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Separation of carotenoids by subcritical fluid chromatography with coated, packed capillary columns and neat carbon dioxide

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Abstract

Different packed capillary columns were evaluated for carotenoid separation by subcritical fluid chromatography with neat CO_2. Packed capillary columns studied involved the use of silica particles deactivated with different methods and particles coated with stationary phases commonly used in gas chromatography, such as SE-54 (95% methyl-, 5% phenylsilicone) and OV-17 (50% methyl-, 50% phenylsilicone). In order to select the appropriate chromatographic conditions to elute carotenoids, a theoretical study of the solubility of β-carotene was performed. Separation of lycopene and β-carotene was obtained with neat CO_2 working at pressures around 300 atm and at a temperature of 10°C (1 atm=101 325 Pa). Evaluation of the columns used in the present study in terms of efficiency and activity was also performed.

Keywords: Capillary columns; Carotenoids

1. Introduction

Carotenoids are pigments naturally found in many different foods such as fruits and vegetables and artificially added in many other foodstuffs as coloring agents. These compounds have also potential anticarcinogenic and provitamin A activities [1,2].

High-performance liquid chromatography (HPLC) analysis of these compounds requires previous sample preparation usually based on laborious extraction methods with numerous steps [3]. Supercritical fluid extraction (SFE) has been suggested as an alternative method for selective isolation of carotenoids in one step and in conditions in which isomerization or degradation of these pigments are avoided. By using this technique, carotene and lutein extracts from leaf concentrates have been obtained at 40°C and pressures ranging from 300 to 700 atm [1] (1 atm= 101 325 Pa). From the analytical point of view SFE is compatible with supercritical fluid chromatography (SFC) because the two techniques can share the mobile phase as well as some instrumental devices like pumps and injection valves which may favor the development of on-line coupled extraction and separation methods. However, the elution strength of pure carbon dioxide (CO_2) with respect to carotenes is weak and, in order to increase their solubility in CO_2, addition of cosolvents in low concentration is usually employed [2,4–7].

SFC with packed columns has been proposed for carotenoid separation [4,5] but binary and even ternary mixtures CO_2-modifiers have been required for their elution.

The use of modifiers for improving separation of compounds in SFC has some limitations related to the complexity of the equipment that must be

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employed and the type of detector that is compatible with the modifiers selected. Also, the separation mechanisms are much more difficult to understand and to extrapolate to other compounds of similar structure.

CO_2 density is highly sensitive to pressure and temperature changes and in some conditions its solubility strength can be high enough to dissolve compounds of high molecular mass. The approach of tuning the polarity of the stationary phase as a way to improve the SFC separation using neat CO_2 has been widely studied [8–12] for different kind of compounds and, in many cases, using packed capillary columns [13,14]. These type of columns show high efficiency and a reasonable sample loadability and are easy to prepare with a wide variety of packings.

The goal of the present investigation was to evaluate the performance of different packed capillary columns for the separation of carotenoids by SFC with neat CO_2. The packed capillary columns studied involved the use of silica particles deactivated with different methods and particles coated with stationary phases typically used in gas chromatography (GC).

In a previous work [15] micropacked columns showed excellent characteristics for SFE–SFC on-line coupling. Based on these results, the present study is presented as the first step towards the development of an on-line SFE–SFC method for carotenoid analysis in foodstuffs with packed capillary columns.

2. Experimental

2.1. Samples

For efficiency measurements, a mixture of pure *n*-alkanes (C_8–C_{30}) (Sigma, St. Louis, MO, USA) was used. For activity evaluation, a mixture of anthracene, fluoranthene, methyl benzoate, menthol, 2,6-dimethylaniline, benzoic acid and 2,3-butanediol (Sigma) was utilized. Lycopene and β-carotene were used as test sample of carotenoids (Sigma). Hexane and methylene chloride used to prepare the solutions were purchased from LabScan (Dublin, Ireland). Carotenoid solutions were maintained at 4°C in dark glass flasks.

