ISSN 1330-9862 *original scientific paper*
(FTB-3046)

The Effect of Extraction Solvents, Temperature and Time on the Composition and Mass Fraction of Polyphenols in Dalmatian Wild Sage (*Salvia officinalis* L.) Extracts

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Received: March 9, 2012
Accepted: August 28, 2012

Summary

The effect of extraction solvents (30, 50 and 70 % aqueous solutions of ethanol and acetone, and 100 % distilled water), extraction temperature (60 and 90 °C) and extraction time (30, 60 and 90 min) on the composition and mass fraction of polyphenolic compounds in Dalmatian wild sage (*Salvia officinalis* L.) extracts has been investigated. The total polyphenolic content of sage extracts was determined spectrophotometrically using Folin-Ciocalteu method, whereas the individual polyphenols were determined by HPLC UV/PDA method. Results indicated that the main polyphenols in sage extracts were vanillic, caffeic, syringic, salvianolic K and salvianolic I acids, methyl rosmarinate, 6-hydroxyluteolin-7-glucoside, luteolin-7-glucuronide, luteolin-7-glucoside, apigenin-7-glucunoride, apigenin-7-glucoside, with rosmarinic acid and luteolin-3-glucuronide as predominant compounds. The mass fractions of total and individual polyphenols significantly depend on the type of extraction solvent, solvent composition and extraction temperature. The results showed that binary solvent systems are more efficient than mono-solvent systems in the extraction of polyphenolic compounds in regard to their relative polarity. The aqueous solutions of ethanol or acetone (30 %), extraction temperature of 60 °C and extraction time of 30 min were the most efficient for the extraction of polyphenols from dry sage leaves.

Key words: sage, phenolic acids, flavone glycosides, extraction parameters

Introduction

Sage (*Salvia officinalis* L.) is a common herbal plant widely cultivated in various parts of the world, but it is native to the Mediterranean region, where it is known as Dalmatian sage. Sage is rich in biologically active compounds, among which the most important are polyphenolic compounds, plant secondary metabolites, which are divided into two basic groups: phenolic acids and flavonoids (*1–3*). More recent studies on biologically active compounds in sage have revealed the presence of a large number of diterpenoids, phenolic acids and flavone glycosides (*2,4,5*). Several extraction methods and chromatographic assays had previously been developed for determination of caffeic acid and its oligomers from aromatic herbs (thyme, balm, mint, rosemary and sage) and the content of polyphenols, *i.e.* phenolic acids, salvianolic acids K and I, methyl rosmarinate, rosmarinic acid, free caffeic acid, flavones, luteolin-7-glucoside, luteolin-7-glucuronide, 6-hydroxyluteolin-7-glucoside and luteolin-3-glucuronide (*6–8*). The antioxidant properties were found to be related to the presence of phenolic compounds, rosmarinic acid, a caffeic acid dimer, and flavone glycosides (*4,7*). Besides antioxidant activities, the importance of phenolic compounds is manifested in their proven antimicrobial and antimutagenic activity (*3,4,8*).

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The extraction of polyphenols is demanding due to their chemical structure and their interaction with other food components. Many factors, such as solvent composition, time of extraction, temperature, pH, solid-to-liquid ratio and particle size, may significantly influence the solid–liquid extraction (*6,9,10*). The polarities of the polyphenols range from polar to non-polar, thus a wide range of solvents (water, acetone, methanol, ethanol, or their mixtures with water) for their extraction has been studied (*1,9,11*). Aqueous mixtures of acetone are good solvents for polar polyphenols as well as other antioxidants, but their application may lead to an unacceptable level of acetone residue in the extracts (*2,3*). Wang *et al.* (*6*) investigated the influence of different solvents like ethanol, methanol, acetone, acetonitrile and water on the amount of the extracted phenolic acids as well as rosmarinic and caffeic acids in aromatic plants. The application of water as extraction solvent results in 20 % less rosmarinic acid compared to other solvents. Among aqueous solutions of ethanol at a volume fraction of 15 to 96 %, best extraction yields of caffeic and rosmarinic acids were obtained with 30 and 60 % solutions. Little difference was found when ethanol, methanol, acetonitrile, acetone or water was used. Water/ethanol mixtures are possibly the most suitable solvent systems for the extraction of sage polyphenols due to the different polarities of the bioactive constituents, and the acceptability of this solvent system for human consumption. The optimal extraction conditions that fulfilled the above constraints were found to be particle diameter of 1 mm, extraction temperature of 40 °C, an extraction time of 1–3 h, solvent-to-sage ratio of 6:1 and ethanol volume fraction of 55–75 % (*10*). There is a growing interest in efficient and environmentally acceptable extraction methods. The desirable features of 'green' extraction methods are low solvent consumption, short extraction time and high extraction yield. Attention is now being directed to the extraction techniques that rely on solvents that are not hazardous to human health (*6,10*).

