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Supercritical carbon dioxide extraction of antioxidants from rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.)

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Abstract: The aim of the present study was to isolate and characterize antioxidant extracts obtained from dried leaves of rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.), originating from the southern Balkan Region. The antioxidant fraction was isolated from the plant material by supercritical carbon dioxide (SC-CO_2) fractional extraction under a pressure of 30 MPa and at temperatures of 40 and 100 °C. In the present study, kinetic data and yields of antioxidant extracts obtained from dried leaves of rosemary and sage under different conditions were determined. Electron spin resonance (ESR) spectroscopy assay on the ability of the extracts to scavenge stable 2,2--diphenyl-1-picrylhydrazyl (DPPH) free radicals and reactive hydroxyl radicals during the Fenton reaction trapped by 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) showed that the investigated extracts had antioxidant activity comparable to that of butylated hydroxyanisole (BHA) and commercial rosemary extract. The antioxidant fractions isolated at the higher temperature had higher antioxidant activities. A tentative analysis of the chemical composition of the antioxidant fractions obtained at the higher temperature was accomplished by LC-DAD and LC-MS analytical methods. Abietane-type diterpenoids, flavonoids and fatty acids were identified in the SC-CO_2 extract of rosemary and sage.

Keywords: rosemary; sage; supercritical extraction; antioxidant; DPPH; hydroxyl radicals.

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INTRODUCTION

Herbs and spices have traditionally been used to impart flavour and aroma to food and for the prevention and treatment of a wide range of diseases. Recently, they have been extensively studied for their 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] In order to produce plant extracts without flavour, odour and colour and with sufficient antioxidant activity to allow usage at levels equivalent to those of synthetic antioxidants (0.01–0.05 %), a number of different techniques for the isolation and concentration of antioxidants from rosemary and sage were proposed: solvent extraction (with polar and non-polar solvents),[2–5] aqueous alkaline extraction,[6] extraction with MCT (medium-chain triglycerides),[7] steam distillation and molecular distillation.[8] Almela *et al*.[3] and Erkan *et al*.[4] investigated the chemical composition and antioxidant activity of methanol extracts isolated from rosemary leaves. Haworth *et al.*[2] showed that a blend of tetrafluoroethane, acetone and methanol improved the total yield while a tetrafluoroethane and acetone blend had a higher efficacy but comparatively lower yields. The study of Tena *et al*.[5] indicated that the hydrogen-bonding ability of acetone and methanol was crucial for the extraction of the phenolic diterpenes responsible for antioxidant activity from rosemary leaves. Solvent extraction, which is generally used for the extraction of antioxidants from plant material, has some drawbacks, including antioxidant transformation, quite low selectivity, the extract is rich in compounds which may interfere with HPLC analysis, co-extracted aroma compounds must be eliminated, and extraction solvent residues are quite often prohibited by food regulations.[9] Molecular and steam distillation used to concentrate active fraction and to remove colour, aroma and flavour components result in different dilution effects due to the presence of the distillation carrier, which has a detrimental impact on the solubility of the extract in fats and oils,[10] while extraction with animal and vegetable oils suffers from low selectivity.[11]

Compared to mentioned methods, supercritical carbon dioxide (SC-CO_2) extraction appears to be an advantageous technology for the isolation of natural antioxidants from rosemary[5,9,10,12–15] and sage.[10,16–18] Tuning the process parameters (pressure, temperature) enables the tuning of the selectivity of SC-CO_2 towards the desirable components, as well as phase separation so that solvent-free extracts are obtained. In order to increase yields of antioxidants from rosemary at similar conditions, some authors added modifiers (co-solvents) such as ethanol. The use of modifiers generally increases the solubility of polar substances in carbon dioxide, although higher concentrations of modifiers can affect the selectivity.[10,12] Modifiers are not recommendable for supercritical extraction of antioxidants from Lamiaceae herbs at higher pressures (*e.g.*, 50 MPa and

