obtained for the separation of vitamin A acetate on polyfunctional C_{30} bonded silica.

3.2. Determination of analytical conditions

Even if interesting and particularly good separations are obtained with the polymeric YMC 30

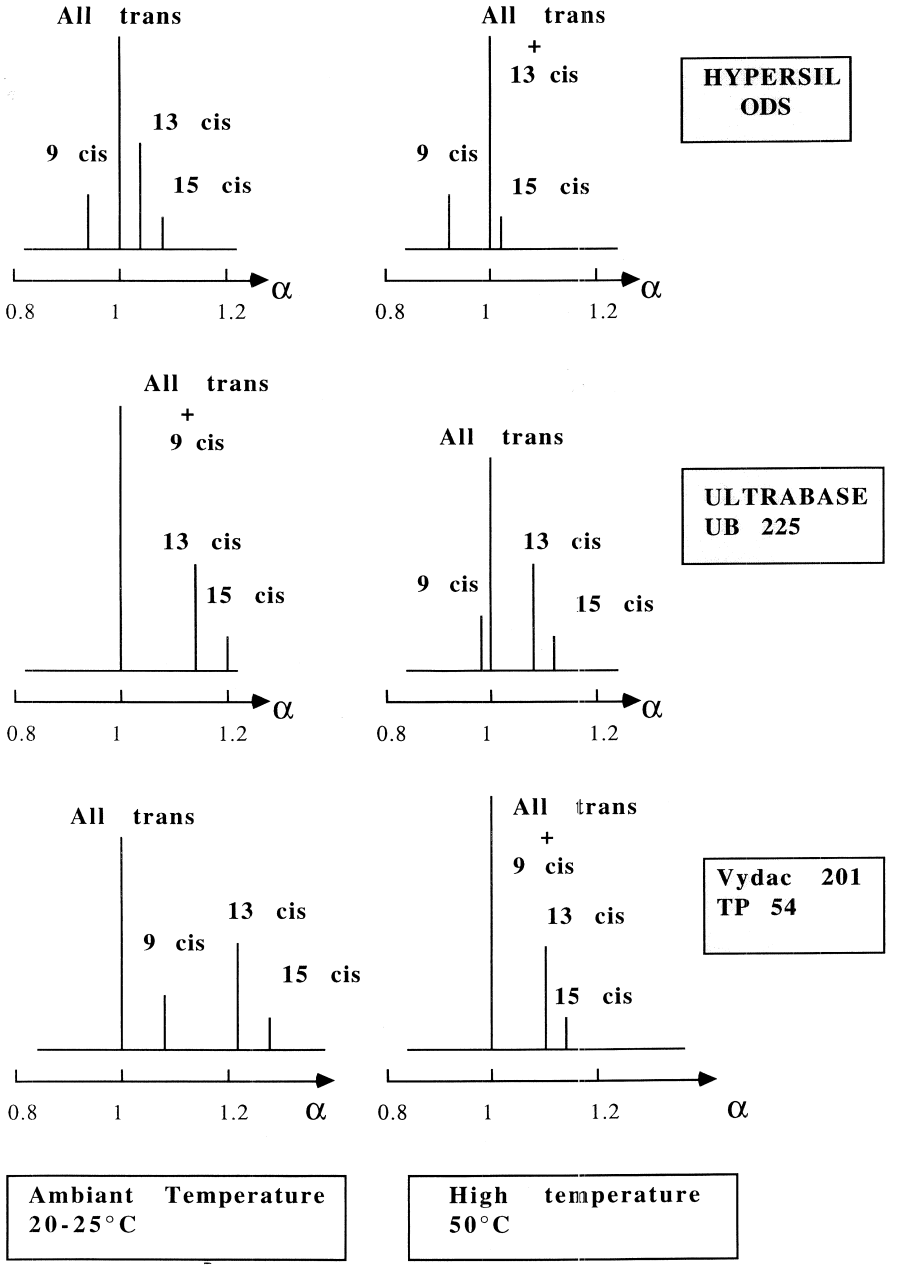

Fig. 3. Variation of the selectivity of the *cis*/*trans* isomers of β-carotene at two temperatures for different stationary phases. Mobile phase: CO_2–methanol (95:5, v/v); outlet pressure, 10 MPa.

silica, it has not been possible in SFC to completely separate the four main isomers of β-carotene (all-*trans*, 9, 13 and 15 mono-*cis*) (Fig. 2).