Furthermore, polyphenols are susceptible to oxidation. High temperature and alkaline environment cause their degradation. Thus the extraction process and further stages of the preparation of a trial to specify the contents of polyphenols require great caution (*12*). In spite of the development of new extraction techniques, conventional extraction dominates in many laboratories mainly due to its simplicity and low economic outlay. The efficiency of the process can be widely regulated by the selection of suitable solvents, optimal temperature, time and pressure.

The aim of this study is to examine the effects of extraction parameters: solvent polarity, temperature and extraction time on sage polyphenols. Extraction was performed with water and aqueous solutions of ethanol and acetone (30, 50 and 70 %), at two different temperatures, 60 and 90 °C, for 30, 60 or 90 min.

Materials and Methods

Chemicals

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

Plant material

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

Extraction of sage polyphenols

For the extraction of sage polyphenols, three different aqueous solutions (30, 50 or 70 %) of ethanol and acetone were used, as well as distilled water. Extraction was performed at two temperatures (60 or 90 °C) for 30, 60 and 90 min. After crushing, the sage leaves were weighed (AND GR 200-EC laboratory scale, A&D Company Ltd, Tokyo, Japan), and approx. 1 g of crushed dried sage leaves was extracted with 20 mL of organic solvent or boiled distilled water at 60 or 90 °C for 30, 60 and 90 min on a horizontal water bath shaker (Memmert WB14, SV1422, Schwabach, Germany). 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).

Spectrophotometric determination of total polyphenols

Total polyphenolic content (TPC) of the extracts was determined by a modified spectrophotometric method using Folin–Ciocalteu reagent, calibrated against gallic acid as the reference standard (*13,14*). Quantification of TPC was made by using the calibration curve of gallic acid, which was prepared by diluting stock standard with extraction solvents to yield 50 to 500 mg/L of TPC. The results were calculated according to the calibration curves for gallic acid and the mass fraction of total polyphenols, derived from triplicate analyses and expressed as mg of gallic acid equivalents (GAE) per 100 g of dry matter (dm). Values were expressed as means (N=3)± standard deviations (S.D.).

Determination of individual polyphenols using HPLC UV/PDA detection

Optimal extraction conditions were selected based on the content of total polyphenols in the obtained extracts. The highest TPC was observed in all extracts obtained at 60 °C and they were selected for the HPLC analysis. Separation of sage polyphenols was performed by HPLC analysis, using a Varian ProStar System (Agilent Technologies, Inc., Santa Clara, CA, USA) 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 (250×4.6 mm, i.d. 5 mm; Agilent Technologies). The composition of solvents and gradient elution conditions had previously been described by Fecka and Turek (*8*) with some modifications. Phenolic compounds were analyzed using an acetonitrile/water gradient with the addition of a 3 % formic acid according to the solvent programme: solvent A, 3 % formic acid in acetonitrile; solvent B, 3 % formic acid in water, commencing with 10 % A in B, raising to 40 % A after 25 min, then to 70 % A after 30 min and then to 10 % A after 35 min. The flow rate was set to 0.9 mL/min. Operating conditions were as follows: column temperature 20 °C, injection volume 20 μL, UV-PDA detection at 278 nm. Before injection, extracts were filtered trough 0.45-μL Supelco Nylon 66 membrane filter (Sigma-Aldrich). Identification of phenolic compounds was carried out by comparing retention times and spectral data with those of authentic standards. Quantitative determinations were carried out using 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 flavonoid standards were made by diluting stock standards (concentration of 0.5–2.0 mg/mL) in extraction solvents (ethanol, acetone or water) to yield 0.001–0.020 mg/mL. Mass fractions of phenolic compounds were calculated from the calibration curves of phenolic acids and flavonoids, and were expressed as mg of phenolic acid or flavonoid equivalent per 100 g of dry matter. 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. Flavonoids 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

Multivariate analyses of variance (MANOVA) and post-hoc Tukey's test were performed using the STATISTICA v. 8.0 (StatSoft, Tulsa, OK, USA) package. MANOVA and Tukey's test were used to compare significant differences in the content of total polyphenols depending on the solvent composition, temperature and extraction time. Differences were considered significant at $p<0.05$. The main influences of solvent type, solvent volume fraction, extraction time and extraction temperature on the phenolic acid and flavonoid content were investigated using MANOVA, whereas individual influences of the mentioned parameters on each analyzed phenolic acid and flavonoid were calculated using analysis of variance (ANOVA; $p<0.05$). Statistically significant differences between each solvent volume fraction and extraction time are expressed using p-values (Tukey's post-hoc test; $p<0.05$).

Results and Discussion

Influence of different extraction parameters on TPC

All sage extracts are a rich source of polyphenols but their mass fractions significantly depend on the extraction conditions, solvent polarity, extraction temperature and time. Therefore, volume fraction of ethanol or acetone in the extraction solvent (30, 50 and 70 %), extraction temperature (60 and 90 °C) and time (30, 60 and 90 min) were evaluated in order to increase the extraction efficiency. Optimal extraction conditions were selected based on the TPC in sage extracts (Table 1).

