Comparison of Conventional and Ultrasound-assisted Extraction Techniques on Mass Fraction of Phenolic Compounds from Sage (*Salvia officinalis* L.)

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An innovative ultrasound-assisted extraction (UAE) is the rapid non-thermal extraction technique, which in comparison to conventional extraction (CE), offers high reproducibility in a short time with simplified manipulation, reduced solvent consumption and lower energy. Optimization of ultrasonic conditions was conducted for devices with nominal output power of 100 and 400 W, including the influence of geometrical parameters of probes regarding ultrasound-assisted extraction. The results showed that the optimal parameters for extraction of total phenols and rosmarinic acid as a dominant compound in sage extracts were as follows: solvent: 30 % ethanol, extraction duration of 11 minutes, and ultrasonic device output power of 400 W. The antioxidant capacity of the obtained extract correlated with the concentration of total phenols and flavonoids, and among individual phenols the rosmarinic acid contributed the most to the antioxidant capacity. The achieved results and statistical analysis ($p \leq 0.05$) have shown how UAE resulted in shorter extraction time, and increased extraction capacity of phenolic compounds by using solvents with a less amount of organic phase.

Key words:

conventional extraction, flavone glycosides, polyphenols, rosmarinic acid, sage, ultrasound-assisted extraction

Introduction

Sage (*Salvia officinalis* L.) is a popular herb which is native to the Mediterranean region and cultivated worldwide. Sage is an important source of antioxidants used as preservatives and has wider implications for the dietary intake of natural antioxidants.[1,2] This antioxidant effect has been attributed to the main phenolic components, caffeic acid dimer – rosmarinic acid[1] and flavonoids, being mostly present as flavones and their glycosides. Flavone glycosides are apparently common in sage, and most of them are flavones 7-glycosides represented by apigenin and luteolin 7-glucoside and their corresponding 3- and 7-glucuronides.[2] In the last few decades, research regarding the extraction of phenolic compounds found in plants have attracted special interest regarding their application in the food industry. Extraction is a very important step in the isolation, identification, and use of polyphenols.[3] The conventional extraction methods, which have been employed for decades, require prolonged extraction times and relatively larger quantities of solvent.[4,5] Therefore, various novel extraction techniques have been developed for the extraction of bioactive compounds from herbs, including ultrasound-assisted extraction,[6–8] microwave-assisted extraction,[9] supercritical fluid extraction.[10] Ultrasound-assisted extraction is an upcoming extraction technique that can offer high reproducibility in shorter time, higher yields of bioactive compounds, simplified manipulation, decreased temperature during processing, reduced solvent consumption, and lower energy input.[11–13] In our previous paper,[5] the influence of solvent polarity and composition, time and temperature of conventional extraction on mass fraction of polyphenols from sage were researched. The mixtures of ethanol and water are possibly the most suitable solvents for the extraction of sage due to different polarities of the active constituents, and acceptability of this solvent system for human consumption.[4,5] Albu *et al.*[3] and Sališova *et al.*[14] have investigated the difference in the application of conventional extraction and ultrasonic-assisted extraction on the concentration of biologically active compounds in sage. They concluded that the content of biologically active compounds is approximately 60 % higher under the influence of ultrasound. Rosmarinic acid is the more active of these antioxidants, but it is relatively easily degraded in solvent and the rate of degradation is solvent-dependant.[6] The ultrasonic-assisted extraction has been widely used for obtaining polyphenols from plants using ethanol, mixture of ethanol/wa-

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ter,[3,15–19] water and acetone.[17] Ultrasound-assisted extraction can also provide the opportunity for enhanced extraction of heat-sensitive bioactive components at lower processing temperatures[15], and is a more effective technique than conventional thermal extraction with most of the plants extracted within 15 minutes.[3] The mechanism of ultrasound in liquids relies on the mechanical effect caused by the implosion of cavitational bubbles. During implosion of micro-sized cavitational bubbles, strong shear forces are created, while both high pressures and temperatures generated as a consequence of the bursting bubbles, cause rapid plant tissue disruption allowing cellular material release and improved mass transfer as well. An important part of ultrasound-assisted processing is overall optimization of the process. The frequency (kHz), amplitude (%), applied cycle (%), nominal output power (W), and geometrical parameters of the sonotrode (length and diameter – mm) should be carefully prepared and taken into consideration.[20–22] The choice of method for the extraction of polyphenols from sage depends on the type of compounds to be extracted, and is found to be dependent on the solvent employed. From literature data,[3] extraction of polyphenols using polar solvents is significantly improved by sonication. It is important to note that extraction time is significantly shortened with the use of ultrasound.

