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ORIGINAL PAPER

**Maarit Ollanketo · Anna Peltoketo · Kari Hartonen
Raimo Hiltunen · Marja-Liisa Riekkola**

Extraction of sage (*Salvia officinalis* L.) by pressurized hot water and conventional methods: antioxidant activity of the extracts

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Abstract The effectiveness of the extraction of antioxidative compounds from sage (*Salvia officinalis* L.) by pressurized hot water extraction, ultrasonication-assisted methanol extraction, hydrodistillation, and maceration with 70% ethanol was evaluated by determining the capability of the extracts to scavenge the free radical DPPH· (1,1-diphenyl-2-picrylhydrazyl) *in vitro*. Pressurized hot water extraction was found to be the most effective extraction procedure, followed by maceration with 70% ethanol, hydrodistillation, and ultrasonication-assisted methanol extraction. In addition to the total extract, special attention was paid to rosmarinic and carnosic acids, carnosol and methyl carnosate. The extracts were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). The identification of compounds was confirmed by coupling RP-HPLC to mass spectrometry with electrospray ionization.

Keywords Sage · *Salvia officinalis* L. · Extraction techniques · Pressurized hot water · Phenolic antioxidants · 1,1-Diphenyl-2-picrylhydrazyl radical · Free radical scavenging activity

Introduction

The past few years have seen a growing interest in natural foods, with increased demand for nonsynthetic, natural antioxidants. The use of synthetic antioxidants in the food industry is severely restricted by law as to both application and level of use. Valued traditionally as a spice, sage is now being studied because of its antioxidant properties [1, 2, 3].

M. Ollanketo · K. Hartonen · M.-L. Riekkola (✉)
Laboratory of Analytical Chemistry, Department of Chemistry,
P.O. Box 55, FIN-00014 University of Helsinki, Finland
e-mail: Marja-Liisa.Riekkola@helsinki.fi
Fax: +358-9-19150253

A. Peltoketo · R. Hiltunen
Division of Pharmacognosy, Department of Pharmacy,
P.O. Box 56, FIN-00014 University of Helsinki, Finland

During recent decades, different plant-derived extracts and phytochemicals have been ascribed a variety of potentially health-promoting biological activities. The linkage between high intake of foods and certain beverages rich, for example, in antioxidative phenolic compounds and a lowered risk of free-radical-related pathological conditions and diseases has been clearly demonstrated [4, 5, 6]. Free radicals are involved in numerous diseases [7], and close attention is being paid to plants and phytochemicals potentially useful in reducing the risk of oxygen-stress-related chronic diseases such as cardiovascular disease and cancer, the two main causes of death in industrialized countries.

Conventional extraction of plant material often involves, for example, steam distillation or various solid–liquid extraction procedures relying on organic solvents [2, 3, 8]. However, solvent extractions may leave prohibited residues in food. An additional drawback in using organic solvents is the chemical transformations that the components of the extract may undergo during the elimination of the solvent residue.

The demand of the food industry for natural antioxidants prepared with safe solvents has directed attention to more efficient extraction methods, such as supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) [9, 10, 11]. SFE with carbon dioxide as extraction fluid and ASE with low solvent consumption are environmentally more acceptable than the traditional extraction methods. Recently, the concern over pollution of the environment with solvents has increased the interest in water as an extraction medium [12, 13, 14, 15, 16, 17, 18, 19]. A change in temperature alters the dielectric constant, surface tension, and viscosity of water, so that, with mere adjustment of the conditions, water can be made to resemble different organic solvents.

In this work we extracted sage, *Salvia officinalis* L., with pressurized hot water and by three conventional solid–liquid techniques. The extracts were analyzed by high-performance liquid chromatography and the identification of compounds was confirmed by mass spectrometry using electrospray ionization. The objectives of the

research were (1) to evaluate the efficiency of the extraction techniques for sage by measuring the antioxidant activity of the extracts as their ability to reduce the stable nitrogen-centered DPPH· (1,1-diphenyl-2-picrylhydrazyl) radical [20], (2) to examine in detail the effectiveness of pressurized hot water extraction (PHWE) for extraction of the sage matrix, (3) to examine the kinetics of PHWE for selected compounds, and (4) to compare the PHWE results with results obtained by conventional solvent extraction techniques.

