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Optimization of the extraction process of polyphenols from *Thymus serpyllum* L. herb using maceration, heat- and ultrasound-assisted techniques

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ABSTRACT

Despite the fact that *Thymus serpyllum* is well-known medicinal plant and its chemical profile and biological activity have been investigated, there is no detailed study regarding the influence of different techniques and conditions on the extraction of polyphenolic compounds from *Serpylli herba*. The aim of this study was optimization of the extraction parameters that improves the efficiency of polyphenols extraction from *T. serpyllum*: particle size, solid-to-solvent ratio, solvent type and extraction time, by using maceration, heat- and ultrasound-assisted extraction (HAE and UAE). The extraction efficiency was expressed via total polyphenol content (TPC) and total flavonoid content (TFC). The statistical analysis (one-way ANOVA and full factorial design) has revealed that the optimal conditions for achieving the best polyphenols yield were particle size of 0.3 mm, 1:30 solid-to-solvent ratio and 50% ethanol, as environmentally friendly extraction medium, while extraction time has not shown statistically significant influence on polyphenols concentration, in all procedures. Under these conditions, the measured TPC was 26.6 mg GAE/L in maceration, 29.8 mg GAE/L in HAE and 32.7 mg GAE/L in UAE, which was in agreement with the predicted values, while TFC was 14.3 mg CE/L, 12.4 mg CE/L and 16.7 mg CE/L for maceration, HAE and UAE, respectively. According to total polyphenols yield, the efficiency of the extraction methods for all variables was ranked by significance in the following order: UAE > HAE > maceration, whereas total flavonoids yield was the highest in UAE, although there was no statistically significant difference between maceration and HAE. According to our results, UAE could be selected as the most successful and suitable technique for extraction of bioactive polyphenolic compounds from *Serpylli herba*. Using LC/MS and HPLC analysis, 9 polyphenolic compounds were identified and quantified: 6,8-Di-*C*-glucosylapigenin, chlorogenic acid, 6-hydroxyluteolin 7-*O*-glucoside, caffeic acid, luteolin 7-*O*-glucuronide, apigenin glucuronide, salvianolic acid K isomer, rosmarinic acid and salvianolic acid I. This study was an initial step in production of polyphenols-rich wild thyme extracts aimed to be used for formulation of foodstuffs and medicines.

1. Introduction

Thymus serpyllum L. (Lamiaceae), well-known as wild thyme, grows in almost all the countries bordering the Mediterranean, in Asia and in parts of Central Europe. It is a perennial subshrub, which bilabiate tube-like calyx and tubular corolla are covered by dense gleaming glands that eradiate essential oil. It belongs to the group of aromatic plants, which have high level of essential oils and polyphenolic compounds [1].

Abbreviations: ANOVA, analysis of variance; CA, caffeic acid derivates; ChA, chlorogenic acid; CE, catechin equivalent; FC, Folin-Ciocalteu reagent; FL, flavonoids; GAE, gallic acid equivalent; HAE, heat-assisted extraction; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; RA, rosmarinic acid; SA, salvianolic acid; TFC, total flavonoid content; TOF, time-of-flight; TPC, total polyphenolic content; UAE, ultrasound-assisted extraction.

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Wild thyme possesses antiseptic, anthelmintic, diaphoretic, antispasmodic, antioxidative, expectorant, carminative and diuretic properties [2–4]. It is also applied as an alexiteric, emmenagogue, analgesic and sedative agent [1]. *Serpylli herba* has been traditionally used in treatment of respiratory, digestive and genitourinary diseases [5]. It has been shown that strong decoction is good for reducing the whooping cough spasms and thinning the phlegm of bronchitis, as well as in preventing of hair loss [6,7]. According to Mihailović-Stanojević et al. [8], aqueous extract from *T. serpyllum* has shown nitric oxyde-scavenging ability and antihypertensive effect. Previous reports have indicated protective effect of the aqueous tea infusions of wild thyme on the copper-induced oxidation of low-density lipoproteins [9]. Moreover, these extracts have antiinflamatory effect on colitis, because of its immunomodulatory properties [10]. Berdowska et al. [11] reported that extracts of wild thyme have showed high cytotoxicity against human breast cancer cell lines.