The aim of this study was to examine and compare CE and UAE of sage leaves (*Salvia officinalis* L.). The research was carried out in two steps: in the first step, conventional extraction and ultrasound-assisted extraction with 30 % ethanol, and setting of the optimum conditions for each method. In second step, the extraction was carried out with different solvents (30 % acetone and water) under optimal conditions for each method set in the first step. The effects of the extraction parameters: solvents polarity (water, 30 % ethanol and acetone) on extraction methods, extraction time (20, 30, 40 min) and temperature (40, 50, 60 °C) for CE, and the influence of different ultrasonic devices (100 and 400 W) and extraction time (8 to 12 min) for UAE, with respect to the amount of extracted polyphenols were investigated. The influence and relevance of the operating parameters required during the extractions were checked and evaluated using a response surface methodology studying phenolic content. The analysis of individual polyphenols of the extracts was made by HPLC with UV/PDA detection.

Material and methods

Chemicals and reagents

Ethanol, sodium carbonate, and sodium nitrite were purchased from Gram–mol Company (Zagreb, Croatia). Folin-Ciocalteu reagent, apigenin-7-glucoside, luteolin-3-glucoside, rosmarinic, caffeic, gallic, vanillic and syringic acid were purchased from Sigma-Aldrich company (Steinheim, Germany). Acetone was purchased from Kemika Company (Zagreb, Croatia). Methanol (HPLC grade) was purchased from J.T. Baker Company (Deventer, the Netherlands).

Material

The plant material (sage) was collected on the Island of Pag (Croatia) in July 2008. The leaves of sage (*Salvia officinalis* L.) were dried immediately after harvesting in a shady and well-aired place for two weeks. Thereupon, the dried leaves were packed in paper bags, sealed, and kept in a dark, dry and cool place. Before use, the dry leaves were crushed using a house blender (Mixy, Zepter International).

Extraction of polyphenols

Conventional extraction was performed in a water bath shaker (Memmert WB14, SV1422, Schwabach, Germany). The crushed dried sage leaves (1 g) were weighed into a 100 mL glass cup, dissolved in 30 % ethanol (20 mL) and extracted in a water bath at 40, 50 and 60 °C for 20, 30 and 40 minutes. Further extraction experiments were performed with 30 % acetone and pure distilled water under optimal extraction conditions (60 °C, 30 min). After the extraction, the flask was removed from the bath and cooled to room temperature with cooling water. The extracts were filtered through Whatman no. 40 filter paper (Whatman International Ltd., Kent, UK) using a Büchner funnel, and the filtrates were adjusted to 25 mL in volumetric flasks with appropriate organic solvent or distilled water. The extracts were stored at –18 °C until analyses (no more than 7 days).

Ultrasound-assisted extraction experiments were carried out with 2 different ultrasonic devices with maximal power of 100 W and 400 W under maximal working amplitude equal to 100 % of maximum nominal output power of the device. The ultrasonic devices were manufactured by "Dr Hielscher", Teltow, Germany). The frequency was constant at 24 kHz for 100 W, and 30 kHz for 400 W ultrasonic devices, and enabled transient cavitations with bubbles implosion effect. The device was working throughout the experiment in continuous mode, i.e. cycle = 1, which means how the ultrasound was propagated in 100 % of the time throughout the medium. As previously mentioned for process optimization, two different ultrasonic devices were used to determine appropriate sonotrode for the used volume of the sample. The setup was not cooled down. The reason for this was to obtain the

best yield in a shorter processing time without additional energy consumption for cooling using ultrasonic in comparison with conventional extraction. The crushed dried sage leaves (1 g) were weighed into a 100 mL glass cup, dissolved in 30 % ethanol (20 mL), and directly sonicated. The extrinsic parameters of extraction time (8, 10, 11, 12 min) and the ultrasonic device (100 and 400 W) were varied. The ultrasonic probe was immersed into the mixture directly. Further extraction experiments were performed with 30 % acetone and pure distilled water under optimal extraction time of 11 minutes. The temperature was in the range of 21.8 to 88.0 °C. After extraction, the extracts were filtered through Whatman no. 40 filter paper (Whatman International Ltd., Kent, UK) using a Büchner funnel, and the filtrates were adjusted to 25 mL in a volumetric flask with appropriate organic solvent or distilled water.

The obtained polyphenolic extracts (CE and UAE extraction) were used for determination of total phenols, flavonoids, antioxidant capacity spectrophotometrically, and individual phenols using HPLC coupled with UV/PDA detector. All treatments were carried out in triplicate.