Consequently, in order to select a suitable octadecyl bonded silica, preliminary studies have been carried out mainly with the UB 225 column to investigate the chromatographic conditions. This column was chosen because it was the intermediate nature of octadecyl bonded phases studied.

The influence of the temperature (between 20 and

Table 1
Variation of different selectivity versus analytical conditions in sub- or supercritical fluid chromatography. Column: Ultrabase UB 225; flow-rate, 3.0 ml/min; detection, 450 nm

Selectivity	All trans/ 9 cis	13 cis/ all trans	15 cis/ 13 cis
T° ↗ (5% of modifier)	↗	↘	↘
P ↗ (T > 35°C)	↘	↗	↗
% modifier ↗ (T > 35°C)	↘	↘	↘

55°C), the outlet pressure (from 10 to 18 MPa) and the percentage of modifier have been assessed. Table 1 displays selectivity variations for three pairs of compounds: all-*trans*/9 mono-*cis*; 13 mono-*cis*/all-*trans*; 15 mono-*cis*/13 mono-*cis*. As previously expected, the all-*trans*/9 mono-*cis* selectivity increases, whereas the 13 mono-*cis*/all-*trans* and 15 mono-*cis*/13 mono-*cis* selectivities decrease with increasing temperature.

Similarly, a decrease of the outlet pressure leads to selectivity variations identical to the one observed with a temperature increase. As the mobile phase density is determined by the outlet pressure, a density modification may involve an aliphatic chain rigidity change, thus explaining the effect of pressure. Increasing the modifier percentage reduces slightly the three studied selectivities. Thus the separation between the 9 mono-*cis* and the all-*trans* is lost. As the other two separations (13 mono-*cis*/all-*trans* and 15 mono-*cis*/13 mono-*cis*) were greater, they are almost unaffected.

The 9 mono-*cis*/all-*trans* separation is more difficult to achieve. Therefore, the choice of analytical conditions should be made mainly to increase this separation. The modifier percentage and the outlet pressure will be low (less than 10% and equal to 10 MPa), and the temperature high (above 40°C).

3.2.1. Choice of octadecyl stationary phase

The most suited support for the separation of the main four isomers of β-carotene is the polymeric Vydac 201 TP column. Two 25-cm columns were necessary to obtain satisfactory separation of the previous isomers owing to the low carbon content of these columns.

Unfortunately, increasing more the column length (until 80 cm), changing mobile phase composition (percentage and nature of the modifier in carbon dioxide), temperature and pressure, have not allowed the separation of the four isomers from other compounds which were produced during the iodine isomerisation.

This has led us to combine different columns, using the specific properties of each of them. The YMC 30 column was not selected because the elution order was very different from that on the other columns (Fig. 2). First, the 13 and 15 *cis* isomers were eluted earlier than the *trans* and the 9 mono-*cis*, and secondly, 15 mono-*cis* was eluted earlier than 13 mono-*cis*.

Similarly, the γ-bond was not selected because of the complete inversion of the mono-*cis* isomers retention order (9, 13 and 15). In these cases, coupling of the YMC-30 or γ-bond columns with a classical C_{18} or with each other should lead to coelution of numerous isomers.

Based on the retention order, two types of associations were used: (1) Vydac 201-UB 225 and (2) Hypersil ODS-UB 225.

For the first selected association, Vydac-UB 225, the retention order was close for the two columns. The C_{18} polymeric bonded silica allowed to separate the 9 mono-*cis* and the all-*trans* β-carotene. The 13 mono-*cis*/15 mono-*cis* separation increases with the high phase density of the C_{18} monomeric bonded silica. However, as reported for the use of the coupled Vydac columns, the presence of isomers other than the four previously noted, always led to coelution of at least one of them.

For Hypersil ODS-UB 225, it was expected that the first column would allow to separate the 9 mono-*cis* from the all-*trans*, the 9 mono-*cis* being less retained, and the separation between the 13 mono-*cis* and the 15 mono-*cis* would increase with UB 225. In addition, as reported in Fig. 3, especially for the UB 225 column, some changes in initial analytical conditions, such as an increase of the temperature, can cause an increase of the separation between the 9 mono-*cis* and the all-*trans* β-carotene. In this case, the separation order with the UB 225 column was identical to that of the Hypersil ODS column.

