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Short communication

# Separation of rosemary antioxidant compounds by supercritical fluid chromatography on coated packed capillary columns

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**Abstract**

Antioxidant compounds in rosemary extracts obtained by supercritical fluid extraction (SFE) were separated by supercritical fluid chromatography (SFC) on packed capillary columns. The columns contained silica particles coated with SE-54 (5% phenyl, 95% methyl silicone) and Carbowax 20 M [poly(ethylene glycol)]. The use of coated packed capillary columns allowed the separation of polar compounds by SFC with neat $CO_2$. The SFC conditions were selected on the basis of previous work. High pressures (up to 370 atm; 1 atm = 10,325 Pa) and moderate temperatures (up to 100 °C) were used to separate the compounds responsible for the antioxidant activity such as carnosic acid and carnosol while lower pressures were sufficient to separate the compounds of the essential oil.


*Keywords:* Antioxidant compounds; Supercritical fluid extraction; Rosemary; Packed capillary columns; Coated particles; Neat $CO_2$

## 1. Introduction

An important trend in food technology is the production of food ingredients with nutraceutical properties from natural sources such as aromatic plants or spices. Among the nutraceuticals, antioxidants receive much attention in the food industry [1], not only as preservatives in food products to prevent or retard oxidation of fats and oils, but also because of their beneficial effects on human health.

Rosemary has been widely accepted as a spice with high antioxidant activity [2]. The most active compounds are the phenolic diterpenes, primarily carnosic acid, and also carnosol, rosmanol, and epi- and isorosmanol [3,4]. Supercritical fluid extraction (SFE) has been suggested as a method for selective isolation of antioxidants from rosemary, mainly because of the mild conditions which avoid oxidation and/or degradation of such phenolic compounds [5,6]. Isolation under SFE conditions can be accomplished through cascade fractionation or using supercritical fluid chromatography (SFC). SFC allows a fractionation of the extracts which is compatible with SFE in terms of mobile phase, instruments and conditions and, what is even more important, maintaining the integrity of the compounds of interest. Preparative-scale SFC is an environmentally clean technology whose main advantage, compared to preparative LC, is the easy recovery of the isolated compounds by a simple decompression of the supercritical fluid [7].

One main problem encountered when separating polar compounds by SFC is the lack of polarity of the $CO_2$ used as mobile phase. An interesting approach to analyze polar compounds without the use of added modifiers is to tune the polarity of the stationary phase to increase the range of polar compounds that can be analyzed using either silica-based or new materials as stationary phase [8,9].

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The objective of the present work was the optimization of the separation of carnosic acid from other antioxidants in rosemary extracts obtained by SFE. In an earlier study in our laboratory, the elution of phenolic diterpenes such as carnosic acid in SFC with pure $CO_2$ using packed capillary columns was studied [10]. To evaluate the influence of the polarity of the coating polymer on the separation of polar compounds, two columns were tested, silica particles coated with SE-54 (5% phenyl, 95% methyl silicone) and Carbowax 20 M (CW20M) [poly(ethylene glycol)].

## 2. Experimental

### 2.1. Samples

The rosemary sample (*Rosmarinus officinalis* L.) consisted of dried rosemary leaves obtained from an herbalist's shop and grown in Murcia, Spain (dried using the traditional method, as follows: once collected, the plant is ventilated to remove humidity, covered with a blanket to avoid sunlight and let dry in a ventilated place for 20–30 days, depending on the season) [6]. Samples were ground under cryogenic carbon dioxide and stored in amber flasks at −20 °C until use.

For efficiency measurements, a mixture of pure *n*-alkanes ($C_{12}$–$C_{28}$) (Sigma, St. Louis, MO, USA) was used. For surface activity evaluation, a mixture of menthol, benzoic acid, anthracene, fluoranthene (Sigma), methyl benzoate and 2,6-dimethylaniline (Fluka, Switzerland) was utilized. Carnosic acid standard from *R. officinalis* (93% purity), camphor, 1,8-cineole and borneol were purchased from Sigma. Hexane and dichloromethane used to prepare the solutions were purchased from LabScan (Dublin, Ireland). Carnosic acid solutions were maintained at 4 °C in dark glass flasks.