Determination of total phenols (TP)

The total phenols content of the extracts obtained by both conventional extraction and ultrasound-assisted extraction were determined by a modified spectrophotometric method using Folin-Ciocalteu reagent, calibrated against rosmarinic acid as the reference standard.[23] Quantification of total phenols was made by using the calibration curve of rosmarinic acid, which was prepared by diluting the stock standard with the extraction solvents to yield 50 to 500 mg per L of TP. The results were calculated according to the calibration curve for rosmarinic acid and the mass fraction of total phenols, derived from triplicate analyses and expressed as mg of rosmarinic acid equivalents (RA) per 100 g dry matter (dm).

Determination of flavonoids (FL)

Flavonoid compounds were determined from the same extracted samples that were used for determination of total phenolic content. The flavonoids were determined by a modified photometric method using $AlCl_3$,[24] calibrated against rutin as the reference standard. Quantification of flavonoids was made by using the calibration curve of rutin, which was prepared by diluting the stock standard with the extraction solvents to yield 50 to 250 mg per 100 mL of flavonoids. The results were calculated according to the calibration curve for rutin and the mass fraction of flavonoids, derived from triplicate analyses and expressed as mg of rutin equivalents per 100 g dry matter (dm).

DPPH radical-scavenging activity

The DPPH radical-scavenging activity was determined using the method proposed by Brand-Williams *et al.*[25] Briefly, 1 mL of phenolic extracts was added to 1 mL of DPPH methanolic solution (0.5 mmol L^{-1}) with 3 mL of methanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 20 minutes. Absorbance was measured at 517 nm. Methanol was used to adjust zero and DPPH methanol solution as a reference sample. The results were corrected for dilution and expressed in mmol trolox equivalents per kg. All determinations were performed in triplicate.

HPLC analysis

Separation of sage phenols was performed by HPLC analysis, using a Varian Pro Star System (Agilent Technologies, Inc., Santa Clara, CA, US) equipped with a ProStar 230 solvent delivery system, Rheodyne® 7125 injector and Pro Star 330 UV-photo diode array detector. Chromatographic separation was performed on a Zorbax ODS column (250 x 4.6 mm i.d. 5 mm; Agilent Technologies). The composition of solvents and gradient elution conditions had previously been described by Fecka *et al.*[26] with modification of formic acid in mobile phases. The mobile phase was composed of solvent A (3 % formic acid in acetonitrile) and solvent B (3 % formic acid in water). The following gradient was carried out: 10 % A in B, rising to 40 % A after 25 minutes, to 70 % A after 30 minutes, and then to 10 % A after 35 minutes. The flow rate was kept constant at 0.9 mL per minute for a total run time of 35 minutes. The injection volume for all the samples was 20 μL. The extracts were filtered through a 0.45 μL diameter membrane filter prior to analysis. The detection wavelength of 278 nm was used for the detection of phenolic acids. Flavones glycosides were detected at 360 nm. Identification of the compounds was achieved by comparing their retention times and UV-VIS spectra with those of authenticated standards. Phenolic compounds were separated in a series of descending polarity, based on the comparison of their retention time and retention time of the standards, and based on a comparison of typical UV spectra. Quantitative determinations were carried out using the calibration curves of the standards. Phenolic acids (rosmarinic, caffeic, vanillic and syringic) and flavonoids (luteolin-3-glucoside and apigenin-7-glucoside) were used as standards. Calibration curves of the phenolic acids and flavone glycosides standards were made by diluting stock standards (concentration of

0.5 to 2.0 mg per mL) in extraction solvents (ethanol, acetone or water) to yield 0.001–0.020 mg per mL. Mass fractions of phenolic compounds were calculated from the calibration curves of phenolic acids and flavones glycosides, and expressed as mg of phenolic acid or flavones glycosides equivalent per 100 g dry matter. The values were expressed as means (N = 3) ± S.D. Salvianolic K and salvianolic I acids and methyl rosmarinate were quantified as equivalents of rosmarinic acid. Flavone glycosides 6-hydroxyluteolin-7-glucoside, luteolin-7-glucuronide and luteolin-3-glucuronide were quantified as equivalents of luteolin-3-glucoside and apigenin-7-glucuronide as the equivalent of apigenin-7-glucoside.

Statistical analysis

All data were expressed as means (N = 3) ± standard deviations (S.D.) of triplicate measurements and analysed by software (Statistica 12.0). One-way analysis of variance (ANOVA)[27,28] was carried out on the significant differences between the extraction methods and the solvents used. Two different extraction methods were examined. The effects of solvent extraction conditions (extraction solvent, extraction time and temperature, power of ultrasonic devices) were investigated. Differences were considered significant at $p \leq 0.05$.