3.2.2. Nature of the modifier

The choice of the nature of the modifier has been investigated with the coupled columns Hypersil-ODS and UB 225, using a β-carotene iodine highly isomerised solution, with a modifier content equal to 5%. Four organic solvents were tested because they presented either similar (methanol, ethanol) or quite different properties in SFC (methanol, acetonitrile,

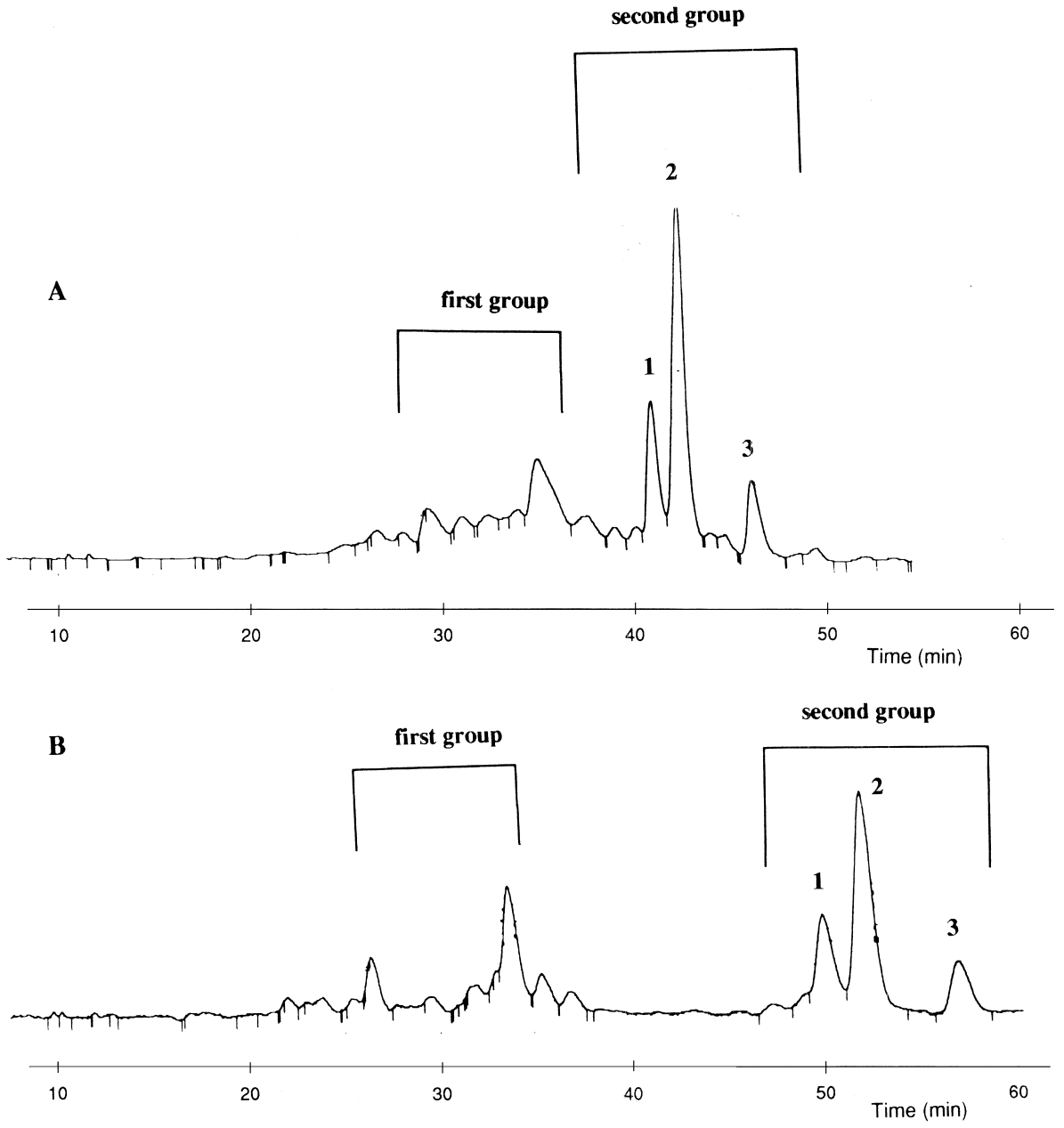

Fig. 4. Chromatogram of a highly isomerised β-carotene solution using different organic modifiers in CO_2. Mobile phase: CO_2–modifier (95:5, v/v); P outlet, 10 MPa; $T°=50°C$. Columns: UB 225 (25 cm)-Hypersil ODS (25 cm)-UB 225 (25 cm). (A) Acetonitrile; (B) methanol; (1) 9 *cis* β-carotene; (2) all-*trans* β-carotene; (3)13 *cis* β-carotene.