### 2.2. Instrumentation

A supercritical fluid chromatograph SFC 3000 (Carlo Erba, Milan, Italy) equipped with a flame ionization detector was used. Sample was loaded by a time-controlled, rotating-valve injection device (Vici, Houston, TX, USA) containing an 1 μL internal loop. The injector temperature was 40 °C; the 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 20 cm × 13 μm i.d. made of fused silica tubing (Composite Metal Services, Ilkley, UK) connected to the column through a zero dead-volume union. The columns were connected to the injection valve via a flow splitter consisting on a silica tubing of 20 cm × 13 μm i.d. SFC conditions of analysis are detailed in figure captions.

### 2.3. Columns

Packed capillary columns were prepared according to a reported procedure [11] by using 500 μm i.d. stainless steel tubing (Symta, Madrid, Spain) of 25 cm length. The stainless-steel tubing was deactivated with 20% polyethyleneglycol (Sigma). The packing procedure was performed at a starting pressure of 80 atm followed by a pressure rate of 3 atm $min^{-1}$ up to 340 atm (1 atm = 10,325 Pa). 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 programme as follows: from 120 to 340 atm at 4 atm $min^{-1}$ and from 40 to 180 °C at 3 °C $min^{-1}$.

### 2.4. Coating of silica particles

Porous silica particles (10 μm, 60 Å, Hichrom, Reading, UK) were used as base material. Particles were conditioned prior to coating by washing them with ethanol and dried by heating at 160 °C for 1 h in a fluidized bed reaction vessel [12] using He as a purge gas.

Silica particles (0.3 g) were placed in the fluidized bed reaction vessel and mixed with 3% (w/w) of SE-54 (5% phenyl, 95% methylsilicone) dissolved in hexane or with 3% (w/w) CW20M dissolved in dichloromethane. When coating with SE-54, dicumyl peroxide (DCUP) was used as crosslinking agent at a concentration of 0.5 mg DCUP/100 mg stationary phase [13]. 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 to 160 °C at 5 °C $min^{-1}$ and maintaining the temperature at 160 °C overnight.

Efficiency measurements provided around 7000 plates $m^{-1}$ at $k' = 10$ (coating with 3% SE-54) and around 20,000 plates $m^{-1}$ ($k' = 3$) (coating with 3% CW20M).

### 2.5. SFE of rosemary

A Suprex PrepMaster (Suprex, Pittsburgh, PA, USA) supercritical fluid extractor was used for all the experiments. Sample (0.85 g, dry weight basis) was placed into a 5 mL stainless-steel extraction cell. The supercritical $CO_2$ flow rate was controlled using a needle valve as variable restrictor; flow rates of 3–4 mL $min^{-1}$ were obtained at the experimental conditions tested.

Sample was extracted by using a two-step method [fractions 1 (F1) and 2 (F2)] according to the experimental conditions shown in Table 1. Extraction time was 5 min static extraction followed by 60 min dynamic extraction for each step. F1 mainly correspond to rosemary essential oil. The residue obtained after the first extraction was re-extracted at conditions selected for F2. The conditions were selected based on previous work in our laboratory [6] and also considering conditions previously suggested by other authors [14,15].

Supercritical fluid extracts were collected in glass vials (2 cm × 0.5 cm) using a device specially designed in our laboratory [6].