Materials and methods

Chemicals and samples

Rosmarinic acid was purchased from ICN Biomedicals Inc. (HPLC-grade, Ohio, USA). The solvents were methanol (J.T. Baker, Deventer, Holland), ethanol (min. 99.5 wt%, Alko, Rajamäki, Finland) and 99.8% acetic acid (Riedel-deHaen, Seelze, Germany). The sage of commercial origin was donated by Pimenta Ltd. (Helsinki, Finland). Distilled water was deionized with a Water I system (Gelman Sciences, Ann Arbor, MI, USA). For the PHWE procedure, sea sand was bought from Riedel-deHaen (Seelze, Germany).

Pressurized hot water extraction and kinetics

The self-constructed apparatus used for the pressurized hot water extractions is presented in Fig. 1. The system consisted of two Jasco PU-980 HPLC pumps (Tokyo, Japan), a Fractovap series 2150 GC oven (Carlo Erba, Milan, Italy), a modified 30–15 HF4-HT high-temperature three-way valve and two 15–11AF1 on/off valves (High Pressure Equipments Co., Erie, PA, USA), a manually adjustable pressure restrictor (Jasco, Tokyo, Japan), an extraction vessel (100 mm×5 mm i.d.) designed for water extraction (Keystone Scientific Inc., Bellefonde, PA, USA), and a solid phase trap (50 mm×2.1 mm i.d.). All connections were made with stainless steel capillaries (1/16 inch o.d., 0.02 inch i.d.). When acetic acid was added as a modifier to improve the recovery, an extra T-junction was added just before the solid phase trap and a third Jasco PU-980 pump (Tokyo, Japan) was used in pumping the modifier.

In PHWE, 0.15 g of dried sage was weighed into the extraction vessel and the vessel was filled with sea sand. The extraction procedure was started by pumping water through the vessel at constant velocity of 1 mL/min, and the variable pressure regulator was adjusted to keep the pressure constant at 100 kg/cm^2. The system was tested for leaks and the temperature was set to either 70, 100, or 150 °C. The extractions were dynamic and took 60 min in all the experiments. Except for the total extract, the extracted analytes were adsorbed onto solid phase trap, which was packed with either C18 or cyclohexyl material (~54 µm, 54 Å, IST, International Sorbent Technology Ltd., Mid Glamorgan, UK). The trap was dried and eluted with two milliliters of methanol at flow rate 1 mL/min. After the extraction the solid phase trap and the capillaries were further flushed with methanol for 2 min and dried with nitrogen. The solid phase trap was not used when the total extract was collected. The volume of the total extract was about 65 mL and the extract was evaporated down on a hot plate and in vacuum to a volume of 50 mL.

The kinetic PHWE experiment was carried out at 100 °C. The 0.15 g of sage in the extraction vessel was extracted in steps of 5–10 min and each fraction was collected separately; final extraction times were 5, 10, 15, 20, 30, 40, 50, 60, and 70 min. Instead of on-line trapping, each water fraction was eluted through a cyclohexyl SPE cartridge with use of two milliliters of MeOH as eluting solvent.