methylene chloride) [18]. Two of them gave satisfactory isomer separation: methanol and acetonitrile (Fig. 4). With these solvents, the chromatograms present two groups of peaks, the main four isomers being located in the second one. If the separation of the isomers does not seem to depend strongly on the nature of the modifier [19], both the analysis time and the two-group separations are greater with methanol.

The latter information has led us to conclude that the first group was composed of oxygenated carotenoids produced during iodine isomerisation.

Thus, a mixture of these two organic solvents was selected as a modifier, because acetonitrile allows to reduce the analysis time and methanol improves the separation of the two groups.

3.3. Final optimization

The last step in the isomer separation has been performed in two ways: an increase of efficiency and an increase of selectivity. Three UB 225 columns were connected to one Hypersil ODS in the goal to improve efficiency. This represents 80 cm in length and allows to obtain 50 000 plates/m. Fig. 5 shows a chromatogram obtained with the four-column system. In the second group, nine peaks can be distinguished. Five of them were unidentified, but they might be β-carotene isomers.

Numerous assays have been carried out with the four-column system, in the range of values of temperature, pressure, percentage and nature of modifiers defined previously. Two modifier mixtures were tested: 90:10 and 80:20 acetonitrile–methanol (v/v), three percentages of modifier (5, 6 and 7%) and four temperatures ranging between 40 and 55°C. We could not obtain values lower than 10 MPa for the outlet pressure owing to a considerably noisy baseline.

Thus, as our goal was to separate most of the

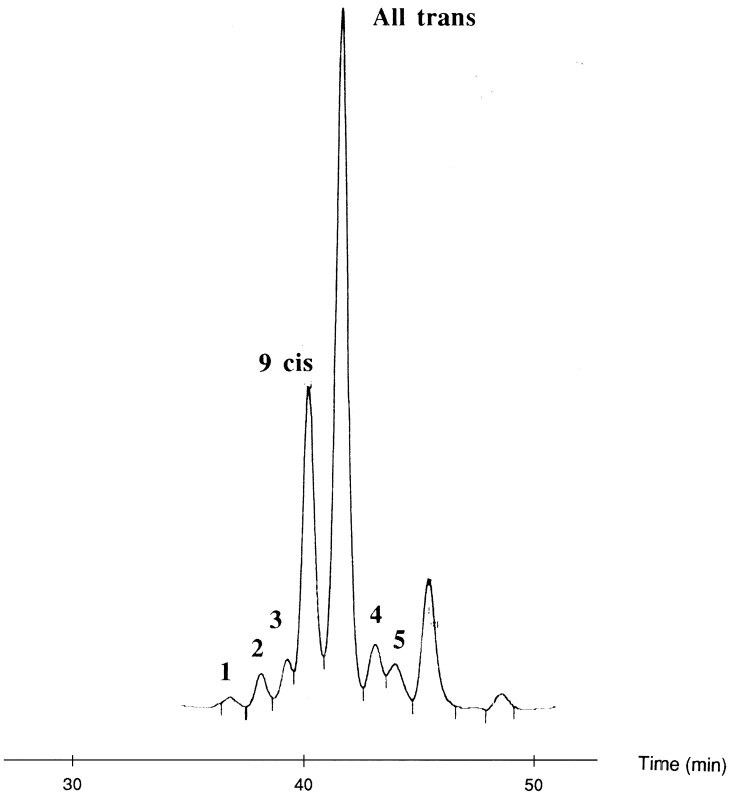

Fig. 5. Assignment of the peaks before the final optimization using the d_o criteria.