Table 1
Experimental conditions used for supercritical fluid extraction of rosemary

| Experiments | Pressure (atm) | Temperature (°C) | Density (g mL$^{-1}$) |
|---|---|---|---|
| Experiment 1 | | | |
| F1 | 140 | 40 | 0.765 |
| F2 | 300 | 60 | 0.831 |
| Experiment 2 | | | |
| F1 | 140 | 50 | 0.674 |
| F2 | 350 | 50 | 0.900 |
| Experiment 3 | | | |
| F1 | 90 | 40 | 0.494 |
| F2 | 250 | 40 | 0.835 |
| Experiment 4 | | | |
| F1 | 100 | 40 | 0.633 |
| F2 | 400 | 60 | 0.891 |

## 3. Results and discussion

### 3.1. Surface activity

Surface activity of the two columns was measured by analizing a polarity mixture. Table 2 shows the asymmetry factors obtained when using pure $CO_2$ as mobile phase. The results show a correct deactivation of the residual silanol groups in the particle surface allowing the separation of alcohols and amines, and, for 3% CW20M stationary phase, of benzoic acid with only slight peak tailing.

### 3.2. Carnosic acid analysis

The retention behavior of carnosic acid on the two columns was studied at various temperatures (Table 3). Elution of carnosic acid is seen to be easier with the non-polar stationary phase which means that both deactivation and stationary phase polarity play a role in the selectivity of the SFC sys-

Table 2
Asymmetry factor ($A_s$) of the two columns evaluated in the study

| | $A_s$ ($n=3$)[a] 3% | |
|---|---|---|
| Compound | SE-54 | CW20M |
| Anthracene | 1.1 | 1.1 |
| Methyl benzoate | 1.3 | 1.1 |
| Fluoranthene | 1.2 | 1.0 |
| 2,6-Dimethylaniline | 1.0 | 1.4 (T) |
| Menthol | 1.4 (T) | 1.5 (T) |
| Benzoic acid | no elution | 1.4 (T) |

[a] *Conditions:* 120 °C, 90–300 atm at 4 atm min$^{-1}$; T, tailing.

Table 3
Retention of carnosic acid in SFC at three oven temperatures ($n=3$)[a]

| $T$ (°C) | $k'$ | |
|---|---|---|
| | SE-54 | CW20M |
| 40 | 7.7 | 9.5 |
| 70 | 11.9 | 12.6 |
| 100 | 13.3 | 14.2 |

[a] For columns see Section 2.3.

