Supercritical Carbon Dioxide Extraction of Natural Antioxidants from Rosemary and Sage

J. Ivanović[1,*], I. Žižović[1], S. Đilas[2], V. Vajs[3], S. Petrović[1, 4] and D. Skala[1, 5]

[1] *Faculty of Technology and Metallurgy, Karnegijeva 4, 11000 Belgrade, Serbia*
[2] *University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia*
[3] *University of Belgrade - Faculty of Chemistry, Studentski trg 12-16, 11000 Belgrade, Serbia*
[4] *Hemofarm group, Vršac, Serbia*
[5] *Texas A&M at Qatar, Education City, 23874 Doha, Qatar*

jasnai@tmf.bg.ac.yu, fax: +381113370-387

ABSTRACT

A number of new separation techniques for natural food ingredients isolation have been recently proposed due to new environmental and public health regulations and restriction. The growing interest in natural food has raised demand for antioxidants obtained from natural sources. Rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) extracts can be used as food additives for different fat products stabilization against lipid oxidation instead of synthetic antioxidants [e.g. Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), etc.]. The goal of this study was oriented to isolation of phenolic antioxidants from dried leaves of rosemary and sage originated from Balkan region using supercritical carbon dioxide fractional extraction. Antioxidant fraction was isolated under pressure of 30 MPa with a supercritical carbon dioxide at 40°C and 100°C. In the present study kinetic data and antioxidant extract's yields obtained from dried leaves of rosemary and sage under different conditions were determined. The antioxidant activity of the rosemary and sage extracts was tested by measuring their ability to scavenge stable 2,2-diphenyl-1-pycrylhydrazyl (DPPH) free radical and reactive hydroxyl radical during the Fenton reaction trapped by 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), using electron spin resonance (ESR) spectroscopy. Thereto, antioxidant fraction obtained from rosemary was characterized chemically by liquid chromatography–mass spectroscopy (LC-MS). Eleven compounds were indentified, including phenolic diterpenes and flavonoids.

INTRODUCTION

Herbs and spices have been traditionally used to impart flavour and odour in food and for prevention and treatment of wide range of diseases. Recently, they have been extensively studied for their antioxidant and antiradical activities as well. Spices can be added to food as whole spices, as ground spices, or as isolates from their extracts. Whole and ground spices contain aromas, pigments, pungent components and other impurities and therefore their use as antioxidants is limited [1]. With a view to produce plant extracts without flavour, odour and colour and with sufficient antioxidant activity to allow the usage at the levels equivalent to the synthetic antioxidants (0.01-0.05%) a number of different techniques for isolation and concentration of antioxidants from natural matter were proposed: solvent extraction (polar and non-polar) [2], aqueous alkaline extraction [3], extraction with vegetable oils or mono- and diglycerides or both [4], steam distillation and molecular distillation [5]. Compared to conventional methods, supercritical fluid extraction with carbon dioxide appears as an advantageous technology for production of natural antioxidants from rosemary [6-8] and sage [9, 10]. It is reported that antioxidant extracts obtained by supercritical carbon dioxide extraction from rosemary and sage have equivalent or stronger antioxidant activity then synthetic antioxidants [8, 10] and extracts obtained by solvent extraction [6, 11]. Supercritical extracts are free of organic solvents and also free of bacteria. Obtained extracts are semi-solid at ambient temperature and can be ground

at temperatures of -18°C and less and be either dissolved or dispersed in animal or vegetable oils and fats [8]. Therewith, by choosing extraction temperature and pressure, a desirable component fraction in the extract can be increased as well.

Rosemary (*Rosmarinus officinalis* L.) is well known for its antioxidant activity due to phenolic diterpenes such as carnosic acid, carnosol, rosmanol, rosmadial [12, 13], rosmanol-9-ethyl ether [10] and phenolic acid such as rosmarinic acid [13] that are also isolated from sage (*Salvia officinalis* L.), methyl carnosate [14], 7-methyl-epi-rosmanol, epirosmanol [12], as well as flavonoids such as genkwanin, cirstimaritin, scutellarein [6, 13].