Polyphenols are large group of plants secondary metabolites often employed as food preservatives, antioxidants and additives, especially in meat and fish [12]. Flavonoids, as important group of polyphenols and natural antioxidants, may reduce oxidative stress in cardiovascular and neurodegenerative diseases, diabetes mellitus, asthma and eye disorders [13–15]. There is a growing interest in extracting these metabolites from plant sources, in order to obtain a safe, natural and low-cost alternative to synthetic antioxidant components, out of which some possess toxic and mutagenic effects [16]. The chemical composition and biological activities of the *Thymus* extracts are the subjects of interest in medicine, pharmaceutical and food industries [2]. Furthermore, in recent studies different methods for the extraction of polyphenols were established [17–20]. These procedures vary in nature of plant material, solvent type, solid-to-solvent ratio, time, temperature, pressure and pH. Considering that polyphenolic compounds are various in structure, it is not simple to establish a standardized extraction method that would extract majority of polyphenols from each plant source [21]. Typical processes for obtaining plant extracts include maceration, percolation and Soxhlet extraction. These methods involve several disadvantages: low yield, long extraction time, large amount of plant material, high solvent consumption and negative environmental impact. In the recent time, application of the novel extraction techniques, such as ultrasound-assisted extraction, have been evaluated [22,23]. According to the literature, these procedures provide numerous benefits (solvent saving, shorter time of extraction and high extract quality). In addition, these new techniques support the concept of "green" solvent, which is aimed to minimize the negative environmental impact from the utilization of large amounts of solvents in extraction process. It has shown that simple alcohols, as well as alcohol-water mixtures are more environmentally favorable solvents [22].

Despite the fact that complete polyphenolic profile of *T. serpyllum* and its activity has been already published by several authors [6,9,14], there is no comprehensive study regarding the effect of different methods and conditions on the extraction of polyphenolic compounds from *Serpylli herba*. In two studies the impact of a single factor (type of solvent and temperature) has been investigated, but the influence of other parameters, as well as the interactions between them, have not been examined [24,25].

The aim of the present study was to optimize extraction of polyphenols from *T. serpyllum* by investigating factors of interest, particle size, solid-to-solvent ratio, solvent type and extraction time. Moreover, three extraction procedures, maceration, heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE) by using ultrasound probe were compared in terms of TP yield. Maceration is traditional and less complex procedure of polyphenol extraction from plant material and it is particularly convenient for thermolabile compounds. On the other hand, high temperature involves faster polyphenol kinetics and improved extraction efficiency [22,25]. Furthermore, extraction of polyphenolic compounds could be enhanced with the application of ultrasound, which stands out as a good alternative extraction method, because of its high efficiency [26]. Since the objective of our study was to obtain extract abundant in polyphenolic compounds that would be further used for food and medicals, the choice of solvent was a critical step. Water and ethanol are, from toxicological point of view, much safer and more suitable than other solvents for the food and pharmaceutical industry [27]. Accordingly, in this study only ethanol, water and their mixtures, were tested. Finally, parameters that could potentially affect the extraction yield were statistically evaluated through full factorial experimental design in order to recognize optimal combination of factor levels for the best extraction result. In addition, qualitative and quantitative analyses of individual polyphenols were performed using LC/MS and HPLC methods.

2. Materials and methods

2.1. Plant material

Air-dried aerial part of *T. serpyllum* in full flowering phenophase was commercially purchased from the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade, Serbia. According to Yugoslav Pharmacopoeia [28], dried herb was grinded in the industrial mill and separated into 5 size fractions (0.3, 0.5, 0.7, 0.9 and 1.5 mm) by analytical sieving. For the purpose of this experiment, only particle sizes of 0.3, 0.7 and 1.5 mm were used, in order to cover the smallest, medium-size and the largest particles.

2.2. Reagents and standards

Ethanol (Fisher Science, UK) and distilled water were used as the extraction solvent. Distilled water was purified through a Simplicity UV® water purification system (Merck Millipore, Merck KGaA, Germany). Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) and sodium carbonate (Fisher Science, UK) were employed for the quantification of total polyphenols, while gallic acid (Merck, Darmstadt, Germany) was used as calibration standard. Sodium nitrite (Alkaloid, Macedonia), aluminum chloride (Sigma-Aldrich, Germany) and 1 M sodium hydroxide (Alfapanon, Serbia) were used for determination of total flavonoids, while catechin (Sigma Chemical, USA) was used for calibration curve. Acetonitrile, LiChroslov gradient grade, was purchased from Merck KGaA (Darmstadt, Germany), formic acid (98%) was obtained from Lachema (Brno, Czech Republic). Rosmarinic acid, caffeic acid, luteolin 7-glucoside and apigenin 7-glucoside were purchased from Sigma-Aldrich, USA.