2.2. Instrumentation

A SFC system (Carlo Erba, Milan, Italy) equipped with a flame ionization detection system was used. Sample was loaded by a time-controlled, rotating-valve injection device (Vici, Houston, TX, USA) containing a 1-μl internal loop. The injector temperature was changed depending on the sample analyzed. When carotenoid mixture was used a temperature of 10°C was maintained, while for working with the other test mixtures a temperature of 45°C was utilized. Detector temperature was kept at 350°C. SFC grade CO_2 (Liquid Carbonic, Madrid, Spain) was pumped by using a SFC300 pump (Carlo Erba). The flow-rate of the mobile phase was set by using a linear restrictor of 25 cm×13 μm I.D. made of fused-silica tubing (Composite Metal Services, Worcestershire, UK) connected to the column through a zero dead-volume union prepared with an epoxy resin. The columns were connected to the injection valve via a flow splitter. Conditions of analysis are detailed in the Figure captions.

2.3. Columns

Packed capillary columns were prepared according to a reported procedure [13] by using 180 μm I.D. tubing (Composite Metal Services) of different lengths. The packing procedure was performed at an starting pressure of 80 atm followed by a pressure rate of 3 atm min^{-1} up to 240 atm. The tubing was introduced into a sonication bath maintained at room temperature. Once filled, the column was allowed to depressurize overnight. The columns were conditioned prior to their use in SFC using a pressure and temperature program as follows: from 80 atm (20 min) to 300 atm at 3 atm min^{-1} and from 80°C (20 min) to 200°C at 2.5°C min^{-1}.

2.4. Deactivation and/or coating of silica particles

Porous silica particles (10 μm, 60 Å, Hichrom, Reading, UK) were used as base material. Particles were washed with ethanol prior to deactivation and

dried by heating at 160°C for 1 h in a fluidized bed reaction vessel [8] using helium as a purge gas.

2.5. Silica particles deactivation

Prior to deactivation, the silanol groups of the silica particles were dehydrated by heating at 250°C for 2 h in a He fluidized bed.

Deactivation was obtained by using D4 (Fluka, Buchs, Switzerland), an octamethyl-cyclotetrasiloxane reagent which structure can be broken at high temperature, forming $O–Si–CH_3$ bonds with the silica particles. Five ml of a mixture of D4–pentane (50:50) was placed in the reaction vessel described above and was allow to mix with 0.4 g of silica particles in bubbling helium for 1 h. Reaction was subsequently performed at 330°C for 12 h.

2.6. Silica particles coating with GC stationary phases

Silica particles (0.3 g) were placed in the fluidized bed reaction vessel and mixed with 3% (w/w) of SE-54 (95% methyl-, 5% phenylsilicone, Supelco, Bellefonte, PA, USA) dissolved in hexane. Dicumyl peroxide (DCUP) was used as crosslinking agent at a concentration of 0.5 mg DCUP/100 mg stationary phase [16]. Coating was performed at room temperature with He as purge gas. Crosslinking was obtained by placing the vessel in a chromatographic oven by heating from 50°C to 160°C at 5°C min^{-1} and maintaining the temperature at 160°C overnight.

The same procedure was used for coating silica particles with 10% (w/w) of OV- 17 (50% methyl-, 50% phenylsilicone, Carlo Erba) and crosslinking using DCUP as the crosslinking agent.