Results and discussion

In the first step of this study, two methods (conventional and ultrasound-assisted extraction) were used to extract polyphenols from sage under the previously described conditions. Table 1 shows the extraction capacity of conventional extraction for total phenols, flavonoids, antioxidant capacity, and quantification results of phenolic acids and flavonoids. Two variables influencing the extraction were investigated by means of the conventional process, which are extraction temperature (40, 50, 60 °C) and time (20, 30, 40 min). In the further experiments, chosen were temperatures below 60 °C and shorter extraction times up to 40 minutes, because higher temperatures may be attributed to thermal degradation of polyphenols.[5] Studying the extraction time (20, 30, 40 min), it was observed that the mass fraction of the extracted phenols slightly

Table 1 – *Quantification of individual phenolic compounds in the sage extracts obtained in ultrasonic bath at 40, 50 and 60 °C for 20, 30 and 40 minutes with 30 % ethanol*

Temp (°C)	Time (min)	Total phenols	Phenolic acids, mg/100 g			Flavonoids	Flavone glycosides, mg/100 g		DPPH (mmol TE kg^{-1})
			rosmarinic acid	Σ hydroxy-cinnamic acids	Σ hydroxy-benzoic acids		Σ luteolin glycosides	Σ apigenin glycosides	
40	20	3698.08±20.12	1226.22±33.34	74.81±11.85	39.41±4.45	1340.38±9.21	611.66±12.12	118.31±9.15	1.69±0.75
	30	5547.71±10.56	1983.25±34.51	102.65±12.21	54.07±5.13	1199.97±8.96	893.51±14.70	221.17±5.47	1.69±0.83
	40	4539.49±12.33	2254.59±11.71	100.69±10.99	58.84±7.66	1186.18±11.72	792.24±15.94	197.12±8.42	1.64±0.95
50	20	3874.10±23.17	2241.71±23.55	100.39±11.09	71.40±10.97	1313.89±7.89	816.10±16.45	205.31±3.49	1.68±1.03
	30	4276.08±12.75	2288.32±32.92	108.45±12.53	67.78±8.85	1443.29±14.63	885.48±12.99	189.32±2.96	1.67±1.04
	40	4221.07±11.45	1906.12±20.68	95.43± 6.69	65.64±9.52	1364.41±9.72	797.31±13.75	180.03±4.69	1.69±0.99
60	20	5203.15±18.55	3032.19±10.92	96.33± 7.88	85.85±10.95	1322.66±14.44	1343.40±21.50	247.31±5.78	1.70±0.87
	30	6399.79±21.85	3499.32±22.78	101.32±12.10	94.16±10.82	1924.60±9.85	1426.14±20.76	267.43±8.45	1.72±0.94
	40	4744.35±13.55	3455.18±13.75	95.03± 9.24	88.61±9.09	2083.36±10.14	1392.77±19.16	248.29±6.75	1.69±0.74

	p-value							
Extraction time	NS	NS	NS	NS	0.028019	NS	NS	NS
Extraction temperature	NS	0.003539	NS	0.000227	NS	0.003452	0.036498	NS

Correlation, *r*	0.7170	0.5070

$p \leq 0.05$ significant statistically; NS, not significant statistically.

Content of phenolic acids and flavone glycosides are expressed as mg per 100 g of dry sage; the sum of hydroxycinnamic acids (caffeic acid, salvianolic K and I acids, methyl rosmarinat); the sum of hydroxybenzoic acids (vanillic and syringic acids); Luteolin glycosides (6-hydroxyluteolin-7-glucoside, luteolin-7-glucuronide, luteolin-7-glucoside, luteolin-3-glucuronide); Apigenin glycosides (apigenin-7-glucuronide, apigenin-7-glucoside).

increased at 30 minutes, and a decrease at longer times of extraction (40 min) at all temperatures. The results of quantification are shown individually for rosmarinic acid as it was the most abundant compound in the sage extracts, while the results for other identified phenolic acids are shown as the sum of hydroxycinammic acids (caffeic, salvianolic K and I acids, methyl rosmarinate) and hydroxybenzoic acids (vanillic and syringic acids) because they were present in very low mass fractions. Also, the results of quantification of flavonoids are expressed as the sum of luteolin glycosides (6-hydroxyluteolin-7-glucoside, luteolin-7-glucuronide, luteolin-7-glucoside, luteolin-3-glucuronide) and apigenin glycosides (apigenin-7-glucuronide, apigenin-7-glucoside), because some of them were present in low mass fractions. As can be observed from the results obtained by conventional extraction, temperature also has a great impact on the extraction of rosmarinic, hydroxybenzoic acids, and flavone glycosides. A temperature increase from 40 to 60 °C had significantly increased the extraction efficiency in conventional extraction (Table 1). When extraction temperature rose to 60 °C, the mass fraction of phenolic acids and flavone glycosides decreased with increased extraction time (30 to 40 min). We suggest that the extraction temperature in the extraction of sage phenols by conventional extraction in a water bath should not exceed 60 °C. As already discussed, the obtained results and the results previously described[5] lead us to conclude that the extraction capacity decreases at higher temperatures. The reduction in yields of extracted phenolics at times longer than 30 minutes and higher temperatures may probably be attributed to the thermal degradation and polymerization reaction occurring due to the combination of various phenols among themselves, having an effect on the analytical quantification.[29] Taking into account these facts, temperature (60 °C) and extraction time (30 min) were selected as the optimum.

