Innovative Food Science and Emerging Technologies 12 (2011) 142–145

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Supercritical CO_2 fractionation of rosemary ethanolic oleoresins as a method to improve carnosic acid recovery

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ARTICLE INFO

Article history:
Received 3 August 2010
Accepted 7 January 2011

Keywords:
Rosemary
Ethanolic oleoresin
$SCCO_2$ antisolvent fractionation
Carnosic acid
Antioxidant activity

ABSTRACT

Supercritical fluid antisolvent fractionation was used to obtain antioxidant compounds, mainly carnosic acid (CA), from high viscous oleoresins derived from dried rosemary leaves (*Rosmarinus officinalis*) extracted with ethanol. Due to the high viscosity of the starting material, which may hinder the mass transfer between the phases, a special nozzle was designed to blend the $SCCO_2$ stream with the high viscous oleoresin. Experiments were conducted at 50 °C and six different pressures in the first separation vessel, ranging from 150 to 400 bar; the best separation was achieved at 300 bar. As a result of the oleoresin two-stage fractionation, the starting material was separated in two fractions. The first one was an insoluble dark green powder, with low concentration of CA (<5 g/100 g extract). The other fraction was an orange colored resinous extract, very soluble in $SCCO_2$, with a high concentration of CA (33 g/100 g extract at 300 bar). The antioxidant effect of this extract was higher to that of BHT when added to soybean oil.

Industrial relevance: The present study adds a possibility for the purification of carnosic acid from rosemary by using $SCCO_2$ antisolvent fractionation. Since the starting material employed (oleoresin) is a fluid phase, the process might be carried out in a continuous or semi-continuous way instead of discontinuous as it should be done if the starting material were a solid (leaves). This feature makes the procedure very promising toward its application at the industrial scale.

1. Introduction

In recent years, rosemary (*Rosmarinus officinalis*) plant species has been largely studied as a source of natural products with diverse biological activities. Rosemary leaf extracts are increasingly used as antioxidants in the food industry, proposed as human dietary factors and investigated for the treatment of cancer and neurodegenerative diseases (Huang et al., 1994; Cuvelier, Richard, & Berset, 1996; Hopia, Huang, Schwarz, German, & Frankel, 1996; Leal, Braga, Sato, Carvalho, Marques, & Meireles, 2003). Several researches have indicated that one of the most active antioxidant component in rosemary leaves is the phenolic diterpene namely carnosic acid (CA, Fig. 1) (Señoráns, Ibañez, Cavero, Tabera, & Reglero, 2000; Munné-Bosch & Alegre, 2001; Thorsen & Hildebrandt, 2003; Terpinc, Bezjak, & Abramovič, 2009). Other substances, such as rosmanol, epi and iso-rosmanol are considered to be minor components resulting from degradation of CA by effect of temperature, light and oxygen exposure (Schwarz & Ternes, 1992; Schwarz, Ternes, & Schmauderer, 1992).

Supercritical fluid extraction (SFE) has been proposed for direct extraction of phenolics from rosemary leaves (Carvalho, Moura, Rosa, & Meireles, 2005; Yesil Celiktas, Bedir, & Vardar Sukan, 2007); however, this methodology may suffer from low rates of mass transfer from solid particles to the supercritical phase and even low solubilities for some types of compounds of interest. In order to increase antioxidant fraction recovery, some works report the addition of modifiers (co-solvents) such as ethanol (Señoráns et al., 2000; Cavero, Jaime, Martín-Alvarez, Señoráns, Reglero, & Ibañez, 2005). The use of co-solvents generally increases the solubility of polar substances in CO_2, but at high concentration they can affect the selectivity of the extraction. Moreover, the use of co-solvents may bring about the problem of their removal from the final product.

One avenue that has not been thoroughly explored is the extraction of the solid material by using an organic solvent, followed by phenolic fraction recovery from the organic resinous extract. In the present work, supercritical carbon dioxide ($SCCO_2$) was used to obtain phenolic extracts, rich in CA, from high viscous oleoresins derived from dried rosemary leaves extracted with ethanol. A home-made nozzle was used to blend the $SCCO_2$ stream with the high viscous oleoresin.