3. Results and discussion

3.1. Column efficiency and surface activity

Before studying the use of the columns for carotenoid analysis, column efficiency and activity were evaluated. Efficiency was measured by separation of an *n*-alkanes solution at a constant pressure of 120 atm and at a constant temperature of 80°C. The expected efficiency depends on the quality of the packing procedure and on the type of material packed. In some cases, the efficiency can be low when the packing material aggregates forming larger particles. The columns studied in the present work included a 50 cm×180 μm column with 10 μm particles coated with 10% OV-17 and crosslinked with 0.5 mg DCUP/100 mg stationary phase, a 35 cm×180 μm column with 10 μm particles coated with 3% SE-54 and crosslinked with 0.5 mg DCUP/100 mg stationary phase, and a 50 cm×180 μm column with 10 μm particles deactivated with D4. The average values obtained for the three columns studied ranged from 20 000 to 30 000 plates/m at $k'=2$ and 6, respectively. Fig. 1 shows the separation of the *n*-alkanes ($C_8–C_{30}$) solution obtained in the D4-deactivated column and in the column containing particles coated with 10% OV-17.

When a modifier is added to the CO_2 to increase its polarity and its solvating strength for polar solutes, the molecules of the modifier interact with the active sites of the column reducing the effect of the surface activity in the separation. However, when neat CO_2 is used the risk of surface activity increases and the behavior of the columns in terms of activity is very important for separating compounds of different polarity. The activity was studied by separating a test mixture containing compounds of different polarity that have different specific and non-specific interactions with either the mobile and the stationary phase. The deactivation of the residual silanol groups in the particles surface by using the procedures described above favor the separation of compounds of different polarity by using neat CO_2. Fig. 2 shows the chromatograms obtained by injection of the test mixture in each of the three columns studied. As can be seen, anthracene and fluoranthene, compounds with non-specific interactions, elute with symmetrical peak shapes in the three columns. The compounds that have strong interactions, such as alcohols (menthol, 2,3-butanediol), and amines (2,6-dimethylaniline) are much more affected by the type of deactivation. If the deactivation is not complete, these polar solutes can interact with the residual silanol groups resulting in tailing and increased retention. Comparing the chromatograms shown in Fig. 2 with the results obtained with non-deactivated C_{18} columns and neat CO_2 (Ref. [17]), it can clearly be seen that the

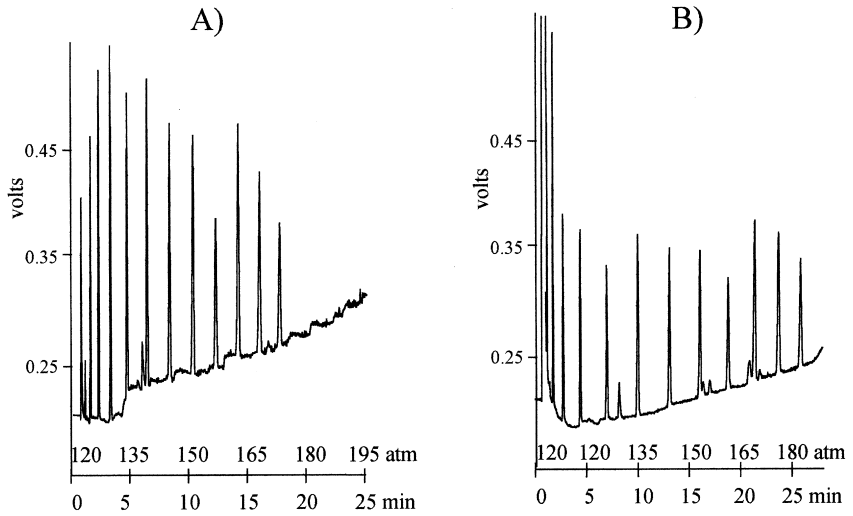

Fig. 1. SFC chromatograms of the *n*-alkanes solution (C_8–C_{30}). (A) 50 cm×180 μm I.D. 10% OV-17 coated silica particles (10 μm), 80°C, 120 atm to 240 atm at 3 atm min^{-1}; (B) 50 cm×180 μm I.D. D4 deactivated silica particles, 80°C, 120 atm (5 min) to 240 atm at 3 atm min^{-1}.