For the ultrasound-assisted extraction, the nominal output power of ultrasonic device with different frequencies (24 and 30 kHz) and the nominal output powers (100 and 400 W) with duration of extraction (8, 10, 11, 12 min) parameters were optimized. The influence of sonication time ranging from 8, 10, 11 to 12 minutes on total phenols and flavonoids mass fraction is shown in Table 2. Our obtained results showed that phenols recovery increased with the time of sonication, reaching a maximum at 11 minutes for the sage total phenols and flavonoids analysed. The extraction efficiencies were lower during first 8 minutes of sonication, indicating that more time of ultrasound propagation was required for cell walls disruption releasing phenols from the cell constituents. Longer sonication times of 10 and 11 minutes improved extraction efficiencies and hence increased the rate of extraction, while prolonged application of 12 minutes did not increase the mass fraction of total phenols and flavonoids to a larger extent. The highest extraction capacity was reached by extraction time of 11 minutes, regardless of the ultrasonic devices (100 and 400 W). The highest mass fraction of TP (7813.69 mg RA/100 g) with Folin-Ciocalteu method was obtained with the following parameters of ultrasonic extraction: device output power of 100 W, sonication times of 11 minutes, while the highest value was not confirmed with conducted HPLC analysis. Considering the above-mentioned, this may have been due to the non-phenolic components in the extract reacting with the Folin-Ciocalteu reagent. Longer sonication times of 10 and 20 minutes improved extraction efficiencies, and hence increased the rate of extraction, while prolonged application of 30 minutes did not benefit greatly in phenol yields. When compared to 30 minutes of extraction, more than 60 % phenolics were extracted in the first 10 minutes. The time increases up to 10 minutes provoked an almost linear rise in total phenols yields, suggesting that maximum recovery was already attained before 10 minutes.[7,13] Statistical evaluation (ANOVA) confirmed that phenol extraction yields were not highly time-dependent ($p \geq 0.05$) (Table 2). The positive effect on the extraction yield of phenolic acids and flavone glycosides was observed with the increase in ultrasonic power. The extraction yield of phenolic acids and flavone glycosides increased, but not significantly ($p \geq 0.05$). The mass fraction of rosmarinic acid was much higher than the others, and had an obvious increasing trend from 100 to 400 W. The total value of phenolic acids and flavone glycosides of ethanol extract to the extraction time of 11 minutes with 400 W ultrasonic devices was obtained in higher mass fraction than using the 100 W devices. The achieved results and the conducted statistical analysis suggested that the extraction time influenced statistically significantly the extraction of phenolic compounds from sage up to 11 minutes, while after that time, a decrease occurred ($p \geq 0.05$) (Table 2). Prolonged extraction time (8, 10 and 11 min) resulted in increased extraction capacity of rosmarinic acid isolation but also of other phenolic acids and flavone glycosides.

When compared to 30 minutes of conventional extraction at 60 °C, most of the polyphenols were extracted in the first 11 minutes. Direct sonication with a probe was more efficient than conventional extraction for sage. Generally, in a directly sonicated system, the energy of the probe unit is directly focused on a localized sample zone, thereby providing more efficient cavitation into the treated solution.[30–32] It is important to note that by using ultrasound, the

Table 2 – *Effect of different ultrasonic devices and sonication times on mass fractions of phenolic acids and flavone glycosides. Extraction was performed with 30 % ethanol under ultrasound working conditions (100 % amplitude/duty cycle) and sonication time (8, 10, 11 and 12 min).*