Table 2
Values of d_o and of the three criteria values used for the determination of optimal conditions[a]

Conditions	d_o 2/3	d_o 3/9 *cis*	d_o 9 *cis*/ *trans*	d_o *trans*/4	d_o 4/5	First criterion d_o average	Second criterion d_o average/ tr *trans*	Third criterion tr *trans* (min)
1	0.462	0.197	0.761	0.292	0.5	0.442	1.029×10^{-2}	43
2	0.581	0.396	0.871	0.111	0.565	0.505	9.8×10^{-3}	51.5
3	0.625	0.434	0.841	0.188	0.589	0.535	1.02×10^{-2}	52.5

[a] Experimental conditions: four columns: UB 225 (4.6×250)-Hypersil ODS (4.6×250)-UB 225 (4.6×250)-UB 125 (4.6×150); outlet pressure, 10 MPa. (1) $T°$=45°C; mobile phase, CO_2–ACN–MeOH (94:5.4:0.6, v/v/v). (2) $T°$=50°C; mobile phase, CO_2–ACN–MeOH (95:4:1, v/v/v). (3) $T°$=50°C; mobile phase, CO_2–ACN–MeOH (95:4.5:0.5, v/v/v).

β-carotene isomers, the six worst separated peaks have been taken into account to determine the optimum for each chromatographic parameter. The calculation of the discrimination factor d_o was used to achieve this determination [29]. For unsatisfactory peak separation, this factor d_o allows to assess the separation quality between two peaks, without a complete separation.

The discrimination factor d_o is equal to $(h_p-h_v)/h_p$, where h_p stands for the height of the smallest peak and h_v stands for the height of the valley between the two peaks. If the resolution is total, h_v is

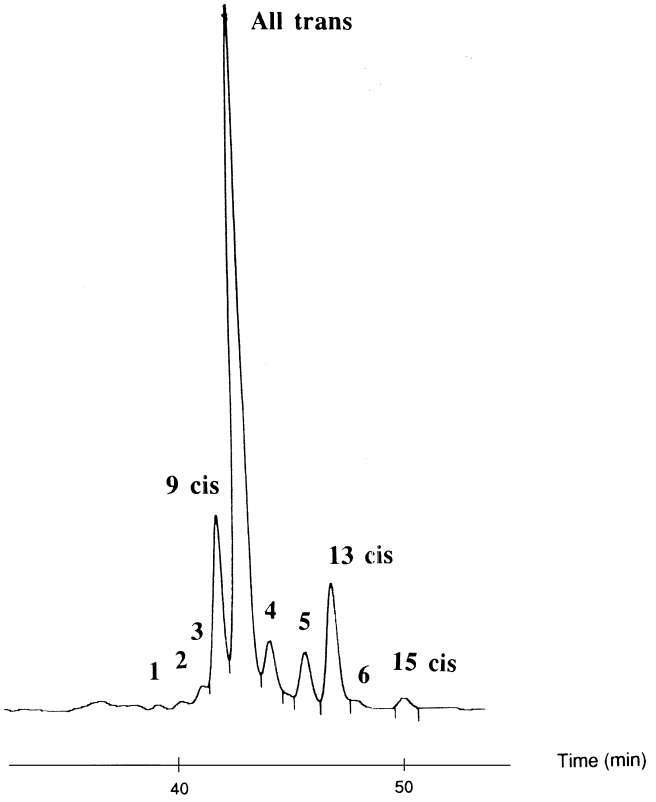

Fig. 6. Supercritical separation of a highly isomerized β-carotene solution under optimal conditions. Columns: UB 225 (25 cm)-Hypersil ODS (15 cm)-UB 225 (25 cm)-UB 225 (15 cm). Mobile phase: CO_2–acetonitrile–methanol (94:5.6:0.4, v/v/v). Outlet pressure, 10 MPa; temperature, 45°C; flow-rate, 3.0 ml/min; detection, 450 nm.

equal to 0 and d_o is equal to 1. This calculation was only realised for the pairs of partially separated peaks: 2/3; 3/9 mono-*cis*; 9 mono-*cis*/all-*trans*; all-*trans*/4 and 4/5.

To select the optimum conditions, three criteria have been successively used. The first one is d_o average. The second one is the d_o average divided by the analysis time of the all-*trans* β-carotene. This second criterion can better evaluate the separation reported by a time unit [30]. The third one is the retention time of the all-*trans* β-carotene.

Following these criteria, three trials have been selected (Table 2), because they presented the higher d_o values. Then the lowest d_o of these three analyses were compared: 0.197 (analysis 1), 0.111 (analysis 2) and 0.188 (analysis 3), and are not obtained for the same pair of peaks. The second analysis was eliminated as it got the worst d_o.