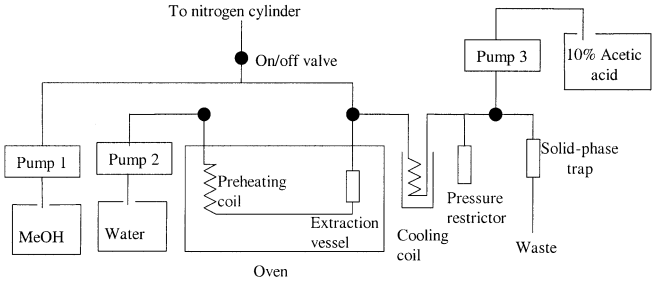

Fig. 1 PHWE instrument constructed in the laboratory

Ultrasonication-assisted extraction with methanol

The dry plant material was milled using a laboratory-scale mill (IKA Labortechnik, Janke&Kunkel Gmbh&Co, Staufen, Germany). 2.5 g of the milled sample was placed in a test tube, 10 mL methanol was added, and the tube was kept in a magnetic stirrer (1000 rpm) for 1 h. The tube was ultrasonicated (Elma Transsonic 460, Elma, Germany) for 20 min and then centrifuged (IEC 4-R Centrifuge, Bedfordshire, UK) at 3000 rpm for 20 min. The supernatant was collected, and the extraction was repeated with 10 mL of MeOH. The combined supernatants were evaporated to dryness under nitrogen, and the dry extract was dissolved in 4 mL of MeOH.

Hydrodistillation

Dried, unmilled sage (25.0 g) was placed in a 1000-mL round-bottom flask, 250 mL of water was added, and the flask was gently swirled to moisturize the sample. The flask was connected to a Ph. Eur. hydrodistillation apparatus (Laborexin Oy, Helsinki, Finland), and the water was left to boil for 2 h. The volatile oil that appeared was collected, and water from the flask was poured through cotton wool into an Erlenmeyer flask. Water (150 mL) was added to the flask and the mixture was left to boil for one hour. The water was poured through cotton wool out into a second Erlenmeyer flask and the plant material was gently pressed to remove most of the water. The water was then filtered through qualitative No. 4 Whatman filter paper (Whatman International Ltd, Maidstone, England). Finally, it was evaporated down on a hot plate and in vacuum and then made up to a final volume of 25 mL in water.

Maceration with 70% ethanol

An amount (5.0 g) of the dried and milled material was placed in an Erlenmeyer flask and, 25 mL 70% EtOH was added; the sample was gently swirled and left to macerate in the dark for 2 days. After this the sample was centrifuged (3000 rpm, 10 min), the supernatant was pipetted out, 10 mL fresh 70% EtOH was added, and the mixture was left to macerate overnight. The sample was again centrifuged, and the supernatants were combined. The solvent was evaporated down in vacuum, and the volume was made up to 20 mL with 70% EtOH.

HPLC/UV analyses

All the extracts were diluted to concentration of 75 mg of herb/mL, except the total extract obtained by PHWE where the concentration was 3 mg of herb/mL. Before the analysis, the extracts were filtered through Gelman (Ann Arbor, NI, USA) Acrodisc 13 CR PTFE (0.45 µm) syringe filters. Extracts were analyzed with an HPLC system consisting of a Hewlett-Packard (Waldbronn, Germany) 1100 system with diode array detector. The injection (20 µL) was done by autosampler with an injection

Table 1 Gradient program for the HPLC analyses

Time (min)	Eluent B, MeOH concentration (%)
0	0
2	0
6	21
14	21
19	39
28	39
30	46
34	46
36	62
48	62
70	100
72	100

needle. Data were collected and analyzed with a Hewlett-Packard computing system (HP ChemStation for LC, Rev. A.06.03). The precolumn (50 mm×2.1 mm i.d.) was packed with Capcell Pak C-18 material (SG, 5 µm, 120 Å, Shiseido, Japan). The analytical column was Luna C-18 (250 mm×3.0 mm i.d., 5 µm, 102 Å, Phenomenex, USA). Analytical separation was achieved at flow rate of 0.5 mL/min with the gradient program presented in Table 1. The program was modified from the original of Cuvelier et al. [21]. Eluent A consisted of 20% methanol (v/v) and 0.8% of acetic acid (v/v) in water. Eluent B was methanol. The temperature was set at 20 °C and the detector monitored wavelength 284 nm in all cases.