The scope of this work was to study kinetics of antioxidant fraction isolation from dried leaves of rosemary and sage originated from Balkan region using supercritical carbon dioxide extraction as well as antioxidant activity of their supercritical extracts by means of 2,2-diphenyl-1-pycrylhydrazyl (DPPH) free-radical and hydroxyl radical scavenging tests. Additionally, both liquid chromatography (LC) and mass spectroscopy (MS) with an electrospray (ES) and diode-array detector (DAD) was used to perform analysis and identification of compounds responsible for antioxidant activity of the rosemary extract obtained at 30 MPa and 100°C which exhibited the highest antioxidant activity amongst investigated supercritical extracts.

MATERIALS AND METHODS

Samples and chemicals

Dried leaves of wild grown rosemary and sage originated from Balkan region were used for experimental studies. Prior to extractions plant material was ground to appropriate particle size (0.315-0.500 mm). Commercial carbon dioxide (99% purity, Tehno-gas, Novi Sad, Serbia) was used for the extractions. 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and BHA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial rosemary antioxidant Flavor'Plus™ (Naturex, France) was obtained from Aleva, Novi Knezevac.

Extraction method

Extractions with supercritical carbon dioxide were preformed in a pilot-plant-scale supercritical fluid extractor (Autoclave Engineers SCE Screening System) with a 150 ml extraction cell described previously [15]. Plant material was fine milled to average particle diameter of 0.4 mm. Fractional extraction was applied in order to obtained antioxidants separately from essential oil. First fraction comprising essential oils was extracted at pressure of 11.5 MPa and temperature of 40°C. Thereupon, antioxidant fraction was extracted at 30 MPa and on temperatures of 40°C and 100°C. The initially used mass of the plant samples was 64.5 g in all experiments.

DPPH and hydroxyl radicals generation and detection

DPPH radicals were prepared in methanol to the final concentration of 1.8×10^{-4} mM. Different concentrations of investigated rosemary extracts (0.05, 0.1, 0.2, 0.3, 0.5 mg ml^{-1}) as well as sage extracts (0.25, 0.5, 1, 1.5, 2.0, 3.0, 5.0) obtained at 30 MPa and different temperatures (40°C and 100°C) were added and mixed with DPPH solution. After that the mixture was stirred for 2 min and transferred to a quartz flat cell ER-160FT. The ESR spectra were recorded on an ESR spectrometer Bruker 300E (Rheinstetten, Germany) under the following conditions: field modulation, 100 kHz; modulation amplitude, 0.256G; receiver gain, 2×10^4; time constant, 40.96 ms; conversion time, 327.68 ms; centre field, 3440.00 G; sweep width, 100.00 G; *x*-band frequency, 9.64 GHz; power, 20 mW; and temperature, 23°C. Magnetic field scanning was calibrated using Fremy's salt. Splitting constants were calculated from computer-generated second derivatives of the spectra after optimising signal-to-noise ratios, were verified by computer simulations. The antiradical activity (AA_{DPPH}) of the extracts was defined as:

$$AA_{DPPH} = 100 \cdot (h_o - h_x)/h_o \, [\%]$$

where h_o and h_x are the height of the second peak in the ESR spectrum of DPPH radicals of the blank and the probe, respectively.

As hydroxyl free radicals are highly reactive, with relatively short half-lives, the concentration found in natural systems are usually inadequate for direct detection by ESR spectroscopy. Spin-trapping is a chemical reaction that provides an approach to help overcome this problem. The Fenton reaction was conducted by mixing 0.2 ml 0.3 M DMPO, 0.2 ml 10mM H_2O_2 and 0.2 ml 10 mM Fe^{2+} (blank). The influence of different types of investigated extracts on the formation and stabilization of hydroxyl radicals was investigated by adding the investigated extracts in the Fenton reaction system at concentrations of 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, and 6.0 mg ml^{-1} for rosemary extracts and 0.25, 0.5, 1.0, 2.0, 3.0, 5.0, 10 mg ml^{-1} for sage extracts. ESR spectra were recorded after 5 min, with the following spectrometer settings: field modulation, 100 kHz; modulation amplitude, 0.512 G; receiver gain, 2×10^5; time constant, 81.92 ms; conversion time, 163.84 ms; centre field, 3440.00 G; sweep width, 100.00 G; *x*-band frequency, 9.64 GHz; power, 20 mW; and temperature, 23° C. The hydroxyl radical scavenging activity (SA_{OH}) of the extracts were defined as:

$$SA_{OH} = 100 \cdot (h_o - h_x)/h_o \, [\%]$$

where h_o and h_x are the height of the second peak in the ESR spectrum of DMPO-OH spin adduct of the blank and the probe, respectively.