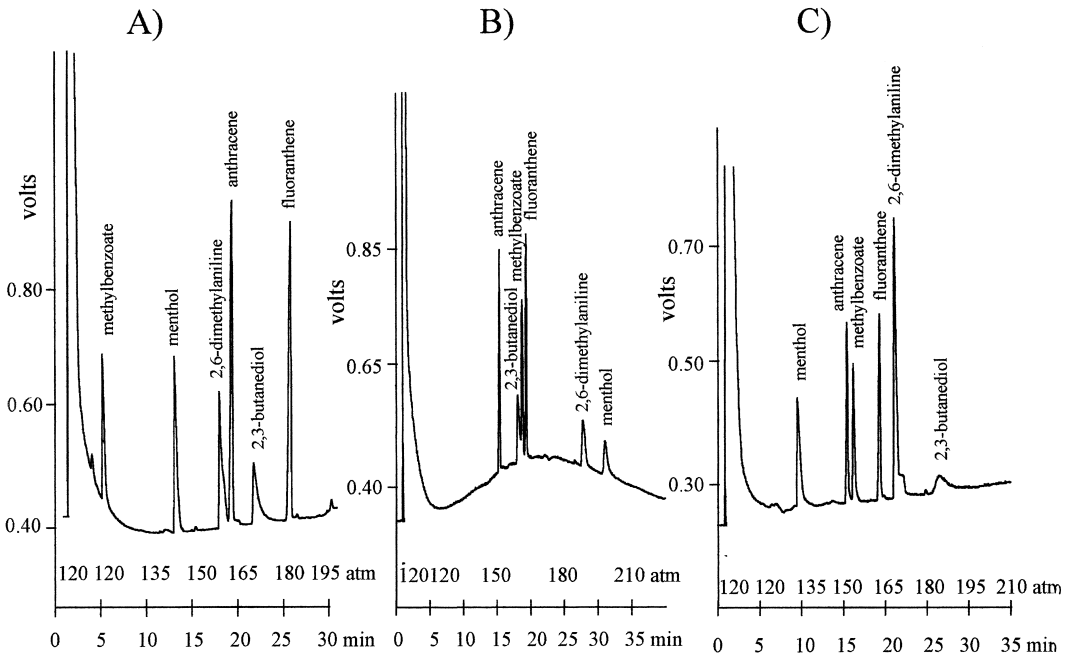

Fig. 2. SFC chromatograms of the test mixture containing methyl benzoate, menthol, 2,6-dimethylaniline, anthracene, 2,3-butandiol and fluoranthene in the three columns studied. (A) 50 cm×180 μm I.D. 10% OV-17 coated silica particles (10 μm), 120°C, 120 atm (5 min) to 240 atm at 3 atm min^{-1}; (B) 35 cm×180 μm I.D. 3% SE-54 coated silica particles (10 μm), 80°C, 120 atm (5 min) to 240 atm at 3 atm min^{-1}; (C) 50 cm×180 μm I.D. D4 deactivated silica particles, 80°C, 120 atm (5 min) to 240 atm at 3 atm min^{-1}.

columns studied in the present investigation offer the advantage of the separation of the polar compounds with, in most cases, only slight peak tailing. Menthol, aniline and even diol can be eluted in all cases although some peak tailing can be seen for 2,3-butanediol with the column deactivated with D4. By working with neat CO_2 and deactivated columns, benzoic acid was not eluted at all indicating that the deactivation was not complete and interactions were still present. This fact has also been described in Ref. [17]. In that case, benzoic acid was the compound most affected by the presence of modifier and was eluted, even using 1.6% of water as modifier, with significant peak tailing.

3.2. Carotenoid analysis

After testing the columns for efficiency and surface activity, carotenoids were eluted with the packed capillary columns using neat CO_2.