Table 1. The mass fraction of TPC extracted from sage in a water bath shaker under different conditions using solvents of different polarity

φ(extraction solvent)/%	Extraction time/min	w(total polyphenols)/(mg of GAE per 100 g of dm), Extraction temperature/°C: 60	90
ethanol 30	30	6278.12±109.08	4612.10±107.81
	60	5578.33±96.11	5154.14±109.32
	90	5468.21±132.01	5210.52±108.39
ethanol 50	30	5841.13±104.36	4727.22±103.53
	60	5037.42±138.84	4592.34±105.32
	90	4294.21±151.14	3512.33±102.28
ethanol 70	30	4016.12±108.86	2457.85±104.53
	60	3928.23±101.50	2010.45±103.65
	90	5207.13±111.22	2341.56±100.19
acetone 30	30	5496.23±100.24	3336.31±146.63
	60	5197.42±103.42	3672.56±108.82
	90	4542.01±139.55	3973.60±133.16
acetone 50	30	4600.22±107.57	2487.42±135.75
	60	5120.22±101.63	2062.63±103.63
	90	5380.12±102.72	1371.45±135.88
acetone 70	30	4467.69±111.46	3138.74±100.83
	60	4514.05±124.40	3774.20±100.22
	90	5121.67±143.13	1760.55±114.81
water	30	5170.62±110.52	5949.71±115.23
	60	4812.13±111.54	5913.10±119.52
	90	6168.01±120.07	6134.88±110.16

GAE=gallic acid equivalent, dm=dry matter

The influence of extraction temperature on TPC

The results in Table 1 showed that ethanol and acetone extracts obtained at the extraction temperature of 60 °C contained higher TPC and they were selected for HPLC analysis. TPC in water extracts increased only slightly with the increase of extraction temperature and time. The mass fraction of total polyphenols significantly depends on the extraction temperature (Table 2), as confirmed by post-hoc analyses (p=0.000173). Koşar *et al.* (*9*) determined the TPC in the crude and hydrolyzed extracts of Lamiaceae herbs (basil, bay, oregano, rosemary, sage, savory and thyme) extracted with 50 % methanol at 60 °C for 20 min in water bath shaker; values of TPC ranged from 7930 to 46 400 mg per 100 g of extracts. Extraction of sage polyphenols at 60 °C gave higher yields due to increased solubility and diffusion coefficients, while the extraction at 90 °C resulted in the decrease of TPC in ethanol and acetone extracts. Temperatures above 60 °C

Table 2. Statistical significance of solvent type and volume fraction, extraction time and extraction temperature on the mass fractions of total polyphenols, rosmarinic acid and luteolin-3--glucuronide

	p-value			
	Solvent type	φ(extraction solvent)	Extraction time	Extraction temperature
		%	min	°C
TPC	0.125706	0.009174	0.958566	0.000058
RA	0.000209	0.000242	0.274972	*
		0.009495		
L3gr	0.057645	0.000104	0.027224	*
		0.016506	0.021850	
			0.216667	

*HPLC analysis was performed only at 60 °C
TPC=total polyphenolic content, RA=rosmarinic acid, L3gr=luteolin-3-glucuronide; p<0.05=statistically significant

probably caused a decrease in the extraction yield due to possible degradation of phenolic compounds, caused by hydrolysis, internal redox reactions and polymerization (*15*). According to Durling *et al.* (*10*) an increase in temperature resulted in the increased extract yields, but at a higher temperature (63 °C) the yield was lower, because more inactive compounds were extracted from sage. Also, increased solvent losses at high temperatures were also reported.

The influence of solvent composition on TPC

Solvents such as methanol, ethanol, acetone, ethyl acetate, *etc.* at different volume fractions in water have commonly been used for the extraction of polyphenols from different plants of Lamiaceae family (*6,8,16*). In this research, distilled water and aqueous solutions of ethanol and acetone (30, 50 or 70 %) for the extraction of sage polyphenols were used. From the results shown in Table 1, it is evident that the recovery of phenolic compounds was dependent on the type of solvent used, its polarity and the solubility of phenolic compounds in the extraction solvents. Akkol *et al.* (*16*) determined the TPC in sage (*Salvia halophila* and *Salvia virgata*) and the values ranged from 2830 to 21 230 mg per 100 g of extract, depending on the applied extraction solvent. Furthermore, solvent polarity plays a key role in increasing phenolic solubility (*17*). On the other hand, an increase in the volume fraction of ethanol or acetone in aqueous solutions did not have positive influence on the extraction efficiency of sage polyphenols, and the yield of TPC was maximized at 30 % ethanol, then followed by a considerable drop with further increases of ethanol as a solvent. The same was observed with acetone, but acetone extracts had overall lower TPC compared to ethanol extracts. Significant influence of volume fractions of ethanol or acetone (30, 50 or 70 %) on the mass fractions of total polyphenols was observed (p=0.009174), while the influence of the type of solvent was not significant (Table 2). The optimum conditions for the extraction of polyphenols from dry sage were 30 % ethanol at 60 °C for 30 min, under which the highest mass fraction of TPC of 6278.12 mg per 100 g of dm was determined.