HPLC analysis of the rosemary extracts with DAD and MS detection

In the present study, chemical characterization of the supercritical fluid extract of rosemary obtained at 30 MPa and 100°C was accomplished using both liquid chromatography (LC) and mass spectrometry (MS) with electrospray (EC). Samples were prepared by dissolving 10 mg of rosemary extract into 1.000 ml methanol (c=9.999 mg/ml). Analysis was preformed with a 6210 Time-of-Flight LC/MS by using ES interface. The separation was carried out in an HPLC apparatus (Agilent Technologies 1200 Series) with an autosampler equipped with a Zorbax Eclipse XDB-C18 column (Agilent Technologies), 5μm particle size, 4.6×150 mm. The mobile phase was mixture of solvent A (0.2% formic acid in water) and solvent B (acetonitrile) according to a step gradient, lasting 60 min, changing from 23 % solvent B at 8 min to 77 % solvent at 20 min, at flow rate of 1 ml/min. Detection was accomplished by using a series 1200 DAD (Agilent Technologies) which stored the signals in a range of wavelength from 190-400 nm. Injection volume was 10 μl and column temperature 25°C. A personal computer system running Mass Hunter Workstation software was used for data acquisition and processing. In the atmospheric pressure ionization ES method, the compounds were mixed with nitrogen in the heated nebulizer interface and the polarity was tuned to positive. Adequate calibration of ES parameters (needle potential, gas temperature, nebulizer pressure, and fragmentator voltage) was required to optimize the response and to obtain high sensitivity of the molecular ion. The selected values were follows: needle potential, 4000 V; gas temperature, 350°C; drying gas, 10 ml/min; nebulizer pressure, 50 psig; fragmentator voltage, 60 V.

RESULTS AND DISSCUSION

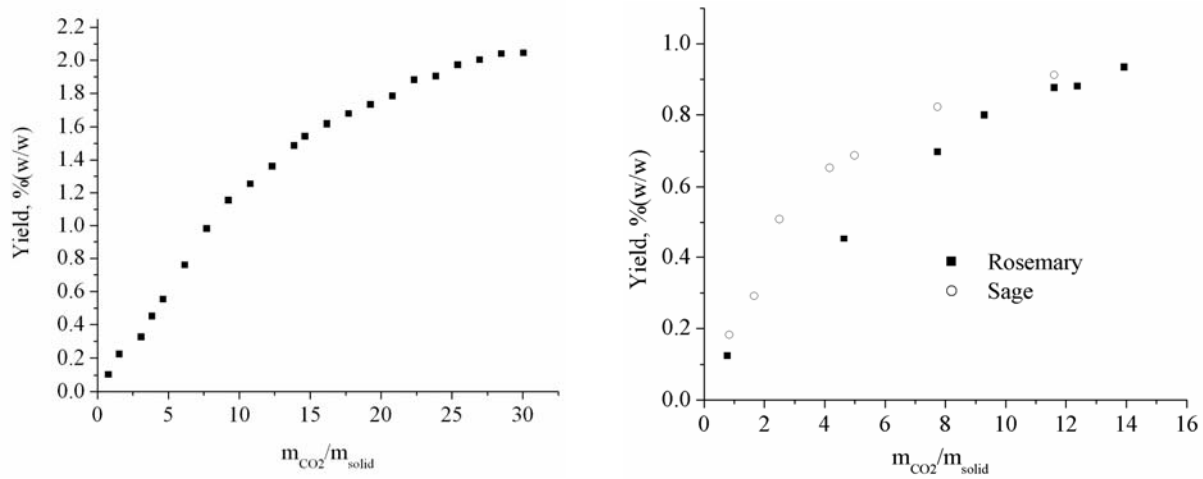
Fractional extraction using supercritical carbon dioxide was carried out with a view to isolate and concentrate antioxidant fraction from rosemary and sage separately from essential oils. First fraction which comprised essential oil was extracted at 11.5 MPa and on temperature of 40°C in order to collect aromatic and highly volatile components, mostly mono- and sesquiterpenes and their oxygenated derivates. Gained yields of first fraction were 0.985 % (w/w) for sage and 0.956 %(w/w) for rosemary. Phenol derivates with antioxidant activity were isolated at 30 MPa and on temperatures of 100°C and 40°C. Extraction yields of antioxidant fraction obtained from rosemary and sage in performed experiments are presented in table 1.