higher) because of the significant decrease of the carbon dioxide selectivity and thus lower antioxidant activity of the supercritical extracts.[10] Nguyen *et al.*[10] isolated antioxidant fractions with a high efficiency from Lamiaceae herbs (rosemary, sage, thyme and oregano) with SC-CO_2 extraction under pressures in the range 50–100 MPa and at temperatures in the range 90–110 °C without using modifiers. In the same study, volatiles were recovered in a second separator at 3––3.5 MPa and 5–20 °C. Cavero *et al.*[12] isolated an antioxidant fraction from rosemary leaves with SC-CO_2 extraction under lower pressures and temperatures (15–35 MPa; 40–60 °C) without modifier and with 4 and 7 % of ethanol. The same authors used fractional separation whereby the volatiles were recovered in a second separator at 2 MPa and 20 °C. Ibanez *et al.*[13] used fractional extraction to isolate volatiles at 10 MPa and 40 °C and the antioxidant fraction at 40 MPa and 60 °C without using a modifier. Senorans *et al.*[14] isolated antioxidant extracts from rosemary at 30–35 MPa and temperatures in the range 40–60 °C with 0–2 % ethanol as modifier, while the volatiles were collected in a second separator at 2––5.5 MPa and 25 °C. Several authors used extraction of crude extracts of rosemary obtained by conventional methods (distillation, solvent extraction) to concentrate the antioxidant fraction.[17,19–21] Thereby, Braida *et al.*[19] concentrated extracts with antioxidative properties derived from Labiatae family herbs by means of an extraction–adsorption–desorption procedure using supercritical carbon dioxide as the solvent. Celiktas *et al.*[20] used SC-CO_2 to extract antioxidant fractions from distilled rosemary leaves collected from different locations and at different harvesting time intervals. In order to improve the antioxidant properties of a bleached alcoholic extract of sage, Djarmati *et al.*[17] used SC-CO_2 extraction to obtain antioxidant fractions at 60 °C and pressures of 20, 30 and 40 MPa and at 100 °C and 50 MPa. It was reported that antioxidant extracts obtained by SC--CO_2 extraction from rosemary and sage have an equivalent or stronger antioxidant activity than synthetic antioxidants.[10,16,17] The obtained supercritical extracts of rosemary and sage are semi-solid at ambient temperature. Furthermore, they can be ground at a temperature of –18 °C and dissolved or dispersed in animal or vegetable oils and fats.[10]

Earlier studies reported that the antioxidant activity of rosemary and sage is attributable to: phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, epirosmanol, 7-methyl-epirosmanol and methyl carnosate, rosmadial;[22,23] rosmaridiphenol and rosmariquinone;[24,25] flavonoids, such as genkwanin, cirstimaritin and scutellarein[12,14] and phenolic acids, such as rosmarinic acid.[26] The list of the components with antioxidant properties isolated from sage is growing, *e.g.*, rosmanol 9-ethyl ether,[17] a range of rosmarinic acid derivatives (salvianolic acid K, salvianolic acid I, sagecoumarin and sagerinic acid) and flavone glycosides (luteolin 7-glucoside, luteolin 7-glucuronide, luteolin 3'-glucuronide, 6-hydroxyluteolin 7-glucoside, apigenin 6, 8-di-C-glucoside).[27] It was reported that SC-CO_2 ex-

traction provides the highest recovery of carnosic acid and carnosol from rosemary leaves compared to acetone, methanol, hexane and dichlormethane extraction.[5,9] Cavero *et al.*[12] and Senorans *et al.*[14] investigated the chemical composition of SC-CO_2 extracts of rosemary isolated under pressures in the range 15–35 MPa and at temperatures in the range 40–60 °C with the addition of ethanol as a modifier. Cuvelier *et al.*[28] studied the chemical composition of the antioxidant extracts of sage and rosemary obtained by different methods, including SC-CO_2 extraction, originating from pilot-plant or commercial sources. The study of Djarmati *et al*.[17] was aimed at isolating and identifying rosmanol-9-ethyl ether from *Salvia officinalis* by SC-CO_2 extraction of ethanol extracts and column-chromatographic isolation.

The present study was aimed at studying the kinetics of the isolation of the antioxidant fraction from dried leaves of wild growing rosemary and sage originating from the southern Balkan region using SC-CO_2 extraction and investigating the antioxidant activity of the SC-CO_2 extracts. The antioxidant activities of the plant extracts were evaluated by the scavenging activities of 2,2-diphenyl--1-picrylhydrazyl (DPPH) and of the hydroxyl radical using electron spin resonance (ESR) spectroscopy. Additionally, both liquid chromatography (LC)-mass spectrometry (MS) with an electrospray (ES) and liquid chromatography (LC) with a diode-array detector (DAD) were employed to perform the analysis and identification of the compounds responsible for the antioxidant activity of the rosemary and sage extract obtained at 30 MPa and 100 °C, which exhibited the highest antioxidant activity amongst the investigated supercritical extracts. As far as a literature survey ascertained, there have been no reports on the chemical composition of SC-CO_2 antioxidant extracts isolated from rosemary and sage at 30 MPa and 100 °C.

EXPERIMENTAL

Plant material

Dried leaves of wild growing rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.), originating from the southern Balkan region, were used for experimental studies. Commercial carbon dioxide (99 % purity, Tehno-gas, Novi Sad, Serbia) was used for the extractions.

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and butylated hydroxyanisole (BHA) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Commercial rosemary antioxidant, Flavor'Plus™, was purchased from Naturex, France. Methanol for HPLC, GC, pesticide residue analysis and spectrophotometry, purchased from Burdick & Jackson (Mashegon, MI, USA), acetonitrile gradient grade for liquid chromatography, purchased from Merck KG (Darmstadt, Germany), formic acid, 85 % pure, purchased from Lach-Ner, s.r.o. (Neratovice, Czech Republic) and Milli Q water 18.2 MΩ cm, obtained from a Millipore Simplicity 185 purification system, were used for the LC-MS

analyses. Carnosol and carnosic acid (≥91 % purity) were purchased from Sigma-Aldrich (USA).