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doi:10.1016/j.ifset.2011.01.004

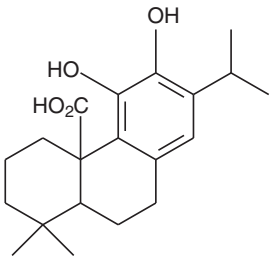

Fig. 1. Carnosic acid, the main diterpenic *ortho*-diphenol presents in rosemary leaves.

2. Materials and methods

Dried rosemary leaves were grounded using a knife mill (Retsch SM100, Germany), and then sieved to obtain very fine particles (0.5–1.4 mm) using sieves of the Standard Tyler series meshes. This material (500 g) was placed in a flat-bottomed flask with 3 L ethanol (96%, v/v), and kept under agitation at room temperature for 2 h. The extract was filtered under vacuum using Whatman No. 1 filter paper. The liquid phase was evaporated (40 °C) under vacuum until a mixture composed by 48% oleoresin and 52% ethanol was obtained. This proportion allowed getting a relatively concentrated crude extract, which could be pumped into the high pressure system (Fig. 2). Preliminary experiments showed that mixtures containing more than 50% oleoresin were more difficult to pump.

A high pressure pilot plant with single stage separation and solvent recycle was used to fractionate the crude extract (Ruetsch, Daghero, & Mattea, 2003). The crude extract was pumped (4.3 mL/min, ISCO pump) into the extractor vessel (2.5 L) *via* a home-made special nozzle (Fig. 3, Table 1). Fractionation experiments were done at six different CO_2 pressures in the first vessel (extractor): 150, 200, 250, 300, 350, and 400 bar. The fluid stream leaving the extractor was sent to the separator unit (0.5 L) (at 100 bar and 50 °C), where a purified extract was recovered.

The composition of rosemary fractionated extracts proceeding from both the extractor and the separator units, was determined by HPLC (Perkin Elmer, Shelton, USA) according to methods described elsewhere (Wellwood & Cole, 2004). Analyses were performed on a reverse phase C18 column (250 x 4.6 mm, 5 μm pore size). The mobile phase was programmed with a linear gradient from 90% A (840 ml of water with 8.5 mL of acetic acid and 150 mL of acetonitrile), 10% B (methanol), to 100% B at 30 min, with a flow rate of 1.5 mL/min. The system was stabilized for 5 min between successive injections. Detection was accomplished by using an UV detector at a wavelength of 284 nm. Identification of CA was carried out by comparison of its retention time with that of the pure standard (Sigma-Aldrich, St. Louis, USA). A Perkin-Elmer UV–vis spectrophotometer was used to determine the amount of CA in the fractionated extracts, after calibration with gravimetrically prepared standard solutions.

The antioxidant capacity of the rosemary purified extracts (RPE, proceeding from the separator unit) was tested following the Oxidative Stability Index (OSI) method according to AOCS standard Cd 12b-92 (1998), using refined soybean oil (RSO) as a substrate for lipid oxidation and butylhydroxytoluene (BHT, 200 ppm) as a reference antioxidant compound. Air flow rate was set at 20 L/h and temperature of the heating block was maintained at 110 °C. Induction time corresponded to the break point in the plotted curve. The antioxidant effectiveness of RPE was expressed as the stabilization factor (F):

$$F = IT_{inh} / \ IT_o$$

where IT_{inh} is the induction time of RSO in the presence of the antioxidant, and IT_o is the induction time of RSO without additives.

3. Results and discussion

Solvent extraction, which is generally used for the extraction of natural antioxidants from plant materials, has some drawbacks including low selectivity and elimination of solvent residues that are often prohibited by food regulations. According to Tena, Valcárcel, Hidalgo, and Ubera (1997) the hydrogen-bonding ability of acetone and methanol is crucial for the extraction of phenolic diterpenes responsible for antioxidant properties of rosemary leaves. In the present work, objected organic solvents such as those mentioned previously, were replaced by ethanol, a wide accepted solvent in the food industry.