It has been previously suggested that optimum rates and yields of supercritical carbon dioxide extraction of antioxidants from Lamiaceae herbs are attained at temperatures between 90 and 110°C at pressures from 35 to 50 MPa [8]. At extraction temperatures above 110°C heat damage can occur to extracted compounds as well as to extracted residue [8].

Table 1. Yields of rosemary and sage antioxidant fractions in performed experiments

Herbaceous material	Pressure, MPa	Temperature, °C	Yield, %(w/w)
Rosemary	30	40	0.934
		100	2.045
Sage	30	40	0.912
		100	1.987

Extraction yield curve of antioxidant fraction extraction from rosemary performed at pressure of 30 MPa and 100°C is presented in Figure 1a. Comparative extraction curves of rosemary and sage antioxidant extracts obtained at 30 MPa and 40°C are presented in Figure 1b. As expected at lower operating temperature lower yields of antioxidant fraction from rosemary and sage were obtained. Lower extraction temperatures are recommendable for economic reasons and especially for fractional separation when aroma fraction is to be used further [16]. In order to increase yields of antioxidants from rosemary at similar conditions some authors added modifiers (co-solvent) such as ethanol. The use of modifiers generally increases solubility of polar substances in carbon dioxide although a higher concentration of modifiers can affect selectivity [6]. Modifiers are not recommendable for extractions at higher pressures (e.g. 50 MPa and higher) because of significant decrease of carbon dioxide selectivity [8].

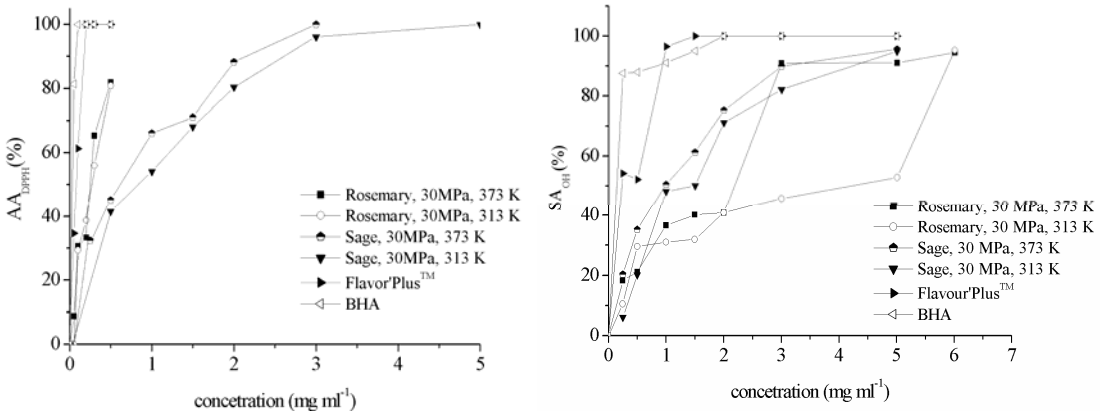


(a) (b)

Figure 1. Yield of antioxidative fraction as function of specific amount of solvent (kg CO2/ kg herbaceous material) for SFE from rosemary at 30 MPa and 100°C (a) and from rosemary and sage at 30 MPa and 40°C (b).

According to the ESR data all investigated extracts scavenged DPPH and hydroxyl radicals in a concentration dependent manner. The antiradical activity (AA_{DPPH}, %) measured by ability of different concentrations of antioxidant fractions isolated from rosemary leaves to scavenge stable DPPH radicals is presented in Figure 2a. The rosemary and sage antioxidant extracts obtained at 30 MPa showed high antiradical activity but they didn't match the strength of synthetic antioxidant (BHA) and commercial rosemary extract (Flavour'Plus^{TM}) when used at the same level. According to this method rosemary antioxidant fractions exhibited higher antiradical activity than sage at low concentrations (<0.5 mg ml $^{-1}$). The antioxidative activity of the rosemary and sage extracts was investigated by the ability of the extracts to scavenge hydroxyl radicals as well (Figure 2b) because of

the fact that hydroxyl radicals were mentioned as the major active oxygen species causing lipid oxidation [17]. Sage extracts showed higher scavenge activity than rosemary extracts at lower concentrations, while at concentrations of 3 mg ml^{-1} scavenging activity (SA_{OH}, %) of sage and rosemary (100°C) antioxidant fraction was similar to BHA and commercial rosemary antioxidant (Flavour'Plus™). Rosemary extract obtained at 40°C showed poor ability to scavenge reactive hydroxyl radicals.