Although at higher temperature and longer extraction time the extraction of polyphenols with distilled water was also high, it was higher with 30 % ethanol or acetone. However, the differences in TPC between ethanol or acetone extracts (30 and 50 %) and water extracts are not significant, so in agreement with green chemistry principles, water can be considered as efficient solvent for the extraction of sage polyphenols. In accordance with previous reports, TPC was maximized at low alcohol volume fraction and contained higher mass fraction of hydrophilic compounds (*10,18*). Wang *et al.* (*6*) also investigated the influence of solvents on the amount of extracted phenolic acids. They concluded that optimal extraction capacity was achieved with 30–60 % solutions of ethanol. Compared with other solvents (30 % methanol, acetone or acetonitrile), the difference in the polyphenolic content was not significant. The amount of water in water/organic solvent mixtures had higher impact on the extraction of polyphenols than the solvent itself. Fecka and Turek (*8*) conducted a conventional extraction of polyphenolic compounds from thyme and marjoram with 30, 50 and 70 % aqueous methanol solutions, in the duration of 15–30 min, and better results were achieved with higher water content.

Our results show that sage TPC varied considerably as a function of solvent composition and the results are in agreement with previous studies which showed that the nature of the solvent exerts a great power on the phenolic extraction capacities in many species (*19,20*). Furthermore, this result was in accordance with previous reports suggesting that a binary solvent system (ethanol/water) is more efficient than a mono-solvent system (water or pure ethanol) in the extraction of phenolic compounds in regard to their relative polarity (*21,22*).

The influence of extraction time on TPC

Experimental results showed that the extraction time (30, 60 or 90 min) did not uniformly influence the recovery of TPC from dry sage. It was observed that the content of total polyphenols in the extracts obtained at 60 and 90 °C with 30 and 50 % ethanol decreased during longer period of extraction (Table 1). Decrease of TPC during longer extraction was observed in the extracts obtained with 30 and 50 % acetone at 60 and 90 °C, respectively. However, in the extracts obtained with 70 % ethanol or acetone, the TPC increased with time and the highest values were observed at 90 min. In their work Durling *et al.* (*10*) conducted an extraction in water bath shaker from 1–3 h. They observed that the content of total polyphenols increased during shorter time of extraction. The increased extraction time potentially increases the loss of solvent by evaporization. It is therefore suggested that an extraction time of no longer than 3 h is employed (*10*). Comparing the different extraction times, it was concluded that the time of extraction did not affect the TPC significantly (p=0.958566, Table 2).

Determination of polyphenolic compounds by HPLC UV-PDA

For HPLC analysis the experimental variables (solvent composition and extraction time) which influenced the extraction polyphenols from the sage were chosen based on the content of phenolic acids (Table 3) and flavone glycosides (Table 4). Seven phenolic acids: vanillic,

Table 3. The results of HPLC-UV PDA determination of individual phenolic acids obtained by the extraction from sage in a shaking water bath at 60 °C for 30, 60 and 90 min using solvents of different polarity