Extraction method

Extractions with SC-CO_2 were preformed in a pilot-plant-scale supercritical fluid system (Autoclave Engineers SCE Screening System) with a previously described 150 ml extraction cell.[29] The plant material was fine milled to an average particle diameter of 0.4 mm. Fractional extraction was applied in order to obtain the antioxidants separately from the essential oil. The first fraction comprising essential oils was extracted at a pressure of 11.5 MPa and at a temperature of 40 °C. Thereafter, the antioxidant fraction was extracted at 30 MPa and at temperatures of 40 and 100 °C. The initially used mass of the plant samples was 64.05 g for rosemary and 56.20 g for sage. The mass flow rate of SC-CO_2 was 0.3 kg/h.

DPPH radical assay

A blank probe was obtained by mixing 400 µl of a 0.40 mM methanolic solution of DPPH and 200 µl of DMF (*N*,*N*-dimethylformamide). A volume of x µl of a 10 mg/ml DMF solution of the investigated extracts was added to a mixture of (200 – x) µl of DMF and 400 µl of 0.40 mM methanolic solution of DPPH radical (probe). The range of concentrations of the investigated extracts was 0.05–1.0 mg/ml for rosemary and 0.25–3.0 mg/ml for sage. Then the mixture was stirred for 2 min and transferred to a quartz flat cell ER-160FT. The ESR spectra were recorded on a Bruker 300E ESR spectrometer (Rheinstetten, Germany) under the following conditions: field modulation 100 kHz, modulation amplitude 0.256 G, 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, temperature 23 °C.

The SA_{DPPH} value of an extract is defined as:

$$SA_{DPPH}\ (\%) = 100(h_0 - h_x)/h_0$$

where h_0 and h_x are the height of the second peak in the ESR spectrum of DPPH radicals of the blank and the probe, respectively.

Hydroxyl radical assay

Hydroxyl radicals were obtained by the Fenton reaction in the system: 0.20 ml of 2.0 mM H_2O_2, 0.20 ml of 0.30 mM $FeCl_2{\cdot}4H_2O$ and 0.20 ml of 112 mM DMPO as the spin trap (blank). The influence of the investigated extracts of rosemary and sage on the formation and transformation of hydroxyl radicals was conducted by adding DMF solutions of the extracts to the Fenton reaction system in the concentrations range 0.25–10 mg/ml. The ESR spectra were recorded after 2.5 min, with the following spectrometer settings: field modulation 100 kHz, modulation amplitude 0.512 G, receiver gain 1×10^4, 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, temperature 23 °C.

The SA_{OH} value of an extract is defined as:

$$SA_{OH}\ (\%) = 100(h_0 - h_x)/h_0$$

where h_0 and h_x are the height of the second peak in the ESR spectrum of the DMPO-OH spin adduct of the blank and the probe, respectively.

LC analysis of the rosemary extracts with DAD and MS detection

In the present study, the chemical characterization of the supercritical fluid extracts of rosemary and sage obtained at 30 MPa and 100 °C was accomplished using both liquid chromatography (LC)-mass spectrometry (MS) with electrospray ionisation (ESI) and liquid chro-

matography (LC) with a diode-array detector (DAD). The samples were prepared by dissolving rosemary and sage supercritical extracts into methanol (c = 5.000 mg/ml). The analysis was performed using an HPLC instrument (Agilent 1200 Series, Agilent Technologies) with a degasser, an autosampler, a Zorbax Eclipse Plus C18 (150 mm×4.6 mm i. d.; 1.8 μm) column and a diode-array detector (DAD) coupled with a 6210 Time-of-Flight LC/MS system (Agilent Technologies). The mobile phase was a mixture of solvent A (0.20 % formic acid in water) and solvent B (acetonitrile) according to a combination of isocratic and gradient modes of elution: 0–1.5 min, 95 % A, 1.5–26 min, 95–5 % A, 26–35 min, 5 % A, at a flow rate of 1.40 ml/min. Detection was accomplished by using diode-array detector system (DAD), storing the signals in the wavelength range from 190–400 nm. The injection volume was 5 μl and the column temperature was 40 °C. A personal computer system running Mass Hunter Workstation software was used for data acquisition and processing. In the atmospheric pressure ionization ESI method, the eluted compounds were mixed with nitrogen in a heated nebuliser interface and the polarity was tuned to negative. An adequate calibration of the ESI parameters (capillary voltage, gas temperature, nebuliser pressure, and fragmentor voltage) was required to optimise the response and to obtain a high sensitivity of the molecular ion. The MS conditions were as follows: capillary voltage, 4000 V; gas temperature, 350 °C; drying gas, 12 ml/min; nebuliser pressure, 45 psig; fragmentor voltage, 140 V; mass range, 100–2000 m/z.