2.3. Extraction procedures

2.3.1. Maceration

Maceration was carried out on the shaker (Unimax 1010, Heidolph, Germany) at room temperature using three particle sizes (0.3, 0.7 and 1.5 mm), three solid-to-solvent ratios (1:10, 1:20 and 1:30), five types of solvent (30%, 50%, 70% and 96% ethanol and water) and five extraction times (5, 15, 30, 60 and 90 min). In our preliminary screening, slight decline in TP yield at 90 min, particularly after 120 and 150 min (data not shown) could be noticed as a result of maceration. This is in accordance with the previous findings that prolonged extraction time causes decrease of TPC in ethanol extracts of wild sage already at 60 and 90 min, as well as in extracts of henna after 90 min [20,29]. Extracts were

obtained by maceration of 2.50 g, 1.25 g and 0.83 g of plant material in an Erlenmeyer flask (100 mL), according to the solid-to-solvent ratios, with 25 mL of every type of extraction solvent. The flasks were covered with aluminum foil to avoid light exposure and ethanol evaporation. Obtained extracts were filtered through a cellulose filter (fine pore, 0.45 μm) and reconstituted filtrates were properly diluted with the solvent to the required concentrations.

2.3.2. Heat-assisted extraction (HAE)

HAE was applied for polyphenol extraction using the same factor levels as in previous case and three extraction times (5, 15 and 30 min) at 80 °C, in the incubator shaker (KS 4000i control, IKA, Germany). In our preliminary tests (data not shown), we have examined influence of increased temperature in fixed maceration conditions: 0.3 mm of particle size, 1:30 ratio, 50% ethanol and 15 min, proposed in Miron et al. [25], where yield of TP became higher with increasing temperature. According to these findings level of 80 °C was maintained for experiment of HAE. In the same preliminary experiment, after 60 and 90 min of exposure to the elevated temperature, there has been a significant drop in the values of TP in *Serpylli herba* extracts. This result is in agreement with Meterc et al. [30], where the amount of polyphenols from green tea started to decrease already after 15 min at 80 °C. Extraction procedure was the same as for the maceration previously described.

2.3.3. Ultrasound-assisted extraction (UAE)

UAE was performed using the ultrasonic processor of 750 W output with a 20 kHz converter (Sonics, USA) and a solid titanium probe of 19 mm diameter at 80% of amplitude and 25 °C (the same particle size, extraction time, solid-to-solvent ratio and type of solvents as in HAE). Since the ultrasound waves have increased solvent temperature, glass flask with the sample was continuously cooled using ice coating during extraction in order to control temperature. In the preliminary experiments, the amplitude was evaluated at three factor levels (20%, 50% and 80%), covering the whole equipment's amplitude working range (data for 20% and 50% of amplitude not shown), while other independent variables were kept constant, like in case of HAE. The maximum response was observed at 80% of amplitude, which is in accordance with the observations of Horžić et al. [31], so this value was appointed for further optimization of the extraction process. The herbal samples (10 g, 5 g and 3.33 g) were extracted with 100 mL of the appropriate solvent (larger volume was applied because of technical properties of the ultrasonic probe), filtered through cellulose filter under vacuum and filled up to 100 mL with appropriate solvents.

All extracts were prepared in triplicate and stored at 4 °C in a dark place until further analyses.

2.4. Determination of the total polyphenols

The total polyphenolic content (TPC) was evaluated spectrophotometrically using modified Folin-Ciocalteu method [32]. Properly diluted sample (100 μL) was added to 6 mL of water and mixed with previously diluted Folin-Ciocalteu reagent with water (1:2 ratio). Subsequently, 1.5 mL of sodium carbonate solution (200 g/L) was added in a flask and the volume was made up to 10 mL with water. The samples were mixed and left in the dark at room temperature for 2 h. The absorbance was measured at 765 nm against a blank probe (water, extraction solvent, FC reagent and sodium carbonate solution in the same ratio as in the samples), using the UV Spectrophotometer UV-1800, Shimadzu, Japan. Triplicate analyses were done for each extract. Gallic acid was used as a standard (100–700 mg/L) for calibration curve, and TPC was expressed as milligrams of gallic acid equivalents per liter of extract (mg GAE/L).