φ(extraction solvent)/%	Extraction time/min	w(phenolic acids)/(mg per 100 g of dm)							
		VA	CA	SA	RA	Sal K	Sal I	MeR	Σ
ethanol 30	30	14.01±0.80	33.21±2.01	70.32±1.78	3634.12±33.30	40.10±1.25	14.21±2.41	34.02±1.02	3839.99
	60	14.05±1.01	29.10±2.02	65.22±1.92	3561.05±34.50	39.02±2.01	14.10±1.03	16.06±0.94	3738.60
	90	13.20±1.22	31.10±2.03	50.12±1.05	3497.09±11.51	39.14±1.98	15.03±1.56	9.06±1.60	3654.74
ethanol 50	30	11.10±0.74	23.12±1.03	67.15±0.99	3141.11±23.05	18.10±1.00	24.21±0.87	26.12±1.95	3310.91
	60	12.01±1.16	14.01±0.68	47.06±0.56	3140.02±32.62	24.17±1.22	22.15±1.65	24.29±1.60	3283.71
	90	9.15±0.74	14.05±1.01	41.09±0.54	3331.02±20.58	22.16±0.69	18.87±1.22	23.20±1.32	3459.54
ethanol 70	30	12.05±1.15	13.20±1.52	33.05±1.02	3224.10±10.42	26.20±0.88	14.11±0.95	16.02±0.96	3338.73
	60	11.92±0.94	11.10±0.74	35.10±0.23	2480.02±22.10	24.98±2.10	12.45±0.82	29.02±1.22	2604.59
	90	11.01±0.68	14.01±0.68	73.12±0.97	2164.13±13.65	27.14±1.24	13.15±1.05	22.02±1.50	2324.58
acetone 30	30	12.10±0.55	22.10±0.61	61.10±0.63	3082.10±10.25	43.12±0.85	26.01±0.82	17.23±0.26	3263.76
	60	9.02±0.34	24.12±0.31	57.10±0.87	2940.06±20.99	50.05±1.01	26.12±0.97	16.13±0.54	3122.60
	90	3.04±0.11	24.11±0.21	57.02±1.06	2840.25±20.65	50.36±0.56	26.12±0.64	16.22±0.31	3017.12
acetone 50	30	9.14±0.60	8.05±0.22	46.21±0.41	2849.12±22.45	38.21±0.85	23.24±1.02	17.06±0.88	2990.94
	60	10.01±0.99	9.02±0.11	42.02±1.02	2296.11±10.55	40.01±1.00	20.11±0.44	12.03±0.78	2429.31
	90	8.45±0.57	9.14±0.56	43.12±0.59	2063.10±11.23	39.12±1.10	20.36±1.04	12.01±0.55	2195.30
acetone 70	30	10.12±0.84	10.15±0.82	43.25±1.75	2181.12±21.04	38.12±0.86	13.56±0.66	12.12±0.47	2308.44
	60	10.31±0.69	8.75±0.71	41.20±0.89	2135.10±10.54	50.14±0.97	14.32±1.01	32.06±0.58	2291.88
	90	8.72±0.50	9.04±0.65	43.31±1.03	1759.10±12.02	45.13±0.67	15.02±0.65	13.02±0.49	1893.34
water	30	10.10±0.25	87.23±1.03	54.12±0.88	2154.02±15.03	28.10±0.74	14.21±0.56	14.10±0.54	2361.88
	60	9.20±0.69	102.10±5.02	64.10±0.98	2455.21±20.69	34.12±0.68	17.21±0.86	100.01±5.47	2781.95
	90	9.10±0.47	125.31±8.32	67.12±0.65	2862.13±20.96	37.12±0.69	13.12±0.99	84.36±1.25	3198.26

VA=vanillic acid, CA=caffeic acid, SA=syringic acid, RA=rosmarinic acid, Sal K=salvianolic K acid, Sal I=salvianolic I acid, MeR=methyl rosmarinate, dm=dry matter

Table 4. The results of HPLC-UV PDA determination of individual flavone glycosides obtained by the extraction from sage in a shaking water bath at 60 °C for 30, 60 and 90 min using solvents of different polarity

φ(extraction solvent)/%	Extraction time/min	w(flavone glycosides)/(mg per 100 g of dm)						
		HLg	Lgr	Lg	L3gr	Agr	Ag	Σ
ethanol 30	30	197.10±10.01	356.20±25.60	180.10±8.98	998.12±20.01	211.20±10.99	129.10±0.87	1073.70
	60	193.25±9.11	335.10±20.87	137.12±10.58	836.36±10.98	159.12±0.97	104.26±0.78	1765.21
	90	144.25±8.96	307.22±18.94	105.12±7.75	767.14±8.41	104.22±1.06	57.22±0.79	1482.17
ethanol 50	30	174.10±10.00	318.00±21.21	101.21±10.48	765.12±10.92	291.10±0.65	56.22±0.74	1705.75
	60	119.12±11.94	226.20±10.88	73.06±5.03	763.23±11.55	287.31±0.69	39.04±1.25	1507.96
	90	102.02±8.48	201.06±10.95	106.51±10.23	736.24±10.88	258.14±1.32	32.12±1.65	1436.09
ethanol 70	30	48.41±3.05	149.21±12.22	69.05±3.69	614.43±10.87	212.72±1.03	42.20±1.74	1136.02
	60	38.10±2.00	145.21±10.89	74.11±4.55	528.14±11.06	246.45±0.99	43.01±1.20	1075.02
	90	42.14±6.01	238.32±20.74	111.10±9.56	387.21±11.24	237.72±1.19	32.07±2.05	1048.56
acetone 30	30	113.11±1.02	266.47±10.89	149.78±10.75	916.12±12.93	69.75±0.66	147.26±1.30	1662.49
	60	113.10±0.99	241.12±10.58	140.63±0.88	796.63±10.44	67.41±0.83	138.22±0.74	1497.11
	90	105.12±0.91	331.62±22.42	135.41±11.21	682.25±6.97	59.55±5.22	92.28±4.28	1406.23
acetone 50	30	89.12±0.56	249.41±20.22	44.24±1.59	723.21±10.49	100.41±0.73	59.22±0.43	1265.61
	60	85.45±0.59	233.14±15.94	43.56±2.83	523.12±9.73	117.89±0.44	54.20±0.77	1057.36
	90	83.52±0.72	255.10±20.49	47.22±1.82	470.31±5.43	108.20±0.77	55.30±0.98	1019.65
acetone 70	30	69.15±3.09	109.63±10.99	25.52±1.23	656.23±11.21	193.22±0.99	50.15±2.25	1103.90
	60	64.11±2.15	140.89±12.54	23.65±1.45	449.62±10.73	160.31±0.88	51.42±0.87	890.00
	90	60.22±0.66	212.31±20.74	58.21±1.05	351.22±5.31	189.93±0.21	22.05±1.27	893.94
water	30	160.41±1.06	245.22±11.00	97.01±4.55	481.32±10.48	106.27±0.83	40.55±2.06	1130.78
	60	183.22±0.44	310.48±10.72	124.56±6.21	635.33±10.73	121.20±0.10	37.40±1.85	1412.19
	90	202.13±0.89	349.41±10.56	233.23±10.73	681.17±11.22	132.05±0.10	39.50±2.20	1637.49