RESULTS AND DISCUSSION

Fractional extraction using SC-CO_2 was performed with the view to isolate and concentrate the antioxidant fraction from the rosemary and sage separately from the essential oils. The first fraction, which comprised the essential oil, was extracted at 11.5 MPa and a temperature of 40 °C in order to collect the aromatic and highly volatile components, mostly mono- and sesquiterpenes, and their oxygennated derivates. The obtained yields of the first fraction were 2.26 % (w/w) for sage and 1.03 % (w/w) for rosemary. The antioxidant fraction was isolated at a pressure of 30 MPa and at temperatures of 100 and 40 °C. The extraction yields of the antioxidant fraction obtained from rosemary and sage in the performed experiments are presented in Table I.

TABLE I. Yields of rosemary and sage antioxidant fractions in the performed experiments

Herbaceous material	p / MPa	t / °C	w / wt. %
Rosemary	30	40	1.10
		100	1.57
Sage	30	40	1.35
		100	1.74

The extraction yield curves of the antioxidant fractions extracted from rosemary and sage, performed at a pressure of 30 MPa and at temperatures of 40 and 100 °C, are presented in Figs. 1 and 2, respectively. Comparative extraction curves of rosemary and sage antioxidant extracts isolated under a pressure of 30 MPa at a temperature of 40 and 100 °C are presented in Figs. 3 and 4, respectively. As expected, at the lower operating temperature, lower yields of the antioxidant fraction from rosemary and sage were obtained. It was previously sug-

gested that the optimum rates and yields of SC-CO_2 extraction of antioxidants from Lamiaceae herbs are attained at temperatures between 90 and 110 °C at pressures above 30 MPa.[10] At extraction temperatures above 110 °C, heat damage can occur to the extracted compounds as well as to the extracted residue.[10] In the mentioned work,[10] the obtained yields of rosemary and sage supercritical extract isolated under a pressure of 50 MPa at a temperature of 100 °C were 5.2 and 5.7 %, respectively. Lower extraction temperatures are recommendable for economic reasons and especially for fractional separation when the aroma fraction is to be used further.[30]

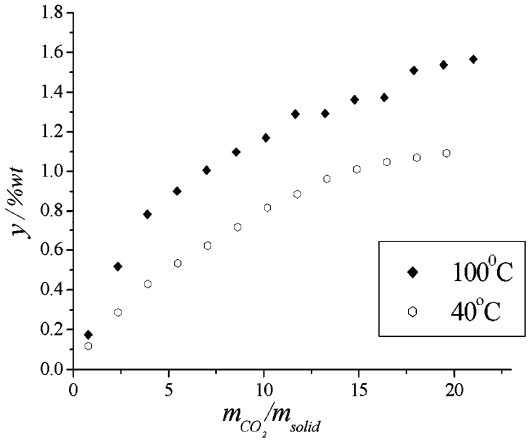

Fig. 1. Yields of rosemary antioxidant fractions as a function of the specific amount of solvent (kg CO_2/kg herbaceous material) for SC-CO_2 extraction at 30 MPa and different temperatures.

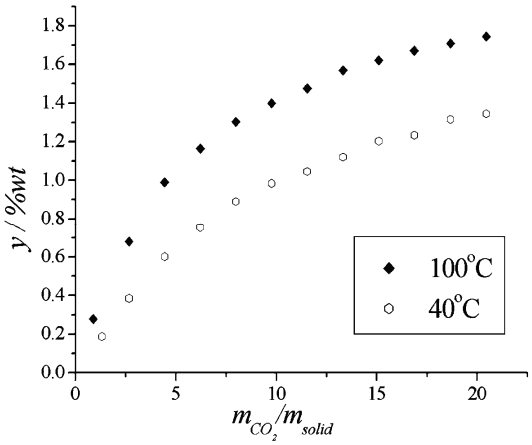

Fig. 2. Yields of sage antioxidant fractions as a function of the specific amount of solvent (kg CO_2/kg herbaceous material) for SC-CO_2 extraction at 30 MPa and different temperatures.