Ultrasonic device (W)	Time (min)	Total phenols	Phenolic acids, mg/100 g			Flavone glycosides, mg/100 g			DPPH (mmol TE kg^{-1})
			rosmarinic acid	Σ hydroxy-cinnamic acids	Σ hydroxy-benzoic acids	Flavonoids	Σ luteolin glycosides	Σ apigenin glycosides	
100	8	6892.67±19.58	3622.96±10.83	38.58±2.36	25.36±3.20	1569.75±8.16	1433.59±11.12	193.47±6.47	1.64±0.98
	10	7146.44±20.47	3915.68±11.14	34.21±0.98	37.55±3.51	2072.21±10.51	1719.19±12.19	232.26±4.83	1.64±0.84
	11	7813.69±14.36	3991.59±12.18	45.14±1.57	43.80±5.35	2122.96±11.84	1689.59±9.45	237.32±4.63	1.64±0.75
	12	7224.21±15.48	3549.36±13.75	42.25±1.89	39.39±4.74	1412.32±14.79	1549.59±8.66	218.41±2.59	1.62±0.97
400	8	5833.88 ±23.45	2461.98±14.58	90.24±14.98	110.63±4.82	1552.11±10.14	1046.60±42.35	144.34±12.11	1.59±1.01
	10	5393.91±24.56	3545.89±14.58	89.60±13.54	115.14±3.89	1884.66±12.42	1562.28±37.79	195.46±14.23	1.59±1.16
	11	6775.52±21.12	4160.31±20.01	117.30±8.47	120.22±4.59	1928.79±14.21	1961.03±49.90	248.20±14.14	1.57±1.25
	12	5595.69±21.76	3699.59±16.88	115.20±13.44	100.49±2.96	1913.77±13.83	1620.05±44.53	216.05±15.34	1.46±1.37

	p-value							
Ultrasonic device	0.015128	NS	NS	NS	NS	NS	NS	0.030183
Extraction time	NS	NS	0.000204	0.000060	NS	NS	NS	NS

Correlation, *r*	0.6742	0.0980

$p \leq 0.05$ significant statistically; NS, not significant statistically.

Content of phenolic acids and flavone glycosides are expressed as mg per 100 g of dry sage; the sum of hydroxycinnamic acids (caffeic acid, salvianolic K and I acids, methyl rosmarinate); the sum of hydroxybenzoic acids (vanillic and syringic acids); Luteolin glycosides (6-hydroxyluteolin-7-glucoside, luteolin-7-glucuronide, luteolin-7-glucoside, luteolin-3-glucuronide); Apigenin glycosides (apigenin-7-glucuronide, apigenin-7-glucoside).

extraction time was significantly shortened compared to conventional extraction. Based on the results from the first step of this research, optimal extraction conditions were defined for optimal extraction conditions for both extraction methods. Optimisation of the extraction procedure was based on total and individual phenols. The optimal extraction conditions selected were as follows: 11 minutes of extraction time with 400 W device, and 30 minutes in water bath at 60 °C with conventional extraction.

In the second step, the extraction was carried out at optimal processing conditions with the other two solvents (30 % acetone and water). Ultrasound-assisted extraction (with 400 W device) in other solvents, 30 % acetone and water, in the same extraction conditions, obtained a somewhat lower mass fraction of TP, 30 % acetone (5202.88 mg RA/100 g), water (4284.64 mg RA/100 g). Table 3 shows the total phenolic and flavonoid content of the three solvent extractions from sage. The difference in polarities of the extracting solvents might influence the solubility of the chemical constituents in a sample and its extraction yield. Therefore, in the selection of a solvent for optimizing the recovery of total phenols and flavonoids from the sample, 30 % ethanol exhibited the highest value of total phenols and flavonoids, hence was is considered as the most efficient solvent system for extracting phenolic compounds from sage. The 30 % acetone extracts of sage leaves ranked next with no significant difference from the 30 % ethanol and water extracts ($p \geq 0.05$, table 3). The highest extraction capacity were achieved by extraction with 30 % ethanol, and significantly less with 30 % acetone and water in the same extraction conditions. In the conventional extraction method, the choice of solvent extraction and extraction method had no significant impact on the mass fraction of total phenols and flavonoids (Table 3). After the photometric determination of total phenols, the extracted samples were subjected to HPLC UV/PDA analysis. The effect of these solvent systems in extracting phenolic compounds from sage were quantitatively measured and compared. Higher mass fractions of rosmarinic acid as the most represented compound in sage, were extracted by implementing 30 % ethanol than with (30 % acetone and water) under same extraction conditions. The statistical analysis showed how the solvent used had no significant influence on the extraction of rosmarinic acid ($p \geq 0.05$) (Table 3).