HLg=6-hydroxyluteolin-7-glucoside, Lgr=luteolin-7-glucuronide, Lg=luteolin-7-glucoside, L3gr=luteolin-3-glucuronide, Agr=apigenin-7-glucuronide, Ag=apigenin-7-glucoside, dm=dry matter

caffeic, syringic, rosmarinic, salvianolic K and salvianolic I acids, and methyl rosmarinate, and six flavone glycosides: 6-hydroxyluteolin-7-glucoside, luteolin-7-glucuronide, luteolin-7-glucoside, luteolin-3-glucuronide, apigenin-7-glucuronide and apigenin-7-glucoside were successfully identified in sage according to the retention times and spectral characteristics of their peaks compared to those of standards using HPLC coupled with UV-PDA detector (Fig. 1). Moreover, Fig. 1, and Tables 3 and 4 show that rosmarinic acid and luteolin-3-glucuronide were the predominant phenolic acid and flavone glycoside in sage extracts, respectively. Results showed that sage phenolic acid and flavone glycoside content varied considerably as a function of solvent composition. Dependence of rosmarinic acid and luteolin-3-glucuronide mass fraction on the volume fraction of ethanol or acetone solution and extraction duration is presented in Figs. 2 and 3.

Compared to water extracts, using 30 % ethanol or acetone as extraction solvents, higher mass fractions of rosmarinic acid and luteolin-3-glucuronide were extracted. However, the differences in mass fractions of rosmarinic acid and luteolin-3-glucuronide between the ethanol and acetone extract were not significant, but in water extracts the values were lower. The extraction with distilled water contributes to higher mass fractions of rosmarinic acid and luteolin-3-glucuronide at longer extraction time, while using other extraction solvents (aqueous solution of ethanol or acetone) longer time of extraction caused the decrease of the mentioned polyphenols.

According to ANOVA results, the type of solvent and solvent volume fraction had a statistically significant influence on the mass fractions of rosmarinic acid and luteolin-3-glucuronide, while the extraction time had significant influence only on the mass fraction of luteolin-3-glucuronide (Table 2). By comparing different times of extraction, the statistical analysis (Tukey's test) showed that the extraction time of 30 min significantly influenced the mass fraction of luteolin-3-glucuronide ($p=0.021850$), while longer extraction time (60 min) caused the decrease of extraction capacity ($p=0.217667$).

Ethanol and acetone solutions with higher volume fraction of water (30 % ethanol or acetone) significantly influenced the mass fractions of luteolin-3-glucuronide ($p=0.016506$) and rosmarinic acid ($p=0.009495$). The choice of solvent (ethanol, acetone or water) had a significant influence on the extraction of rosmarinic acid ($p=0.000209$) but not of luteolin-3-glucuronide ($p=0.057645$). The obtained results lead to the conclusion that water could be used as adequate solvent for the extraction of rosmarinic