Daukşas *et al.*[16] investigated the influence of modifier (0–2 % of ethanol) on the yield of the SC-CO_2 extract of sage isolated under pressures of 25 and 35 MPa and at a temperature of 100 °C. They reported a significant increase in the total yield of SC-CO_2 extract after the addition of 1 % ethanol, while further

addition of ethanol was not efficient. In the same study,[16] the results clearly show that a large part of the sage substances is soluble at 30 MPa and higher pressures. A pressure between 25 and 30 MPa can be considered as a critical one in terms of the solubility of approximately 50 % of the sage extractives isolated at 35 MPa with CO_2 enriched with 1 % of ethanol.[16]

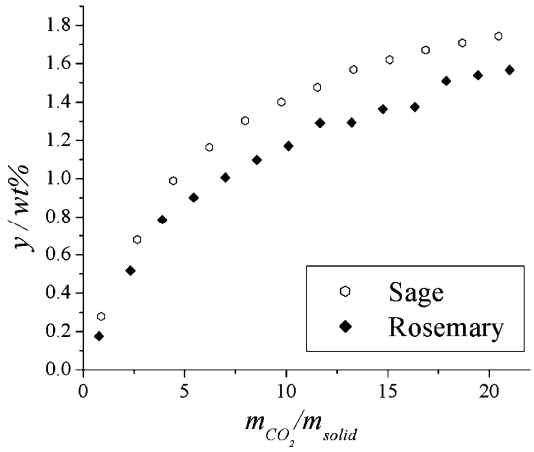

Fig. 3. Yields of antioxidant fraction as a function of the specific amount of solvent (kg CO_2/kg herbaceous material) for SC-CO_2 extraction from rosemary and sage at 30 MPa and 100 °C.

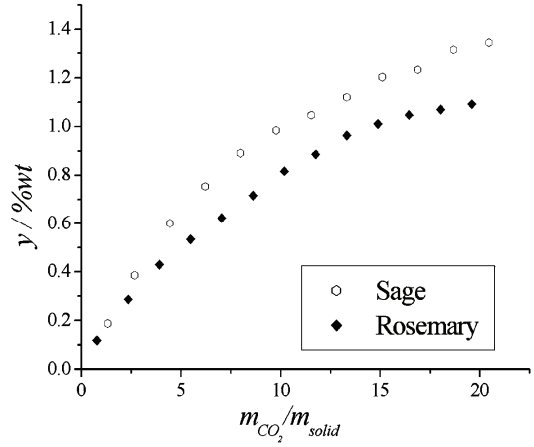

Fig. 4. Yields of antioxidant fraction as a function of the specific amount of solvent (kg CO_2/kg herbaceous material) for SC-CO_2 extraction from rosemary and sage at 30 MPa and 40 °C.

In the previously published paper, it was reported that the yield of rosemary antioxidant extract obtained by a one-step supercritical extraction under a pressure of 30 MPa was 3.3 and 5 % at a temperature of 30 and 40 °C, respectively.[16] Ibanez *et al.*[13] isolated rosemary antioxidant extract by a two-step extraction at a pressure of 40 MPa at a temperature of 60 °C and obtained 1.0–1.8 % yields. Under similar conditions (pressures in the range 15–35 MPa and temperature in the range 40–60 °C), Cavero *et al.*[12] obtained yields of rosemary antioxidant

extract of 3.93–6.78 %. The yield of SC-CO_2 sage extract (Lithuania) isolated by Dapkevicius *et al.*[18] at a pressure of 30 MPa and temperature of 40 °C was 5.02 %.

The yields of antioxidant extracts of rosemary and sage reported in this study were lower than the yields of antioxidant extracts of rosemary and sage reported in previously published papers. This could be explained in terms of different cultivation conditions, geographical locations and climate conditions. Celiktas *et al.*[20] also reported that the geographical location and seasonal variation have a great influence on the amount of active components in SC-CO_2 extracts. In this study, wild growing rosemary and sage from the southern Balkan region were used to obtain antioxidant extracts. Therewith, in order to achieve higher selectivity of the SC-CO_2 extraction and thus higher antioxidant activity of the SC--CO_2 extracts, no modifiers were used in this study. This could also be the reason for the lower yields of antioxidant extracts reported in this study.

According to the ESR data, all the investigated extracts scavenged DPPH and hydroxyl radicals in a concentration dependent manner. The scavenging activity (SA_{DPPH}, %) measured by the ability of different concentrations of antioxidant fractions isolated from rosemary leaves to scavenge the stable DPPH radicals is presented in Fig. 5. When the concentration of the SC-CO_2 extract from rosemary was increased from 0.010 to 1.0 mg ml^{-1}, the scavenging effect on the DPPH radicals was increased from 30 to 100 %. In the case of sage extracts, when the concentration was increased from 0.25 to 5.0 mg ml^{-1}, the scavenging effect on the DPPH radicals was increased from 32 to 100 % (for the extract isolated at a temperature of 100 °C) and from 18 to 100 % (for the extract isolated at a temperature of 40 °C). As can been seen, the scavenging activity of the rosemary and sage antioxidant extracts obtained at 30 MPa showed the same scavenging activity as a synthetic antioxidant (BHA) and a commercial rosemary extract (Flavor'Plus^{TM}) at a concentration of 1.0 mg ml^{-1} (for the rosemary extracts) and at concentrations in the range of 3.0–5.0 mg ml^{-1} (for the sage ex-