HPLC/UV/ESI/MS analyses

A mass spectrometer (ESQUIRE, Bruker Daltonics, GmbH, Bremen, Germany) was used for detection of the analytes. The mass spectrometer included an ion beam focusing hexapole and a nonlinear multiple Paul RF ion trap with resonance ejection. The MS instrument was used in positive mode with voltages for the MS inlet capillary, end plate, and cylinder electrode of –4600 V, –4100 V, and –3500 V, respectively. Nitrogen (200 L/h, 150 °C) was used as drying gas. Ten raw spectra were averaged for each spectrum. The maximum allowed accumulation time was 200 ms. The scan range of positive ion detection was 50–400 u. The HPLC gradient program described above was used unmodified.

Measurement of radical scavenging activity

The antioxidant activity of the sage extracts was determined by measuring their abilities to reduce the stable nitrogen-centered $DPPH^{\cdot}$ (1,1-diphenyl-2-picrylhydrazyl) radical *in vitro*. The sample was diluted to a concentration series in MeOH. One milliliter of $DPPH^{\cdot}$ solution (60 µM in MeOH) was added to 1 mL of each diluted sample. The mixture was vortexed (Vortex-Genie 2, Scientific Industries Inc., NY, USA) and the resulting solution was allowed to develop for 30 min in the dark at ambient temperature [22]. The absorbance caused by the $DPPH^{\cdot}$ radical at 517 nm was determined by Unicam UV 500 Spectrophotometer (Unicam, UK) for a reference sample (A_r) and each test sample (A_t). The antioxidant activity, expressed as percentage inhibition (PI) of the radical absorption, was calculated relative to the reference absorption using the equation $PI(\%)=[1–(A_t/A_r)]\times100$. The IC_{50} (concentration of sample producing 50% reduction of the radical absorbance) values (µM) were calculated using the equation $IC_{50}=C_1–\Delta C$, where $\Delta C=[(C_1–C_2)\times(PI_1–50)]/(PI_1–PI_2)$ and PI_1 is the antioxidant activity value superior to 50% inhibition and PI_2 is inferior to 50% inhibition; C_1 and C_2 are the concentrations corresponding to PI_1 and PI_2, respectively. All data are the means of 4–12 replicates.

Table 2 Antioxidant activity of extracts expressed as IC_{50} values, and time and amount of starting material used in the different extraction methods

Extraction method	$1/IC_{50}$ (mg/mL)	Extraction time (hours)	Amount of herb (g)
70% EtOH maceration	19.3	36	5
Hydrodistilled sage extract	14.3	3	25
Ultrasonication-assisted MeOH extraction	12.0	2.7	2.5
PHWE at 100 °C:			
Total extract, no trap	25.0	1	0.15
Cyclohexyl trap, acetic mod.	6.8	1	0.15
C18 trap, acetic mod.	5.6	1	0.15
Cyclohexyl trap	5.1	1	0.15
C18 trap	4.7	1	0.15

Results and discussion

Identification of compounds

The main antioxidative effect of sage has been reported to relate to the presence of phenolic diterpenes (such as carnosic acid and carnosol) and phenolic acids (such as rosmarinic and caffeic acids) [2, 3], and thus special attention in this study was paid to rosmarinic acid, carnosol, carnosic acid, and methyl carnosate. These compounds were characterized in terms of their retention times and UV and mass spectra. In addition, published data [2, 3, 23] or a commercial standard (rosmarinic acid) were used in the identification of compounds. Carnosol, carnosic acid, and methyl carnosate showed molecular ions $[MH]^+$ at *m*/*z* 331, 333, and 347, respectively. Carnosic acid and methyl carnosate had major fragments at *m*/*z* 287 and 301, respectively, corresponding to the loss of HCOOH group.