Table 3 – *Effect of extraction solvent type on mass fractions of total phenols and phenolic acids under optimal extraction conditions of both extraction methods. Ultrasound extraction was performed under ultrasound working conditions (100 % amplitude/duty cycle, ultrasonic device of 400 W) and sonication time of 11 min. Conventional extraction was performed at 60 °C for 30 min.*

Extraction method	Solvent type	Phenolic acids, mg/100 g					Flavone glycosides, mg/100 g	
		total phenols	rosmarinic acid	Σ hydroxy-cinnamic acids	Σ hydroxy-benzoic acids	Flavonoids	Σ luteolin glycosides	Σ apigenin glycosides
Conventional extraction	30 % ethanol	6399.79±21.85	3499.32±22.78	101.32±7.42	43.80±5.35	1924.60±9.85	1426.14±45.32	267.43±12.11
	30 % acetone	6192.09±20.18	3820.17±18.54	92.10±11.27	39.81±4.01	1597.13±12.41	1177.36±34.25	282.46±19.40
	water	3493.34±15.83	625.22±19.98	80.35±6.52	26.59±3.08	866.05±6.42	902.53±39.12	205.16±20.27
Ultrasound extraction	30 % ethanol	6775.52±21.12	4160.31±20.01	117.30±8.47	94.16±4.54	1928.79±14.21	1961.03±49.90	248.20±14.14
	30 % acetone	5202.88±21.48	3937.13±15.32	99.57±14.37	65.41±4.22	1712.00±10.45	1736.48±44.94	217.84±22.40
	water	4284.64±25.76	2654.14±14.88	83.88±8.82	46.21±5.11	1459.01±12.52	1323.42±49.52	210.33±21.37

	p-value						
Extraction method	NS	NS	NS	0.030195	NS	0.010377	NS
Extraction solvent	NS	NS	0.011312	0.049781	NS	0.013783	NS

$p \leq 0.05$ significant statistically; NS, not significant statistically.

Content of phenolic acids and flavone glycosides are expressed as mg per 100 g of dry sage; the sum of hydroxycinnamic acids (caffeic acid, salvianolic K and I acids, methyl rosmarinate); the sum of hydroxybenzoic acids (vanillic and syringic acids); Luteolin glycosides (6-hydroxyluteolin-7-glucoside, luteolin-7-glucuronide, luteolin-7-glucoside, luteolin-3-glucuronide); Apigenin glycosides (apigenin-7-glucuronide, apigenin-7-glucoside).

In the extracts obtained by the probe system of 400 W under optimal conditions, the mass fractions of all identified polyphenols were higher than in extracts obtained with the probe system of 100 W, and especially with regard to conventional extraction. Improvement was obtained using a 400 W probe system. Here, additional stirring was not required since the probe itself provided sufficient mixing of the heterogeneous mixture. The mass fraction of rosmarinic acid was the highest in ethanol extracts (4160.31 mg/100 g), followed by acetone (3937.13 mg/100 g), while it was the lowest in the water extracts (2654.14 mg/100 g). Other phenolic acids were determined in higher mass fractions in ethanol extracts with regard to acetone and water extracts. Luteolin and apigenin glycosides were the highest in the ethanol extract, followed by acetone, and the lowest in the water extracts. The mass fractions of all identified phenols in the water extracts were low compared to ethanol and acetone extracts, although ultrasound enhanced the extraction, so the mass fractions of all identified phenols were higher in ultrasound-assisted extraction than conventional extraction (Table 3). Statistical analysis showed a significant influence of the extraction method and solvent on the amount of hydroxybenzoic acids and luteolin glycosides, while only the extraction solvent had a significant influence on the amount of hydroxycinamic acids ($p \leq 0.05$, Table 3). Several studies have also revealed the efficiency of ethanol and acetone water mixtures in extracting polyphenols from samples of plant materials. Ethanol is possibly a preferable solvent because of its nontoxic, environmentally safe and inexpensive features, while acetone may lead to an unacceptable level of residue in the extracts.[3,6,10] Therefore, ethanol and water are possibly the most suitable solvent system for the extraction of sage due to the different polarities of the active constituents, and the acceptability of this solvent system for human consumption.

By ultrasound-assisted extraction for 11 minutes, using 30 % ethanol, the mass fraction of TP (6775.52 mg RA/100 g) was 20 % higher than with conventional extraction for 30 minutes with the same solvent, TP (6399.79 mg RA/100 g). By using other extraction solvents (acetone and water), the mass fraction of TP was lower. Under ultrasonic extraction, ethanol showed a greater enhancement in the mass fraction of total phenols and flavonoids compared with acetone and water, which suggested it could be a viable solvent for extraction under these conditions. Sonication also appeared to reduce the dependence on the extraction solvent itself, with yields greatly enhanced when employing ethanol.[3] From the results of individual phenols obtained, it appeared that 30 % ethanol was a better extraction solvent for rosmarinic acid under the conditions studied (Table 3). This is probably due to the more polar nature of rosmarinic acid favouring the more polar ethanol solvent. Wang *et al.*[17] investigated the effect of extraction time on the content of rosmarinic acid using 30 % ethanol as the solvent. They found that 10 minutes of sonication was sufficient to extract the phenols. It was found that there was little difference using 30 % ethanol or