The extraction of rosemary leaves with ethanol yielded a dark green high viscous oleoresin. The HPLC analysis of this crude extract gave a total of 20 peaks, among which CA represented 14% of the total peak areas. This percent represented about 3.3 g CA/100 g crude extract; it was lower than that obtained by Erkan, Ayranci, and Ayranci (2008) using Soxhlet extraction with methanol as solvent.

The crude extract was then subjected to $SCCO_2$ antisolvent fractionation with the aim of increasing CA recovery. Thanks to the special nozzle employed (Fig. 3, Table 1) a proper mixing between the two fluid streams was achieved at the entrance of the first separation vessel (extractor) in which the heavier compounds were selectively precipitated because of the antisolvent effect of CO_2. The fraction soluble in CO_2 continued in the fluid phase and moved to the second unit (separator), where most of it precipitated as a result of the pressure reduction, while only the most volatile compounds remained soluble in CO_2 + ethanol light phase.

In previously published works, it was reported that the yield of rosemary antioxidant extracts obtained by a one-step supercritical extraction under a pressure of 300 bar, increased significantly when the extraction temperature was risen from 30 to 40° C (Dauksas, Venskutonis, Povilaityte, & Sivik, 2001; Carvalho et al., 2005). From these data, an increase in the extraction temperature would be also

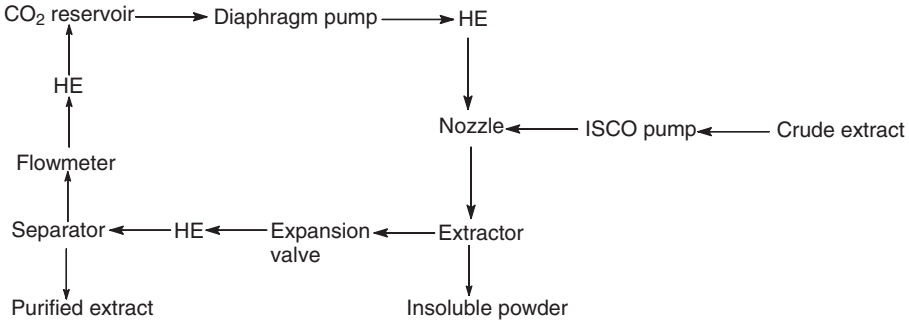

Fig. 2. Scheme of the supercritical fluid extraction process used in this study. HE, heat exchanger.

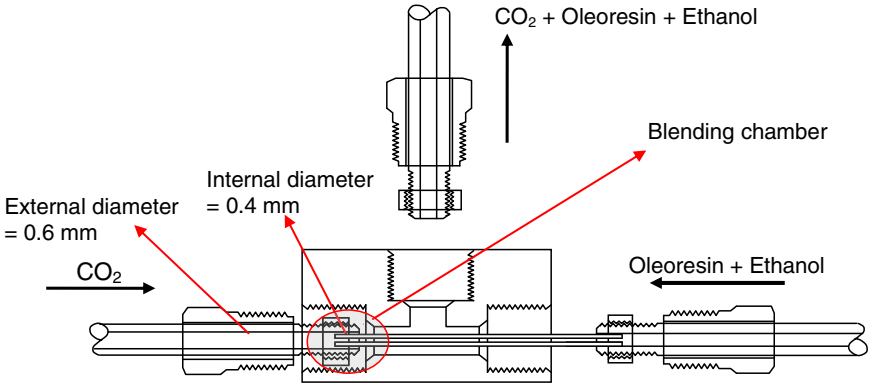

Fig. 3. Scheme of the home-made nozzle designed to blend the CO_2 stream with the crude extract.

expected to enhance the CA yield. With this hypothesis in mind, preliminary experiments were carried out to test the effect of temperatures higher than 40 °C (at the isobaric condition of 300 bar) on the CA recovery. The highest one was obtained at 50° C (Table 2). An increase in the extraction temperature dramatically decreased the CA concentration. This fact could be due to thermal degradation of CA. It has been reported that CA is quite unstable and can be degraded or converted to other diterpenes (Ramírez, García-Risco, Santoyo, Señoráns, Ibáñez, & Reglero, 2006).