Antioxidant activity

In evaluating the antioxidant activities, it should be noted that the antioxidative performance of plant extracts depends not only on the extraction method [1, 24], but also on the quality of the original plant, its geographic origin, the harvesting date, its storage and the processing prior to extraction [3, 23, 24]. In this work the IC_{50} values were used in evaluating the efficiency of the extraction. The maximum total recovery of the compounds was not the most important factor for the extraction efficiency; because of the selectivity of the extraction, the antioxidant activity was sometimes higher where the total recovery was lower than the maximum.

The results of the antioxidant activity measurements are presented in Table 2. The relative standard deviations (RSD) for the measurements were 4%–10% (n=4–12). The lowest IC_{50} values (i.e., highest radical scavenging activities) were obtained for the total PHWE extract (without solid phase trapping). The essential oil

obtained from sage by hydrodistillation was analyzed in our earlier work and its effect on the total radical scavenging activity was found to be almost insignificant [25]. Comparison can therefore be made between the aqueous extract obtained by hydrodistillation and the extracts obtained by other techniques. Antioxidant activities of the extracts obtained by hydrodistillation and ultrasonication-assisted MeOH extraction were similar and slightly weaker than the activity of the extract obtained by maceration with 70% EtOH.

Selected analytes were investigated in this study, but also many other compounds, including diterpenes, triterpenes, and flavonoids, have been isolated from sage [2, 3, 25, 26, 27, 28, 29]. Because most of these are phenolic compounds they may make a significant contribution to the total antioxidant activity. The antioxidative behavior and synergistic action of most of these compounds remain unknown.

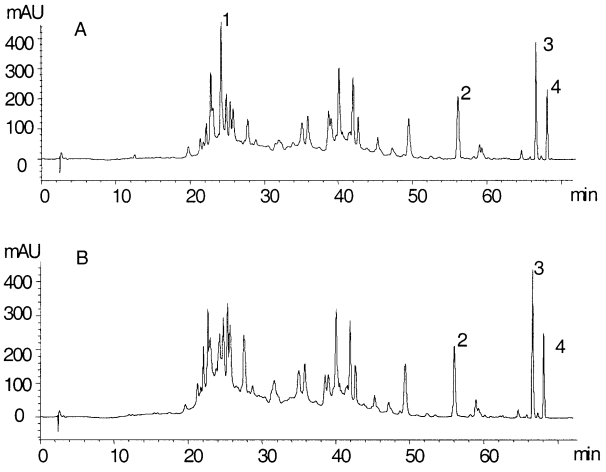

Fig. 2A, B LC chromatograms of extracts obtained by PHWE, where extract was modified with acetic acid and trapped onto **A** C18 adsorbed material, and **B** cyclohexyl adsorbent material. Compounds: *1*, rosmarinic acid; *2*, carnosol; *3*, carnosic acid; and *4*, methyl carnosate

Pressurized hot water extractions

The highest recovery of extracted analytes at the tested temperatures (70, 100, and 150 °C) was achieved at 150 °C, but the antioxidant activity was at a maximum already at 100 °C and this temperature was chosen for the further experiments. The selected pressure (100 kg/cm^2) was high enough to keep the water in liquid state at the tested temperatures.

The water-based total extract was collected without use of the solid phase trap (LC profile of the total extract obtained by PHWE (not shown) was similar to the one obtained by ultrasonication-assisted MeOH extraction). The evaporation of this extract (from 65 to 50 mL) was both time-consuming and laborious. Moreover some analytes of the extract may undergo transformations in the procedure. Two different trapping materials were thus tested, as a means of concentrating the extracts more effectively. Because the sage extract consists of many compounds with a variety of polarities, the choice of material required a compromise. In addition to C18 material, which was successfully used by Rovio et al. [15] in PHWE (T=150–300 °C) in collecting relatively polar eugenol and eugenyl acetate, we also tested cyclohexyl adsorbent. Both materials adsorbed the nonpolar material, but only a small part of the rosmarinic acid and compounds of similar polarities. These compounds appeared to be too polar for the extraction conditions employed. At higher temperatures, water becomes significantly less polar, affecting the extraction and trapping. Thus, in our study, the trapping of rosmarinic acid would be expected slightly to improve at higher temperature due to decreased solubility of the compound in water, but most likely this would be at the expense of its extractability.