2.5. Determination of the total flavonoids

The total flavonoids content (TFC) in selected water and ethanol extracts was determined by a colorimetric assay described by Barros et al. [33]. Properly diluted extract (250 μL) was mixed with 5% sodium nitrite solution (75 μL) and water (1.25 mL). After 5 min, 8.8% aluminum chloride solution (150 μL) was added and after 6 min 1 M sodium hydroxide solution (500 μL), and the mixture was adjusted to 3 mL with water. The absorbance was measured at 510 nm against the blank (all reagents except the extract), using the UV Spectrophotometer UV-1800, Shimadzu, Japan. Catechin monohydrate was used to calculate the calibration curve (0.05–0.3 g/L), and results were expressed as milligrams of catechin equivalents per liter of extract (mg CE/L).

2.6. LC/MS analysis

LC/MS analysis was performed on an Agilent MSD TOF coupled to an Agilent 1200 series HPLC, and reverse phase Lichrospher RP-18 (Agilent) analytical column (250 × 4 mm i.d., 5 μm particle size) was used. The mobile phase consisted of solvent A (0.2% solution of HCOOH in water) and mobile phase B (acetonitrile). The injection volume was 2 μL, and elution at 1.4 mL/min with gradient program (0–2 min 5–20% B, 2–20 min 20–55% B, 20–25 min 80% B, 25–27 min 80–100% B). Mass spectra were acquired using an Agilent ESI-MSD TOF. Drying gas (N_2) flow was 12 L/min; nebulizer pressure was 45 psig; drying gas temperature was 350 °C. For ESI analysis, the parameters were: capillary voltage, 4000 V; fragmentor, 140 V; skimmer, 60 V; Oct RF V 250 V, for negative mode. The mass range was from 100 to 3200 *m*/*z*. Processing of data was carried out with the software Molecular Feature Extractor. The identification of individual phenolic acids and flavonoids was done in selected wild thyme extract (50% ethanol as solvent; 1:30 solid-to-solvent ratio; 0.3 mm particle size and 15 min of UAE).

2.7. HPLC analysis

HPLC analyses were carried out on Agilent series 1200 RR HPLC instrument (Agilent), with DAD detector, on a reverse phase Lichrospher RP-18 (Agilent) analytical column (250 × 4 mm i.d., 5 μm particle size). The mobile phase and gradient elution were the same as for LC/MS analysis. The injection volume was 10 μL, flow was adjusted to 0.8 mL/min and detection wavelengths were set at 280 and 330 nm. Identification of caffeic and rosmarinic acid was based on comparison of retention time and UV spectra with those from the authentic standards, while the flavonoids were identified according to its characteristic UV spectra. Quantification of caffeic and rosmarinic acid was done using calibration curves of authentic standards, whereas detected flavonoids were quantified as equivalents of luteolin 7-glucoside or apigenin 7-glucoside, and salvianolic acids as equivalents of rosmarinic acid. The quantification of individual phenolic acids and flavonoids was done in selected wild thyme extracts (30% and 50% ethanol as solvent; 1:20 and 1:30 solid-to-solvent ratio; maceration, HAE and UAE; 0.3 mm particle size and 15 min of extraction). All analyses were done in triplicate. The results are presented as micrograms per mL of the extracts.

2.8. Statistical analysis

Separate statistical analysis for each extraction mode (maceration, HAE and UAE) were done, where each analysis covered preliminary screening of process levels and two experimental design methods.

2.8.1. Preliminary screening of process levels

The selection of the process levels of each factor that have significant influence on the extraction of total polyphenols has been performed. Statistical significance among factor levels has been estimated on triplicate samples through one-way ANOVA followed by Duncan's multiple range test at $p < 0.05$ level. Means followed by different letters in charts and tables differ significantly, based on Duncan's test at $p < 0.05$ level. Selected two levels with the highest yields of polyphenols were subjected to further factorial designs.

2.8.2. Factorial design

Two experimental design methods were used for the screening and optimization of process factors. In the first factorial design (Plackett–Burman design), four independent variables particle size, solid-to-solvent ratio, solvent type and extraction time, each at two levels, were screened forming the 2^4 full factorial design (Table 1). The purpose of this step is to identify which variable have significant effect on the total polyphenols yield. Based on the results obtained in the first factorial design, a new 2^3 full factorial design was employed to investigate the effect and choose the optimum values of particle size (1), solid-to-solvent ratio (2) and solvent type (3) on the TPC (dependent variable). Each factor was tested at two most promising levels using the upper and lower limits chosen on the basis of preliminary screening.