Finally, after a comparison between analyses 1 and 3, the first one was selected because the retention time of the all-*trans* β-carotene was only 43 min against 52.5 min for the third one. Fig. 6 shows the chromatogram of the second group, containing the β-carotene isomers, obtained with the selected analytical conditions. Then, with these conditions, two more compounds can then be detected, the first one between the peaks at 44.1 and 45.7 min, and the second one after the 13 mono-*cis* isomer. The overall analysis is carried out in 50 min.

3.4. Compound identification

The identification of different β-carotene isomers has been made by injection of standard molecules. During the analysis of these standards, their UV-Visible spectra were recorded. Thus the identification of the compounds present in the iodine isomerised solution was achieved by comparing both the retention time and the spectra with the one of the standards.

The all-*trans* β-carotene and the 9, 13 and 15 mono-*cis* isomers were identified at the beginning of the analyses. For the other compounds, di-*cis* isomer standards were analysed, added, alone or mixed, to the isomerised mixture (Fig. 7). The chromatographic results showed that the 9–9′ di-*cis* has the same retention time as peak 2. Since the spectra, recorded by the diode array detector, for peak 2 and the di-*cis* standard were very close, peak 2 is probably this di-*cis* isomer.

The retention time of the 9–15 di-*cis* isomer is identical to that of peak 4, but in this case, the spectra are different, and do not prove that peak 4 is that of the 9–15 di-*cis*. The 13–15 di-*cis* isomer is eluted between the all-*trans* β-carotene peak and peak 4. However, for the isomerised solution, the very low absorption of the baseline between all-*trans* β-carotene and peak 4 does not reveal the formation of the 13–15 di-*cis* isomer during iodine isomerisation (Fig. 6).

4. Conclusion

Regardless of the stationary phase used, the complete separation of the *cis*/*trans* β-carotene isomers remains difficult to carry out. In HPLC, as in SFC, coelution of 13 mono-*cis* and 15 mono-*cis* occurred with the C_{30} *polymeric bonded silica*.

In SFC, satisfactory separation is obtained by combining different stationary phases. Two C_{18} monomeric bonded phases were selected, each of them providing a specific increase in the separation.

Moreover, some analytical conditions have permitted to obtain the same retention order for the two selected columns. Thus, the separation of the four classical isomers has been carried out in 50 min.

No coelution with the 13 mono-*cis* and the 15 mono-*cis* isomers has been detected. Numerous other isomers have been separated, among which the 9–9′ di-*cis*. The identification of others still has to be done.

Following this work, studies should be done with other important carotenoids: lycopene, α-carotene or β-cryptoxanthine.

Acknowledgements

The authors gratefully thank Dr. B. Ludwig (Hoffmann La-Roche, Basel) for providing β-carotene isomers.

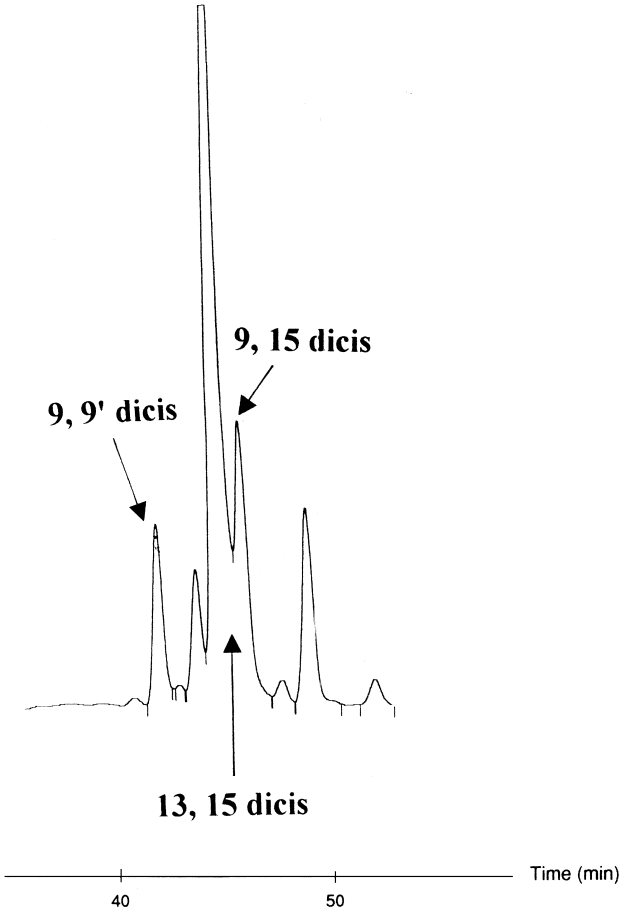

Fig. 7. Chromatogram of an isomerized β-carotene solution spiked with three di-*cis* β-carotene isomers. Analytical conditions as in Fig. 6.