The antioxidant activity was consistently higher with cyclohexyl than with C18 as trapping material. This was slightly surprising since rosmarinic acid, one of the main antioxidant compounds in sage [2, 3], is much less strongly retained on cyclohexyl material. On the other hand, the total amount of compounds retained between 20 and 30 min was greater with cyclohexyl adsorbent than with C18, as can be seen in the LC profiles of the acetic-acid-modified runs presented in Fig. 2 A and B. It should be kept in mind that one or several of the compounds present in timescale 20–30 min may be present in only small amount but have high antioxidant activity. The addition of acetic acid improved the antioxidant activity of the extracts with both adsorbent materials. In part, the effect may be explained by the increased stability. Consider, for example, the stability of carnosic acid, which is rather unstable particularly in polar solvents. It is also converted into carnosol by air oxidation or by heating [2, 8, 31]. The stability of carnosic acid is also strongly affected by pH [32, 33] and its antioxidant activity is significantly greater in an acidic medium.

A temperature of 100 °C was chosen for the kinetic experiments because the highest antioxidant activity was observed at 100 °C. A kinetic curve (relative recovery % as a function of extraction time) was drawn for each compound investigated. The curves (not shown) indicated that 100% recovery was achieved for all compounds within 60 min.

Comparison of the extractions

The differences in extraction time and amount of starting material for the different extraction procedures are presented in Table 2. It is worth noticing that PHWE takes just 1 h, whereas the maceration with 70% ethanol requires 3 days. There is also a significant difference in the amount of starting material. However, there were no appreciable differences in RSD values for the recoveries of the different extractions, the values being 5%–17% (n=3). In the maceration with 70% EtOH and ultrasoni-