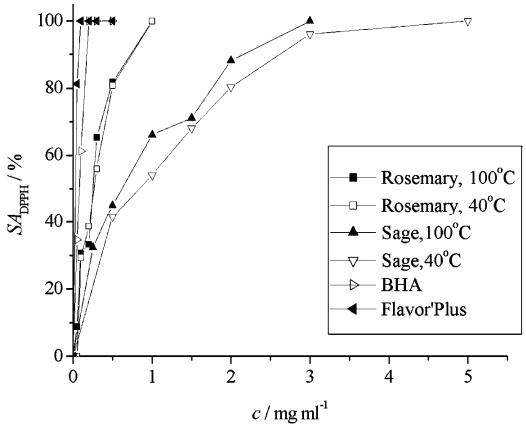

Fig. 5. The scavenging activity (SA_{DPPH}, %) of different concentrations of rosemary and sage antioxidant fractions, Flavor'Plus^{TM} and BHA on DPPH radicals.

tracts). According to this method, rosemary antioxidant fractions exhibited higher antioxidant activities than the sage ones. It can be seen that even a concentration of 0.5 mg ml^{-1} of rosemary extracts reduced 81 % of the DPPH radicals. Sage extracts reduced 80–88 % of the DPPH radical molecules at a concentration of 2 mg ml^{-1}.

The antioxidative activities of the rosemary and sage extracts were investigated by the ability of the extracts to scavenge hydroxyl radicals as well (Fig. 6) because of the fact that hydroxyl radicals were mentioned as the major active oxygen species causing lipid oxidation.[31] To test the reactions of hydroxyl radicals with the investigated extracts, the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} +$ $+ OH^- + {}^{\bullet}OH$) was used as a source of hydroxyl radicals. Using a spin trap such as DMPO, it is possible to convert the reactive hydroxyl radicals into stable nitroxide radicals (DMPO–OH adducts). The relative intensity of the free radical formation can be determined because the intensity of the ESR spectroscopy signal is directly related to the concentration of the spin adducts. The scavenging activity (SA_{OH}, %) increased in the presence of 0.25–10 mg ml^{-1} SC-CO_2 extracts of rosemary from 18 to 100 % (for the extract isolated at 100 °C) and from 11 to 100 % (for the extract isolated at 40 °C). The scavenging effect (SA_{OH}, %) of the same concentrations of sage extracts increased from 20 to100 % (for the sage extract obtained at 100 °C) and from 6 to 100 % (for the sage extract obtained at 40 °C). The scavenging activities (SA_{OH}, %) of the sage and rosemary antioxidant fractions were the same as those of BHA and a commercial rosemary antioxidant (Flavor'Plus^{TM}) at concentrations from 5 to 6 mg ml^{-1} and higher. The rosemary extract obtained at 40 °C showed a much lower ability to scavenge reactive hydroxyl radicals in comparison to the other extracts at concentrations from 3 to 6 mg ml^{-1}. The rosemary extract isolated at 100 °C and the sage extracts showed satisfactory scavenging activity (82–91 %) at a concentration of 3 mg ml^{-1}.

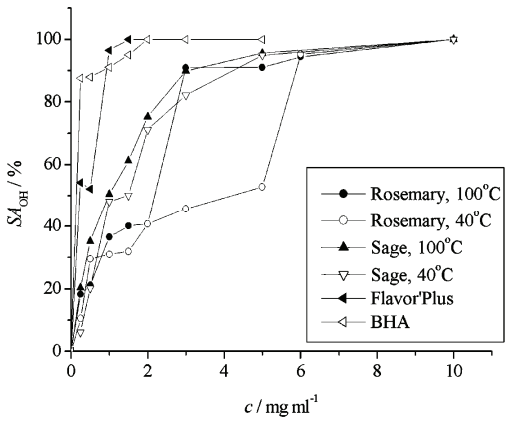

Fig. 6: The scavenging activity (SA_{OH}, %) of different concentrations of rosemary and sage antioxidant fractions, Flavor'Plus^{TM} and BHA on the DMPO--OH spin adduct.

Antioxidant fractions from rosemary and sage isolated at the higher temperature (100 °C) showed higher scavenging activities (SA_{DPPH} and SA_{OH}, %) than those obtained at the lower temperature. This is in accordance with previously published results on the antioxidant activity of SC-CO_2 extracts of Lamiaceae herbs.[10] Namely, the supercritical antioxidant extracts of rosemary and sage isolated at higher pressures 35–50 MPa at a temperature of 100 °C showed the highest levels of antioxidant activity, at least equal to BHA/BHT (1:1).[10] The SC-CO_2 extracts of rosemary isolated by Peng *et al.*[32] at 34.5 MPa and 80 °C showed a higher antiradical activity than BHA, trolox and ascorbic acid at all concentration levels according to the DPPH radical assay.

The rosemary and sage antioxidant fractions isolated at 30 MPa and 100 °C were chemically characterized by means of LC-MS.