In order to select the appropriate chromatographic conditions to elute carotenoids, a study of the solubility in CO_2 of β-carotene was performed based on the work developed by Favati et al. [1]. Solubility parameter theory predicts that the maximum solubility is attained when the solubility parameter of the solvent and the solute are equal. First of all, the β-carotene solubility parameter was obtained by using the group contribution method in which specific energy and volume increments are assigned to individual structural units comprising the molecular structure [18]. The sum of the individual group contributions for β-carotene yielded a solubility parameter (δ) of 8.71 $cal^{1/2}/cm^{3/2}$ (1 cal=4.184 J). In the same paper [1], it was shown that in order to extract β-carotene with neat CO_2, the following conditions are needed: T=40°C and P=500 atm (δ=8.66 $cal^{1/2}/cm^{3/2}$) and T=40°C and P=700 atm (δ=9.12 $cal^{1/2}/cm^{3/2}$). These values were obtained by using the equation proposed by Giddings et al. [19], where the solubility parameter (δ) of the solvent can be calculated as follows:

$$\delta_{gas} = 1.25P^{1/2}\left(\frac{\rho_{r,gas}}{\rho_{r,L}}\right) \quad (1)$$

where P=critical pressure of the gas; $\rho_{r,gas}$=reduced

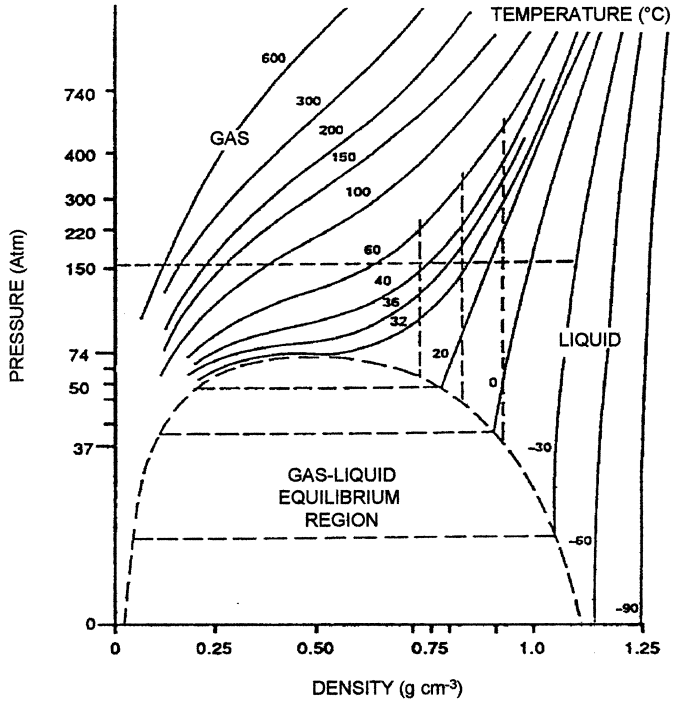

Fig. 3. Dependence of carbon dioxide density on pressure at different temperatures. (Reproduced by permission from Ref. [21]).

Table 1
Values of pressure (P) and temperature (T) obtained by interpolation in the graph shown in Fig. 3 at two different constant densities: $\rho = 0.98$ and $\rho = 1.04$ g/ml

$\rho = 0.98$ g/ml		$\rho = 1.04$ g/ml	
T (°C)	P (atm)	T (°C)	P (atm)
36	490	32	635
32	400	20	550
20	330	0	275
0	125		

density of the gas; $\rho_{r,L}$ = reduced density of the gas at infinite compression. δ provides a measurement of the solvent polarity and elution power [20].

The equipment used in this work for SFC does not allow pressures greater than 350 atm. Therefore, new chromatographic conditions (P, T) must be calculated in order to obtain the same solubility parameter as previously described above for CO_2. By using the graph representing pressure vs. density (Fig. 3) [21] it was possible to obtain different set of conditions (P, T) that provide the same solubility parameter as presented above. The corresponding densities were calculated transforming the reduced densities and were as follows: 0.98 g/ml (40°C, 500 atm) and 1.04 g/ml (40°C, 700 atm). Subsequently, the different conditions were obtained moving through the lines at constant density.