(a) (b)

Figure 2. The antiradical activity (AA_{DPPH}, %) of different concentrations of rosemary and sage antioxidant fractions, Flavor'Plus™ and BHA on DPPH radicals (a) and scavenging activity (SA_{OH}, %) of different concentrations of rosemary and sage antioxidant fractions, Flavor'Plus™ and BHA on DMPO-OH spin adduct (b).

Antioxidative fractions from rosemary and sage isolated at higher temperature (100°C) showed higher antiradical activity (AA_{DPPH}, %) as well as scavenging activity (SA_{OH}, %) than those obtained at lower temperature. It was reported that supercritical antioxidant extracts of rosemary and sage isolated at higher pressures 35-50 MPa showed the highest levels of antioxidant activity at least equal to BHA/BHT (1:1) [8].

Table 2. Results of preliminary LC-MS analysis of chemical composition of rosemary antioxidant fraction isolated at 30 MPa and 100°C.

Compounds	t_R (min)	formula	mass
cirsimaritin	16.10	$C_{17}H_{14}O_6$	314.08
genkwanin	17.32	$C_{16}H_{12}O_5$	284.07
rosmarinic acid	18.16	$C_{18}H_{16}O_8$	360.08
7-methyl-epirosmanol	18.74	$C_{22}H_{30}O_6$	390.20
rozmadial	20.07	$C_{20}H_{24}O_5$	344.16
epirozmanol methyl ether	20.31	$C_{21}H_{28}O_5$	360.19
carnosic acid	20.63	$C_{20}H_{28}O_4$	332.20
4'-methoxytectochrysin	21.25	$C_{17}H_{14}O_5$	298.08
rosmanol, epirozmanol, epiisorozmanol	23.05	$C_{20}H_{26}O_5$	346.18
methyl carnosate	24.39	$C_{21}H_{30}O_4$	346.21
carnosol, carnosol isomer	50.83	$C_{20}H_{26}O_4$	330.18

Rosemary antioxidant fraction isolated at 30 MPa and 100°C which exhibited higher antioxidant activity (AA_{DPPH}, %) was chemically characterized by means of LC-MS. Preliminary results of LC-MS analysis are given in table 2. Eleven compounds, including phenolic diterpenes (e.g. carnosic acid, carnosol, rosmadial, rosmanol, 7-methyl-epirosmanol, etc.), flavonoids (cirstimaritin and genkwanin) and phenolic acid (rosmarinic acid) were indentified by means of tentative analysis of chemical composition.

CONCLUSION

This study showed that at higher operating temperature of supercritical CO_2 extraction (100°C; 30 MPa) higher yields of antioxidant fraction from rosemary and sage could be obtained. Rosemary and sage supercritical extracts possess significant free radical scavenging activity on stable DPPH and high reactive hydroxyl radicals. Therewith, antioxidant fractions of rosemary and sage isolated under 30 MPa at higher temperature of CO_2 SCE (100°C) had a higher antioxidant activity than those obtained at lower temperature (40°C) but still lower than BHA and commercial rosemary antioxidant. Rosemary antioxidant fractions (CO_2 SCE at 40°C and 100°C; 30 MPa) had a higher antioxidant activity then sage on stable DPPH radicals when used at same level. Rosemary antioxidant fraction obtained at CO_2 SCE 100°C and 30 MPa as well as sage antioxidant fractions (CO_2 SCE at 40°C and 100°C; 30 MPa) had similar ability to scavenge hydroxyl radicals as BHA and commercial rosemary antioxidant at concentrations of 3 mg ml^{-1} and higher, while rosemary extract obtained at 40°C and 30 MPa had poor scavenging effect. In conclusion, this study indicates that supercritical extracts of rosemary and sage can be promising alternative to synthetic antioxidants although they should be tested further in the actual food under realistic conditions prior to practical use.

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