References

[1] E. Lesellier, A. Tchapla, C. Marty, A. Lebert, J. Chromatogr. 633 (1993) 9–23.
[2] C. O'Neil, S.J. Schwartz, J. Chromatogr. 624 (1992) 235–252.
[3] M. Vecchi, G. Englert, R. Maurer, V. Medun, Helv. Chim. Acta 64 (1981) 2746.
[4] E. Meyer, W.E. Lambert, A.P. De Leenheer, J. Chromatogr. 570 (1991) 149–156.
[5] M. Vecchi, E. Glinz, V. Meduna, K. Schiedt, J. High Resolut. Chromatogr. 10 (1987) 348–351.
[6] K. Tsukida, K. Saiki, T. Takii, Y. Koyoma, J. Chromatogr. 245 (1982) 359–364.
[7] L.A. Chandler, S.J. Schwartz, J. Food Sci. 52 (1987) 669–672.
[8] H.H. Schmitz, S.J. Schwartz, G.L. Catignani, J. Agric. Food Chem. 42 (1994) 2746–2750.
[9] H.H. Schmitz, C. Emenhiser, S.J. Schwartz, J. Agric. Food Chem. 43 (1995) 1212–1218.
[10] N. Katayama, H. Hashimoto, Y. Koyama, T. Shimamura, J. Chromatogr. 519 (1990) 221–227.
[11] E. Lesellier, C. Marty, C. Berset, A. Tchapla, J. High Resolut. Chromatogr. 12 (1989) 447–454.
[12] K.S. Epler, L.C. Sander, R.G. Ziegler, S.A. Wise, N.E. Craft, J. Chromatogr. 595 (1992) 89–101.
[13] K. Jinno, Y. Lin, Chromatographia 41 (1995) 311–317.
[14] L.S. Sander, K. Epler, N.E. Craft, S.A. Wise, Anal. Chem. 66 (1994) 1667–1674.
[15] C. Emenhiser, L.S. Sander, S.J. Schwartz, J. Chromatogr. A 707 (1995) 205–216.
[16] M.C. Aubert, C.R. Lee, A.M. Krstulovic, E. Lesellier, M.R. Pechard, A. Tchapla, J. Chromatogr. 557 (1991) 47–58.

[17] E. Lesellier, A. Tchapla, M.R. Pechard, C.R. Lee, A.M. Krstulovic, J. Chromatogr. 557 (1991) 59–67.
[18] E. Lesellier, A.M. Krstulovic, A. Tchapla, Chromatographia 36 (1993) 275–282.
[19] E. Lesellier, A.M. Krstulovic, A. Tchapla, J. Chromatogr. 641 (1993) 137–145.
[20] E. Lesellier, A. Tchapla, A.M. Krstulovic, J. Chromatogr. 645 (1993) 29–39.
[21] E. Lesellier, A. Tchapla, Poster A6, SEP 95, Lyon, 1995.
[22] E. Lesellier, A. Tchapla, in: C. Berger, K. Anton, J. Stubenrauch (Eds.), Packed Column Supercritical Fluid Chromatography, ch. 7, Marcel Dekker, New York, 1997.
[23] L.C. Sander, S.A. Wise, LC·GC 5 (1990) 377.
[24] C. Emenhiser, G. Englert, L.C. Sander, B. Ludwig, S.J. Schwartz, J. Chromatogr. A 719 (1996) 333–343.
[25] D.E. Martire, R.E. Boehm, J. Phys. Chem. 91 (1987) 2433.
[26] J.F. Weeler, T.L. Beck, S.J. Klatte, L.A. Cole, J.G. Dorsey, J. Chromatogr. A 656 (1993) 317–333.
[27] A. Tchapla, S. Heron, E. Lesellier, H. Colin, J. Chromatogr. 656 (1993) 81–112.
[28] M. Pursch, S. Strohschein, H. Handel, K. Albert, Anal. Chem. 68 (1996) 386–393.
[29] M.Z. El Fallah, M. Martin, Analusis 16 (1988) 241.
[30] A. Tchapla, Analusis 20 (1992) M 71.