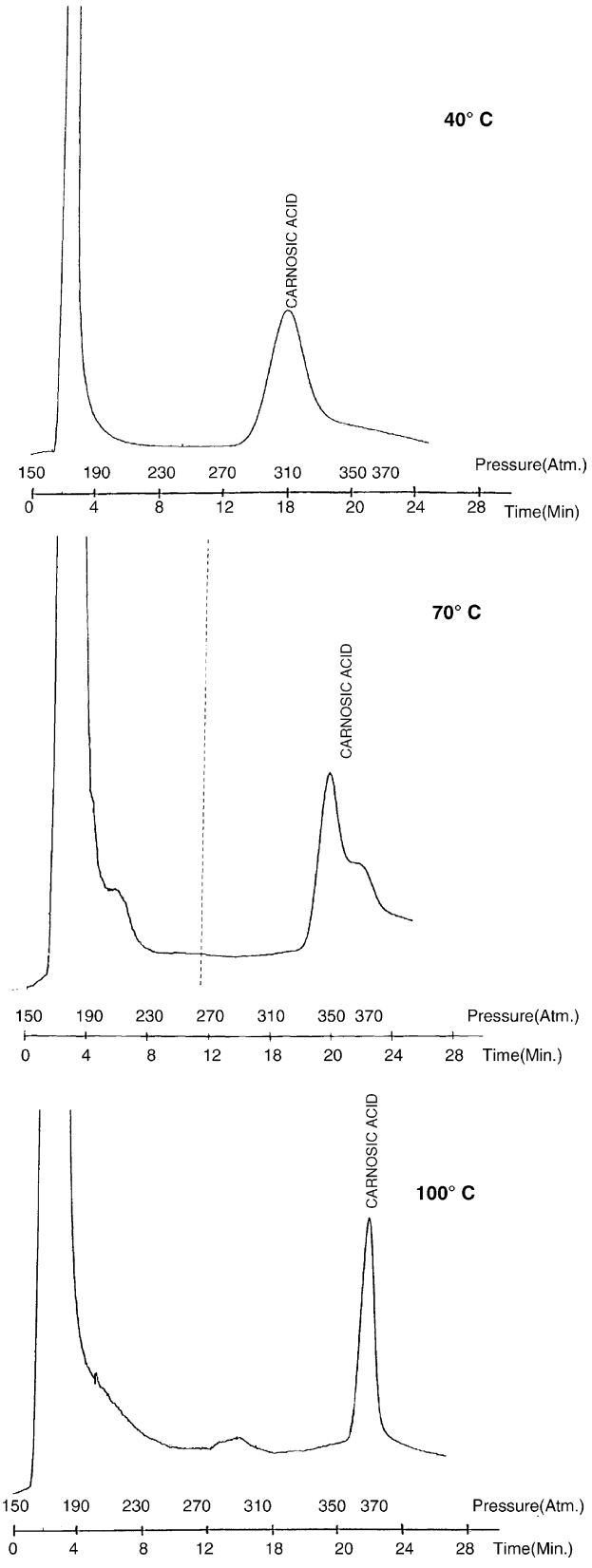


Fig. 1. SFC–FID of carnosic acid in the 25 cm × 500 μm i.d. column packed with 3% SE-54 coated silica particles (10 μm). Pressure program from 150 to 370 atm at 10 atm min$^{-1}$. Column temperatures tested: 40, 70 and 100 °C.

tem when using neat $CO_2$. The behavior of carnosic acid was found to be similar on the two columns; Fig. 1 shows results for SE-54. At lower temperatures the broad peak shape suggests the co-elution of carnosol (the standard contains 7% of carnosol). When the temperature increases from 40 °C via 70 to 100 °C the separation of carnosic acid and carnosol (appearing as a small peak at around 15 min) becomes possible.

### 3.3. Separation of SFE rosemary extracts

For an appropriate separation of phenolic antioxidants in rosemary, both column efficiency and selectivity were taken into account. While the CW20M column provides higher efficiencies, the SE-54 column allows a faster analysis. Therefore, the latter was selected in this study.

The separation of SFE experiments 2 and 4 (Table 1) are shown in Fig. 2 (F2, antioxidant fraction and F1, essential oil fraction). Fraction 2 does not show a very complex profile, and the major compound detected in all experiments was carnosic acid which is extracted at the highest extraction density (350 atm and 50 °C). It is interesting to note the high resolution achieved between carnosic acid and most other compounds in the extracts. This will become important when developing packed columns with selectively coated particles for preparative SFC.

The F1 fraction also contain carnosic acid, but the profiles are more complex in terms of the number and type of compounds extracted by SFE. An important group of compounds is eluted close to the dead time of the solvent; those being the most volatile components of the essential oil. Major constituents of the essential oil (camphor, 1,8-cineole and borneol) were identified by comparing retention times with those of standards. These compounds can play an important role as natural antimicrobials; that is, their preparative-scale purification by SFC could be of interest. With the columns tested, baseline separation of 1,8-cineole was possible but borneol and camphor co-eluted. Obviously, further research will be required if preparative-scale SFC becomes desirable.