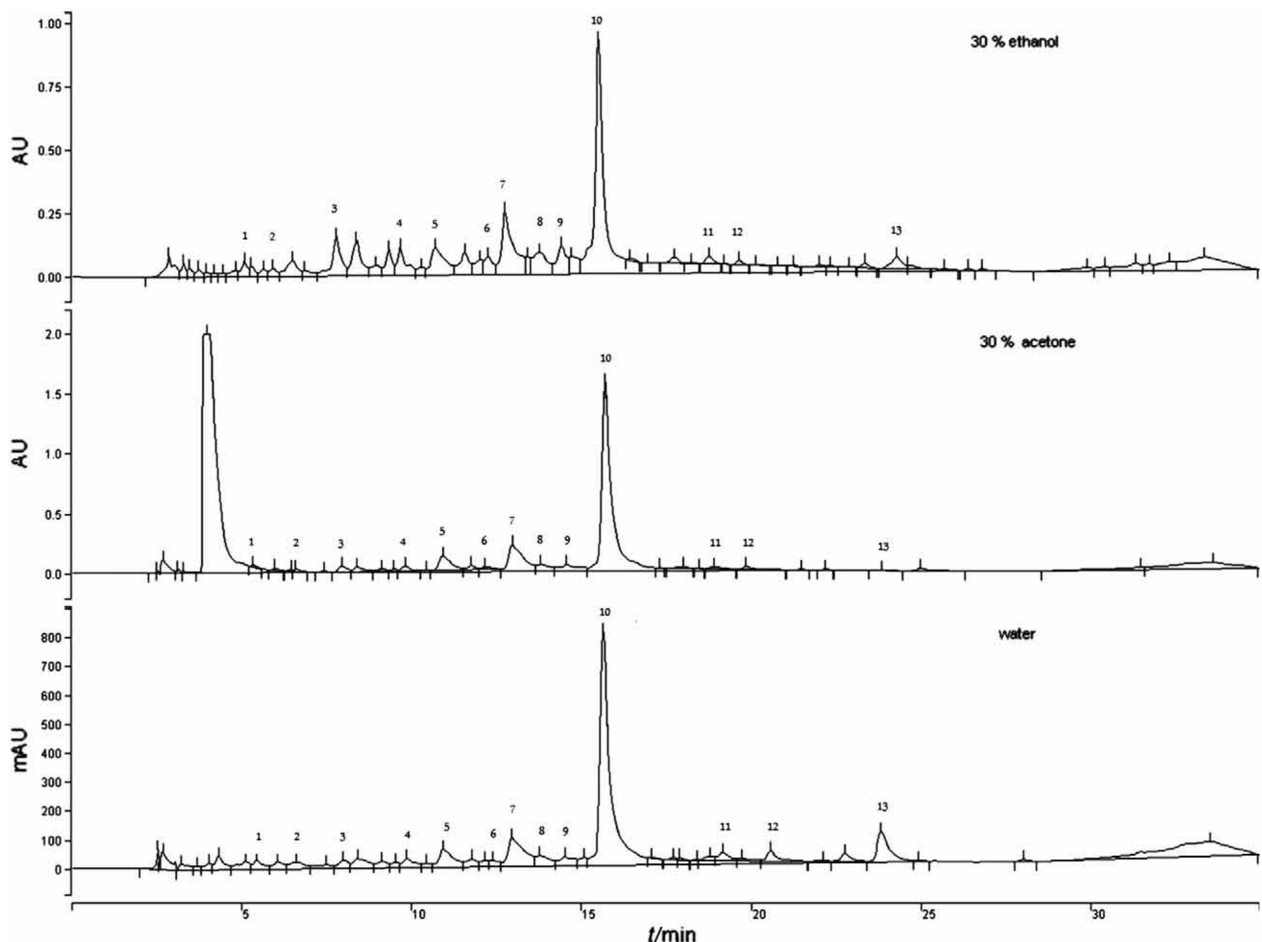

Fig 1. HPLC UV-PDA chromatogram of sage polyphenols obtained using three different solvents in water bath shaker at 60 °C for 30 min. Peaks: **1**=vanillic acid, **2**=caffeic acid, **3**=syringic acid, **4**=hydroxyluteolin-7-glucoside, **5**=luteolin-7-glucuronide, **6**=luteolin-7-glucoside **7**=luteolin-3-glucuronide, **8**=apigenin-7-glucuronide, **9**=apigenin-7-glucoside, **10**=rosmarinic acid, **11**=salvianolic K acid, **12**=salvianolic I acid, **13**=methyl rosmarinate

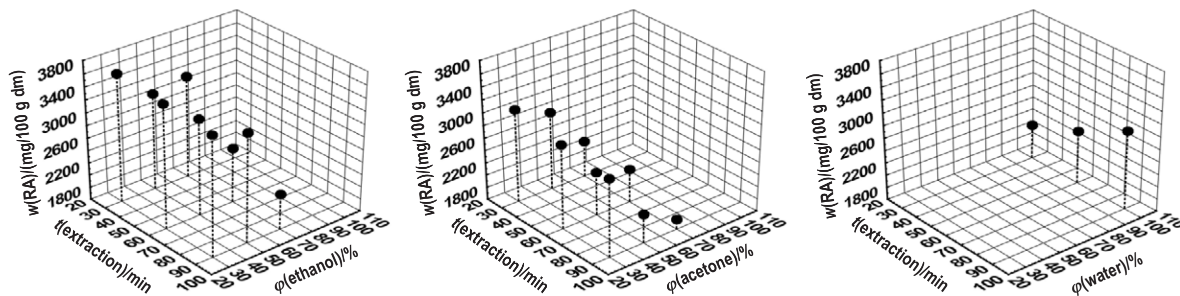

Fig. 2. The 3D surface plot results of HPLC-UV PDA determination of rosmarinic acid (RA) in sage obtained by extraction in a water bath shaker at 60 °C for 30, 60 and 90 min using solvents of different polarity