Considering the results mentioned above, experiments were done to check the effect of different extraction pressures (150, 200, 250, 300, 350, and 400 bar) at the isothermal condition (50° C). Results are showed in Table 3. As a result of the oleoresin fractionation at the different extraction pressures checked, the starting material was separated in two fractions. The first one, precipitated in the extractor vessel, was an insoluble dark green powder, with low concentration of CA. The other fraction, precipitated in the separator unit by decreasing the CO_2 pressure at 100 bar, was an orange colored resinous extract, very soluble in $SCCO_2$, with higher concentration of CA (9.47–33.0 g/100 g extract) (Table 3). The highest yield (33% in average) was obtained at 300 bar. This means that the CA recovery enhanced ten times with respect to CA content presents in the crude extract. The analysis of published data on extraction yields of antioxidant compounds from rosemary shows lower CA recovery percentages. For example, Carvalho et al. (2005) achieved the largest CA yield (1.07%) from dried leaves extracted at 300 bar and 40 °C, whereas Babovic et al. (2010) recover up to 4.76 g CA/100 g extract obtained at 350 bar and 100 °C.

Table 1
Dimensional parameters of the home-made nozzle designed to blend the CO_2 stream with the crude extract.

Internal diameter	0.4 mm
External diameter	0.6 mm
Hydraulic radius	0.05 mm
Annulet area	0.157 mm^2
External tubing area	0.282 mm^2
Internal tubing area	0.125 mm^2

Table 2
Carnosic acid concentrations obtained at 300 bar and different extraction temperatures.

Temperature (°C)	Carnosic acid (g/100 g extract)	
	Extractor	Separator
50	5.51	33.0
60	2.35	7.90
70	3.45	6.08
80	3.14	2.82

The $SCCO_2$ extract obtained at 300 bar was tested for its antioxidant capacity when added to RSO (Table 4). A protection factor greater than that obtained with a conventional synthetic antioxidant (BHT) was determined. The induction time (IT) of RSO with 200 ppm RPE duplicated that of the pure RSO. The addition of 400 and 600 ppm RPE extended the IT from 3.21 until a value of 7.52 h. No significant differences were observed among treatments containing 200, 400 or 600 ppm RPE indicating that the optimum antioxidant activity could be reached with a relatively low amount of RPE.

4. Conclusions

The results indicated that the process employed to extract phenolic antioxidant compounds from rosemary leaves using $SCCO_2$ antisolvent fractionation of high viscous ethanolic oleoresins, is useful as a purification method to obtain fluid extracts with elevated CA concentration. A special home-made nozzle was used to improve the mass transfer between both the oleoresin and the $SCCO_2$ fluid phases. Tuning the process parameters (pressure and temperature) enabled the tuning of the $SCCO_2$ selectivity towards the desirable component (CA), as well as phase separation so that solvent-free extracts could be obtained. Data showed that the selectivity of the $SCCO_2$ can be optimized by adjusting the pressure in the extractor (first stage).

Table 3
Carnosic acid concentration obtained at different extraction pressures.

CO_2 Pressure (bar)	Carnosic acid (g/100 g extract)	
	Extractor	Separator
150	1.84	9.47
200	2.33	16.5
250	2.56	13.7
300	5.51	33.0
350	3.65	19.6
400	2.10	11.8

Table 4
Induction times (IT) and stabilization factors (F) of refined soybean oil without additives (RSO), RSO plus BHT, and RSO plus rosemary purified extract (RPE) at different concentrations.

Treatment	Antioxidant (ppm)	IT	F
RSO		3.21^{a}	
RSO + BHT	200	5.96^{b}	1.85^{a}
RSO + RPE	200	6.38^{c}	1.98^{b}
	400	7.52^{cd}	2.34^{bc}
	600	7.52^{cd}	2.34^{bc}

All measurements were replicated three times. Values in each column with different superscript letters present statistically significant differences ($P \leq 0.05$).

Extractions made at 300 bar and 50 °C yielded the highest CA content. Since the starting material is a fluid phase (oleoresin), the extraction procedure can be carried out in a continuous or semi-continuous way instead of discontinuous as it should be done if the starting material were a solid (leaves). This feature makes the procedure very promising toward its application at the industrial scale.

Acknowledgement

This work was financed with a grant from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT)—PICT 09-25673.

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