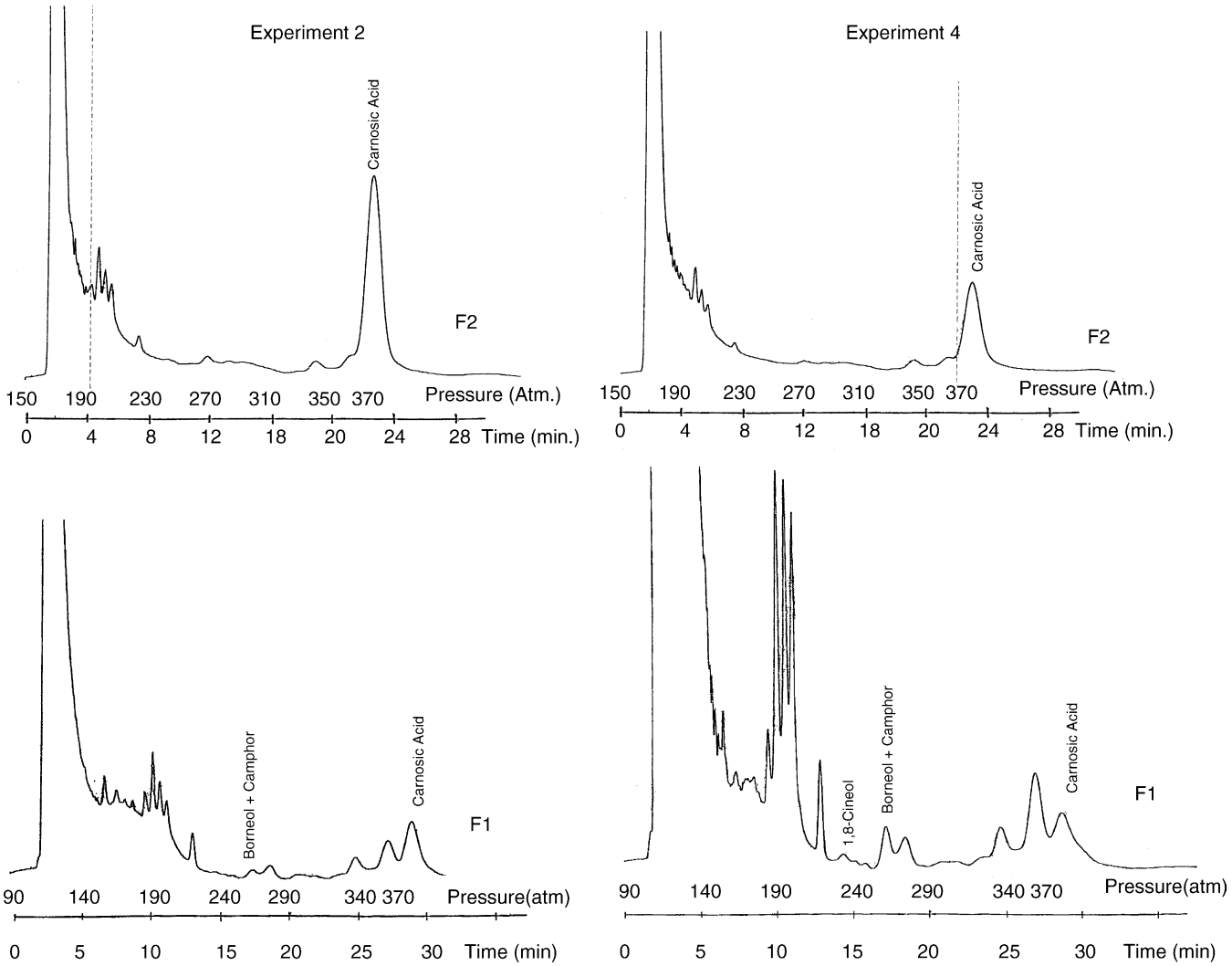


Fig. 2. SFC–FID of SFE rosemary on SE-54-coated silica columns. Extracts corresponding to F2 and F1 (experiments 2 and 4, Table 1). Column temperature: 100 °C, pressure program for F2 from 150 to 370 atm at 10 atm $min^{-1}$, and for F1 from 90 to 370 atm at 10 atm $min^{-1}$.

In conclusion, this work shows the feasibility of SFC with coated packed capillary columns as a method to separate rosemary extracts obtained by SFE with pure $CO_2$ as mobile phase. The next step will be preparative-scale SFC to purify carnosic acid. At present, these coated (packed) columns are being manufactured at a large scale for the fractionation and purification of antioxidants of high activity and high added value such as carnosic acid.

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