By using this approach, the conditions presented in Table 1 provide the same CO_2 density as 40°C/500 atm and 40°C/700 atm. Similar densities can be obtained at lower pressures if the temperature is reduced sufficiently. In order to operate at pressures lower than 300 atm, the temperature must be maintained between 20 and 0°C, therefore, subcritical conditions must be used. As a first approach, 10°C was used as the column temperature for eluting carotenoids, and pressures ranged from 275 to 300 atm. Fig. 4 shows the chromatograms obtained at 10°C for β-carotene and lycopene separation with the three columns evaluated. As can be seen, very short analysis times were obtained in all cases, with retention times for lycopene ranging from 2 to 10 min and for β-carotene around 4 to 6 min. Lycopene is eluted, in all the columns studied, with a relatively symmetrical peak shape. However, β-carotene shows appreciable tailing in the column with silica particles coated with 3% SE-54. Nevertheless, the peak shape for β-carotene is improved with particles coated with 10% OV-17 and those deactivated with D4.

The procedure and the columns studied in the present investigation suggest a very interesting possi-

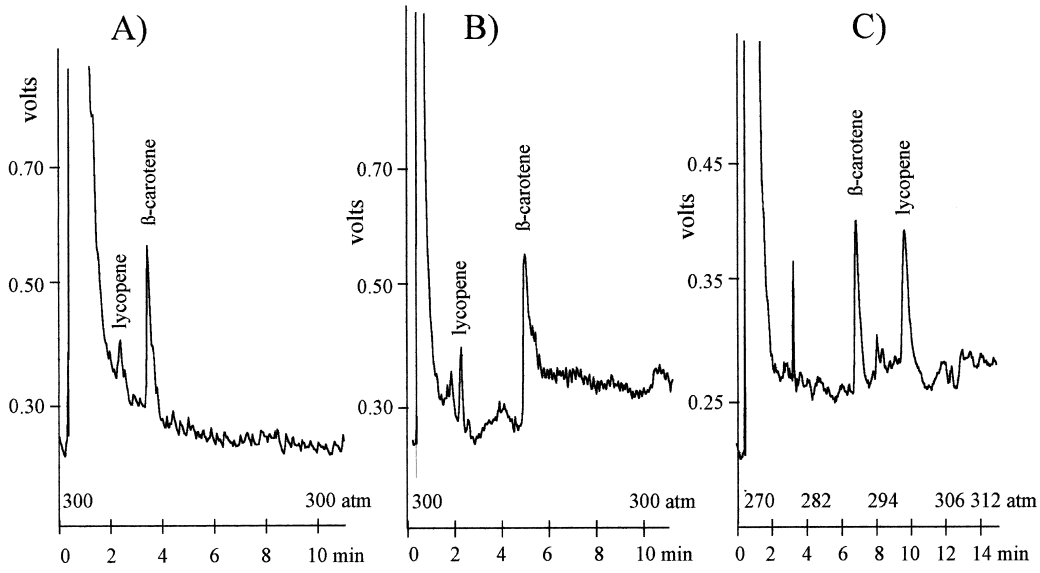

Fig. 4. SFC chromatograms of carotenoids in the three columns studied. (A) 50 cm×180 μm I.D. 10% OV-17 coated silica particles (10 μm), 10°C, 300 atm constant pressure; (B) 35 cm×180 μm I.D. 3% SE-54 coated silica particles (10 μm), 10°C, 300 atm constant pressure; (C) 50 cm×180 μm I.D. D4 deactivated silica particles, 10°C, 270 atm to 325 atm at 3 atm min^{-1}.

bility towards the optimization of a very fast and convenient method for carotenoid analysis that can be potentially applicable to the separation of other lipophilic food components.

At present, studies are being conducted in our laboratory in order to develop the interface for an on-line coupled SFE–SFC system with packed capillary columns.

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