acetone as an extraction solvent, while the content of rosmarinic acid was about 20 % higher than with water. With water alone as an extraction solvent, the content of rosmarinic acid was about 30 % lower than with the other solvents. From our results, water is not a good solvent for extracting phenols from sage, but it has been observed that the addition of small percentages of water to the extraction solvent helps increase the effectiveness of extraction of the analytes of interest from the sample.[5] All the factors had positive effects on the rosmarinic acid content. Research results are in accordance with literature data stating that caffeic acid and their derivates,[33] salvianolic K and [5,9,34] sagerinic,[1] caffeic acid,[34–36] methyl rosmarinate,[26,37] play a central role in the composition of *Lamiaceae*. The content of free caffeic acid in our extracts was lower compared to syringic and especially rosmarinic acid. The results of our study showed that rosmarinic acid was present in all these sage extracts. Rosmarinic acid was the dominant compound, while other phenolics were present in significantly lower mass fractions. HPLC analysis showed higher mass fractions of phenolic acids and flavone glycosides in samples obtained by ultrasound than by conventional extraction under the same polarity of solvents with significant shortening of treatment time (threefold).

In recent years, there have been several reports on the application of UAE in the isolation of various phenolic compounds from plant materials.[3,13,14,16] The mass fractions of rosmarinic acid and other identified phenols in ethanol extracts obtained by applying ultrasonic-assisted extraction with directly immersed probe (output power of 400 W, 11 minutes) achieved about 20 % higher extraction capacity compared to conventional extraction in a water bath (60 °C, 30 minutes). These results are consistent with studies of other authors.[3,14] It is important to emphasise that ultrasound-assisted extraction shortened the extraction time threefold. During sonication, the cavitations process causes the swelling of cells or the breakdown of cell walls, which allows high diffusion rates across the cell wall in the first case, or a simple washing out of the cell contents in the second.[15] Besides the solvent, temperature and pressure, better recovery of cell contents can be obtained by optimising ultrasound application factors, including frequency, sonication power and time, as well as ultrasonic wave disruption.[11] Ultrasound as a technology and ultrasound-assisted extraction can be called an ″environment-friendly″ or ″green″ technique.[19] Overall, ultrasound-assisted extraction of polyphenols by using food grade solvents has strong potential for its industrial development as an efficient and environment-friendly process for preparation of extracts rich in natural antioxidants aimed at replacing synthetic antioxidants.[16] UAE has been recognised for application in industry to improve efficiency and reduce extraction time.[15]

A good correlation was obtained between antioxidant properties of sage extracts and their total phenols content, while the sage extracts possessed higher radical scavenging ability for both extraction methods. The results obtained for flavonoids and DPPH assays showed a low degree of correlation. Tables 1 and 2 show the linear correlation between the mass fraction of total phenols, flavonoids and antioxidant capacities of sage extracts obtained by different extraction temperature and time. It can be concluded that increased temperature increased the antioxidant capacity of the sage extracts. This parallel relationship proves that the total phenol content directly affects the antioxidant capacity of sage extracts.

Conclusion

By applying different extraction methods, the ultrasound-assisted extraction employing an ultrasonic device with direct agitation, resulted in the highest recovery of total and individual polyphenols coupled with lower solvent consumption compared to conventional extraction. Direct sonication with a probe system was more efficient than conventional extraction. The energy of the probe unit was directly focused on a localised sample zone thereby providing efficient cavitations into the extracting solution. The values of polyphenols extracted with ultrasound-assisted extraction under optimal conditions (output power of 400 W, 11 minutes) using 30 % ethanol were 20 % higher than with conventional extraction (60 °C, 30 min) with a meaningful (up to threefold) shortening of processing time. Improvement of all identified polyphenols was obtained using a 400 W probe system under optimal conditions, especially relative to the probe system of 100 W. The results showed that rosmarinic acid was the dominant compound in the sage extracts, while other phenolics were present in significantly lower mass fractions. Total and individual polyphenols were determined in higher mass fractions in ethanol extracts relative to acetone and water extracts.

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Abbreviations

CE – Conventional extraction
dm – Dry matter
FL – Flavonoids
TP – Total phenols
RA – Rosmarinic acid
UAE – Ultrasound-assisted extraction

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