The preliminary results of the LC–MS analysis are shown in Tables II and III. According to the tentative analysis of the chemical composition by means LC–MS, the most abundant components in the rosemary SC-CO_2 extract were abietane-type diterpenoids (*e.g.*, carnosic acid, carnosol, rosmadial, cafestol, rosemaridiphenol, methyl carnosate, 12-methoxycarnosic acid, *seco*-hinokiol, *etc*.) and flavonoids (wogonin, 7-methylapigenin, oroxylin A, biochanin A, genkwanin, negletin, acacetin, 5,6-dihydroxy-7-methoxyflavone). Similar components were identified in rosemary extracts isolated at pressures of 15–35 MPa and at temperatures of 40–60 °C [12,14] with ethanol as modifier and sub-critical water under pressures of 4–7 MPa and at temperatures of 25–200 °C.[65] Among the compounds in the SC-CO_2 extract of sage identified by the tentative analysis of chemical composition by means of LC–MS, abietane diterpenoids (carnosol, epiisorosmanol, royleanonic acid, royleanone, epirosmanol methyl ether, rosmanol, rosmadial, galdosol, carnosol *p*-quinone, safficinolide), fatty acids (C_{18}) and a triterpene (allobetulonlactone-1-en-2-ol) were identified.

TABLE II. Results of a preliminary LC-MS analysis of the chemical composition of rosemary antioxidant fraction isolated at 30 MPa and 100 °C

Formula	M_r / g mol^{-1}	t_R /min	Compounds
$C_{12}H_{18}O_3$	210	12.33	Jasmonic acid[33], vanillyl butyl ether[34]
$C_{16}H_{12}O_5$	284	15.08	Wogonin[35], genkwanin[12,36,37], oroxylin A[35], Biochanin A[38], acacetin[35], prunetin[39], 5,7 dihydroxy-6-methoxyflavone[35]
$C_{18}H_{32}O_4$	312	17.27	Oxiraneoctanic acid[40]
$C_{19}H_{28}O_4$	320	17.89	Ubiquinol-10[41]
$C_{20}H_{26}O_4$	330	18.41	Carnosol
$C_{20}H_{26}O_4$	330	18.64	Carnosol isomer
$C_{20}H_{24}O_5$	344	19.16	Rosmadial[42]
$C_{20}H_{28}O_3$	316	19.98	Rosemaridiphenol[43], cafestol[44], *seco*-hinokiol[45]
$C_{20}H_{28}O_4$	332	20.53	Carnosic acid
$C_{21}H_{30}O_4$	346	21.75	Methyl carnosate[46], 12-methoxycarnosic acid[46]
$C_{20}H_{30}O_3$	318	22.83	[9]-Shogaol[47]

TABLE III. Results of a preliminary LC-MS analysis of the chemical composition of sage antioxidant fraction isolated at 30 MPa and 100 °C

Formula	Mass	t_R / min	Compounds
$C_{10}H_{16}O_3$	184.23	9.62	*α*-Camphlonic acid[48], *cis*-pinonic acid[49]
$C_{20}H_{26}O_5$	346.42	13.96; 14.50; 15.19; 18.16	Rosmanol[43], epirosmanol[50], isorosmanol[51], royleanonic acid[52], epiisorosmanol[28]
$C_{20}H_{28}O_4$	332.43	14.66	Horminone[53], hydroxyroyleanone[54]
$C_{20}H_{24}O_5$	344.16	17.72;19.27; 19.77	Rosmadial[28], galdosol[55], carnosol *p*-quinone[56], safficinolide[57]
$C_{21}H_{28}O_5$	360.44	18.27	7-Methoxyrosmanol[52], epirosmanol methyl ether[28]
$C_{20}H_{26}O_4$	330.18	18.53	Carnosol (picrosalvin)
$C_{20}H_{26}O_4$	330.18	19.07	11,12-di-*O*-Methyl-picrosalvin[58]
$C_{20}H_{28}O_3$	316.44	19.90	Royleanone[59], rosmaridiphenol[48], 20-deoxocarnosol[60]
$C_{20}H_{28}O_4$	332.20	20.64	Carnosic acid (salvin)
$C_{21}H_{30}O_4$	346.21	21.88	12-*O*-Methylcarnosic acid[55], Methyl carnosate[43]
$C_{20}H_{30}O_3$	318.22	21.96; 22.97	2-Hydroxy-6-((6*Z*)-6-tridecenyl)-benzoic acid[61]
$C_{20}H_{28}O_2$	300.44	23.66	Retinoic acid[62], dehydroabietic acid[63], dehydro-4-epiabietic acid[63]
$C_{18}H_{30}O_2$	279.44	23.97; 25.36	Linolenic acid[64], trans-10-*cis*-12-octadecadienoic acid, *trans*-11-*cis*-9-Octadecadienoic acid[65]
$C_{30}H_{44}O_4$	468.32	28.68; 30.12; 30.49	Allobetulonlactone-1-en-2-ol[66]