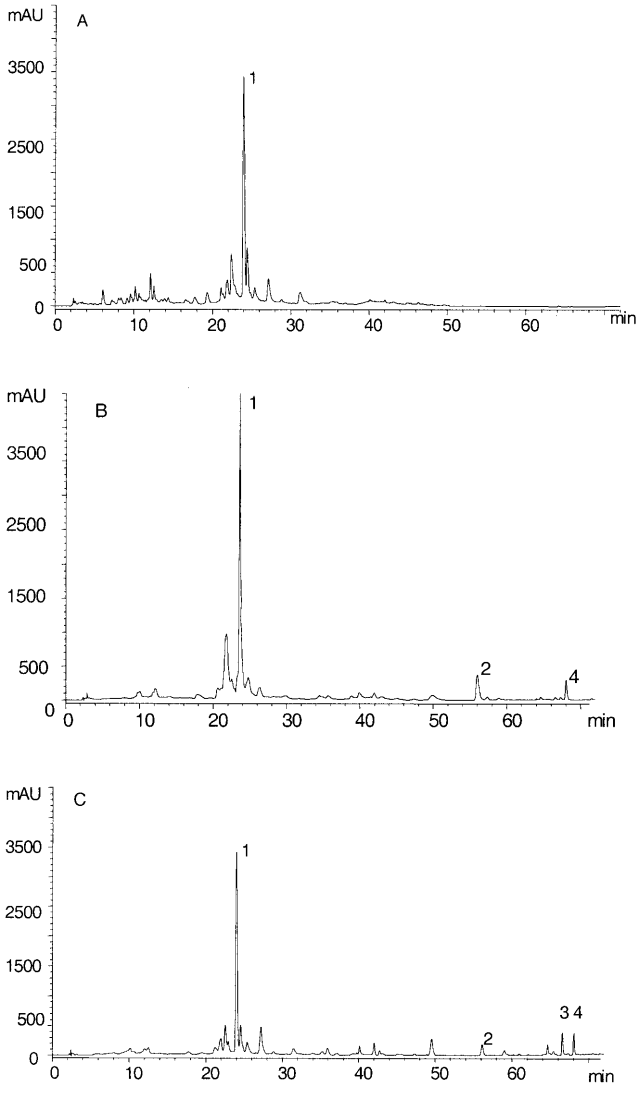

Fig. 3A–C LC chromatograms of extracts obtained by **A** hydrodistillation, **B** maceration with 70% ethanol, and **C** ultrasonication-assisted methanol extraction. Compounds: *1*, rosmarinic acid; *2*, carnosol; *3*, carnosic acid; and *4*, methyl carnosate

cation-assisted MeOH extraction the sage was milled, whereas in hydrodistillation and PHWE the sage was used as such. Usually smaller particle size enhances recovery and speeds up the extraction. In the hydrodistillation procedure herbs are normally used as such (unmilled). When test was made of milled sage in PHWE, the extraction was completed in less time, but the antioxidant activity was only slightly greater than with unmilled herb. Thus, we used unmilled sage in subsequent PHWE experiments. Our use of only a small quantity of unmilled material may explain why PHWE gave the highest RSD values for recoveries (17%).

The LC chromatograms for the hydrodistillation, maceration with 70% ethanol, and ultrasonication-assisted methanol extraction are presented in Fig. 3A, B, and C, respectively. In the conventional extraction procedures the composition of the extract varies with the solvent employed and its polarity. The most polar solvent, water (as used in hydrodistillation, Fig. 3A), extracted only the polar compounds. The essential oil contains the volatile compounds but not, for example, the diterpene compounds (carnosol, carnosic acid, and methyl carnosate) [34, 35]. The second most polar solvent, 70% EtOH, also extracted some of the less polar compounds. This is shown by the presence of compounds (2) and (4) in Fig. 3B. Methanol is the least polar solvent of those tested and the less polar compounds were easily extracted with it (Fig. 3C). However, among conventional extraction techniques the highest antioxidant activity was achieved in the maceration with 70% EtOH, and also the highest recovery of rosmarinic acid was achieved with this solvent (Fig. 3B).

As noted, the extract from the hydrodistillation contained only polar compounds. When the hydrodistilled material was further treated by maceration with 70% ethanol (the same procedure as applied to the milled sage material), the extract also contained the less polar diterpenes. The LC profile was similar to the one obtained by ultrasonication-assisted MeOH extraction, differing only in the lower intensity.

PHWE exhibited better extraction power than hydrodistillation at the same temperature (100 °C). The high pressure in PHWE has only a minor effect on the dielectric constant and the solvent strength of water. However, the high pressure may help the water to penetrate better into the sage matrix (or otherwise physically alter the matrix) and to extract also the less polar compounds. In addition, PHWE is a dynamic process, where the solvent flows through the sample, whereas hydrodistillation resembles the static extraction. Because of the large sample amount and relatively small solvent volume in hydrodistillation, the solvent is probably saturated with the analytes. This could also help to explain the lower recoveries of the extracted compounds and especially the lower antioxidant activity. Carnosic acid, for example, was extracted from sage by PHWE but not by hydrodistillation or maceration with 70% ethanol alone. However, it was successfully extracted with a combination of hydrodistillation and maceration.

Conclusions

This study has shown that pressurized hot water extraction is a highly promising alternative to conventional solid–liquid techniques in the extraction from plant matrixes of natural compounds with a variety of polarities. Highest antioxidant activities did not correspond with the maximum recoveries, but the antioxidant activity was highest when pressurized hot water (PHW) was used as the extracting solvent. PHW was a more effective solvent than either methanol or 70% ethanol. In addition, no prohibited solvent residues were present in the final PHWE extract. PHWE demonstrated better extracting power than hydrodistillation performed at the same temperature. The total analysis time was shorter in PHWE

than in the conventional extraction techniques. In future, attention needs to focus on the collection of more concentrated extracts in PHWE.

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