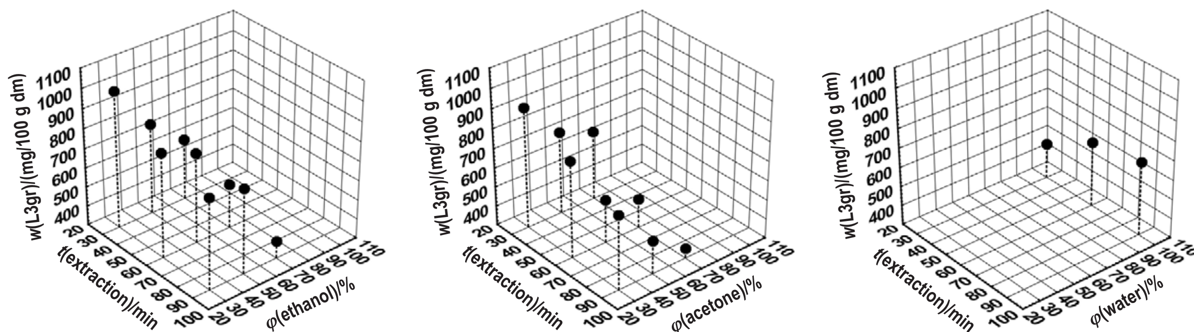

Fig. 3. The 3D surface plot results of HPLC-UV PDA determination of luteolin-3-glucuronide (L3gr) in sage obtained by extraction in a water bath shaker at 60 °C for 30, 60 and 90 min using solvents of different polarity

acid and luteolin-3-glucuronide, although binary solvents (30 % ethanol solution) showed even better results.

Applying higher volume fractions of organic solvents (50 or 70 % ethanol or acetone), and prolonging the extraction time from 60 to 90 min, the mass fractions of rosmarinic acid and luteolin-3-glucuronide decreased. It is evident from the results that the highest mass fractions of rosmarinic acid (3634.12 mg per 100 g of dm) and luteolin-3-glucuronide (998.12 mg per 100 g of dm) were obtained with 30 % ethanol at 60 °C for 30 min. The mass fractions of rosmarinic acid and luteolin-3-glucuronide in sage determined in this research were comparable to the results of other researchers (*3,6,10*). Wang *et al.* (*6*) determined the content of rosmarinic acid in sage, and concluded that the difference between the used solvents was small. They used ethanol at a volume fraction of 15 to 96 % and obtained the highest content of rosmarinic acid with 30 % ethanol, which is consistent with our results. Using water as a solvent, the content of rosmarinic acid was about 20 % lower. They identified rosmarinic acid in the range from 850 to 1410 mg per 100 g and found that ethanol volume fractions between 30 and 60 % gave the highest extraction yield of phenolic acid. Durling *et al.* (*10*) determined that the highest content of rosmarinic acid was extracted with 69 % ethanol at 40 °C in 1 h. Koşar *et al.* (*9*) identified rosmarinic acid as the major component in the sage extracts (extracted with aqueous methanol solution) at 9790 mg per 100 g.

Besides rosmarinic acid and luteolin-3-glucuronide, sage contains other phenolic acids such as vanillic, caffeic, syringic, rosmarinic, salvianolic K and salvianolic I acids, and methyl rosmarinate. Koşar *et al.* (*9*) and Zgórka and Głowniak (*23*) confirmed the presence of rosmarinic and caffeic acids. Lu and Foo (*2*) identified salvianolic acid I. The most abundant flavone glycoside determined in sage was luteolin-3-glucuronide, whereas other flavone glycosides such as 6-hydroxyluteolin-7-glucoside, luteolin-7-glucuronide, luteolin-7-glucoside, luteolin-3-glucuronide, apigenin-7-glucuronide and apigenin-7-glucoside were determined at remarkably lower mass fractions. The presence of luteolin-3-glucuronide, as well as other flavone glycosides, in sage was confirmed by other authors (*3,7,24*).

Conclusions

This study demonstrates that it is essential to optimize systematically the extraction solvent composition, temperature and time for accurate and reproducible assay of sage polyphenols. Our results showed that binary solvent systems are more efficient than mono-solvent systems in the extraction of polyphenolic compounds in regard to their relative polarity. The mass fractions of sage total polyphenols, rosmarinic acid and luteolin-3-glucuronide as well as other sage polyphenols varied considerably as a function of the type of solvent (ethanol, acetone or water), solvent composition (water/organic solvent) and extraction temperature, while extraction time had a significant influence only on the mass fraction of luteolin-3-glucuronide. This study confirmed that the aqueous solutions of ethanol or acetone of 30 %, extraction temperature of 60 °C and extraction time of 30 min were the most efficient for the extraction of polyphenols from dry sage leaves.

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