As can been seen, both the rosemary and sage antioxidant fraction contained carnosic acid and its derivative carnosol. Miura *et al.*[55] reported that the antioxidant activity of carnosic acid, carnosol, rosmanol, isorosmanol, epirosmanol and galdosol (isolated from sage), measured by the OSI method using methyl linoleate at 90 °C and the DPPH method, were comparable to those of *α*-tocopherol and ascorbic acid. According to previously published results of Cavero *et al.*[12] carnosic acid is considered the main component in the rosemary extract isolated by SC-CO_2 responsible for the antioxidant activity determined by the DPPH test and the *β*-carotene bleaching assay.

The identification of carnosic acid and of carnosol was based on the retention time and authentic samples. The other compounds were tentatively identified in accordance with the molecular formula and data found by the Substance Identifier and Molecular Formula Search in SciFinder Scholar. The most probable compounds found in *R. officinalis* and *S. officinalis* related to adequate molecule formulas of detected compounds are given in Tables II and III.

CONCLUSIONS

This study showed that the yields of the antioxidant fraction from rosemary and sage obtained by SC-CO_2 extraction at 30 MPa and 100 °C were much higher than those obtained at a lower temperature (40 °C). Despite the somewhat lower yields, the SC-CO_2 extracts isolated from wild growing rosemary and sage from the southern Balkan region at a pressure of 30 MPa and at temperatures of 40 and 100 °C showed significant free radical scavenging activities towards the stable DPPH and highly reactive hydroxyl radicals, comparable to those of BHA and a commercial rosemary antioxidant. According to the DPPH assay, rosemary and sage antioxidant extracts obtained at 30 MPa showed the same scavenging activity as a synthetic antioxidant (BHA) and a commercial rosemary extract (Flavor'Plus^{TM}) at a concentration of 1.0 mg/ml (for the rosemary extracts) and at concentrations in the range 3.0–5.0 mg ml^{-1} (for the sage extracts). The hydroxyl radical assay showed that the rosemary and sage antioxidant fractions had a scavenging activity the same as those of BHA and a commercial rosemary antioxidant (Flavor'Plus^{TM}) at concentrations from 5 to 6 mg ml^{-1} and higher. Thereby, the antioxidant fractions of rosemary and sage isolated under a pressure of 30 MPa at the higher temperature (100 °C) of SC-CO_2 extraction exhibited somewhat higher antioxidant activities than those obtained at the lower temperature (40 °C). The rosemary antioxidant fractions had a higher antioxidant activity than those of sage towards stable DPPH radicals when used at same level. However, the rosemary and sage antioxidant fractions had a similar ability to scavenge hydroxyl radicals. In conclusion, this study indicates that supercritical extracts isolated from wild growing rosemary and sage from the southern Balkan region can be promising alternatives to synthetic antioxidants, although they need to be tested for the specific application in food.

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И З В О Д

НАТКРИТИЧНА ЕКСТРАКЦИЈА АНТИОКСИДАНАСА ИЗ РУЗМАРИНА (*ROSMARINUS OFFICINALIS* L.) И ЖАЛФИЈЕ (*SALVIA OFFICINALIS* L.)

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Циљ овог рада био је изолација и карактеризација антиоксидативних екстраката рузмарина (*Rosmarinus officinalis* L.) и жалфије (*Salvia officinalis* L.) са подручја јужног Балкана.

Антиоксидативна фракција изолована је из биљног материјала применом фракционе екстракције са наткритичним угљеник(IV)-оксидом на притиску од 30 MPa и на температурама од 40 и 100 °C. У овом раду су приказани резултати испитивања кинетике наткритчне екстракције антиоксидативних фракција из рузмарина и жалфије на различитим условима. Електрон спин резонантна (ESR) спектрална анализа утицаја антиоксидативних екстраката рузмарина и жалфије на трансформацију стабилних 2,2-дифенил-1-пикрилхидразил (DPPH) радикала као и на стварање и трансформацију реактивних хидроксилних радикала образованих у Фентоновој реакцији у присуству «спин-трапа» 5,5-диметил-1-пиролин-*N*-оксида (DMPO), показала је да испитивани екстракти имају антиоксидативну активност упоредиву са бутилованим хидроксианизолом (BHA) и комерцијалним рузмаринским антиоксидансом. Антиоксидативне фракције рузмарина и жалфије изоловане на вишој температури показале су већу антиоксидативну активност. За прелиминарну анализу хемијског састава антиоксидативних екстраката изолованих на вишој температури коришћена је течна хроматографија (LC) са детектором са низом диода (DAD) и течна хроматографија (LC) са масеном спектрометријом (MS). Екстракти рузмарина и жалфије садржали су абијетанске терпеноиде, флавоноиде и масне киселине.

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