For statistical analysis of factorial design multivariate ANOVA has performed using STATISTICA 7.0 software, where factor influence on total polyphenols was observed through absolute values of standardized estimated effects, and presented on Pareto charts with level of significance set at $p < 0.05$ for the first factorial design. For the second factorial design, observed and predicted means for each dependent variable are presented in Table 3. The effects and corresponding regression coefficients of factors and factor interactions are listed in Table 4.

3. Results and discussion

3.1. Factor effects on total polyphenols

One-way ANOVA analysis followed by Duncan's multiple range test were implemented to determine how significant the influence of the extraction conditions (particle size, solid-to-solvent ratio, solvent type, extraction time, temperature and ultrasound) was on TP yield. Two most promising levels of each observed factor were selected for further experimental design. Also, this statistical tool was used to show the effect of solid-to-solvent ratio, solvent type and extraction technique on concentration of total flavonoids.

3.1.1. Effect of particle size on total polyphenols

One of the tested factors affecting the extraction results was found to be particle size. The impact of particle size (0.3, 0.7 and 1.5 mm) on the amount of TPC is shown in Table 2. The highest TPC values were achieved during extraction of 0.3 mm diameter particles, in all tested procedures: maceration, HAE and UAE (18.1, 22.1 and 22.8 mg GAE/L, respectively). The lowest extraction yield was obtained with 1.5 mm particles, in maceration (15.0 mg GAE/L). In the extracts obtained by maceration statistically significant improvement of TPC was noticed as particle size decreased, HAE favored the smallest particle size, while in UAE there was no statistical difference between analyzed levels regarding this factor. Enhancement of TP yield in HAE and UAE comparing with maceration strongly depends on size of plant particles, while the internal diffusion may be the limiting step during the extraction process [26]. Furthermore, according to Rai et al. [34] if the internal mass transfer constitutes dominant mechanism in the extraction process compared to external mass transfer, the degree of fragmentation can significantly influence the extraction yield. The highest yield of polyphenol is principally achieved with the finest particles, because of the increased active surface area and the enhanced contact of plant material with solvent, which both consequently also lead to the reduction of extraction time [35]. In addition, it was previously reported that the maximal TP yields were produced using the highest degree of grinding, i.e. the highest level of destroyed cells, which indicated that the convective mass transfer had the dominant effect [36]. However, the use of very small fragments may cause technical difficulties, during the mixing and filtration process. Thus, it is important to investigate the size of herbal particles that will provide the best extraction yield [35]. The previous studies of polyphenols extraction have pointed that decreasement in particle sizes of grinded *Ginkgo biloba* leaves, *Punica granatum* peels, as well as *Vitis vinifera* seeds and steams induced enhancement in TPC [19,36,37]. Furthermore, temperature and particle size, as well as their interaction had significant impact on extraction kinetics [35]. According to the literature as the temperature of extraction increases higher content of TP was recorded during extraction of the smallest particle fraction of grape seeds and parsley flakes [38,39]. As it was mentioned earlier, only UAE procedure did not result in statistical difference among observed levels regarding the influence of particle size on TPC yield (Table 2). This could be explained by the mechanism of ultrasound extraction, which includes reduction of the particle size (approximately 5%), damage of the cell walls and modification of microstructure, intensification of the solvent penetration in herbal cells and the instantaneous release of the polyphenolic compounds into the surrounding medium, caused by acoustic cavitation and thermal effects, regardless of the initial size of plant particles [18,40].

In a conclusion, since TP have achieved maximal yield with particle size of 0.3 mm, followed by 0.7 mm, these factor levels were selected for further factorial design.

3.1.2. Effect of solid-to-solvent ratio on total polyphenols

The results of the influence of solid-to-solvent ratio on the extraction efficiency are listed in Table 2. Comparing the matching solid-to-solvent ratio factor levels' TPC for each extraction procedure we can conclude that extracts obtained by maceration had the lowest TPC (13.7–18.9 mg GAE/L), while extracts obtained by UAE had the highest TPC (16.9–27.5 mg GAE/L). For each extraction procedure TP yield was lower as solvent volume decreased. The reason could be in the excessive amount of herbal drug, which caused the rise of viscosity and thus inhibited diffusion of polyphenols through the extraction medium. Results of our experiment showed that all tested levels of solid-to-solvent factor in all extraction procedures differ significantly. These differences in maceration could be explained by high solubility of polyphenols in hydroalcoholic solution, particularly in their glycoside form, which provided larger diffusion rate at 1:30 solid-to-solvent ratio [20,22]. Regarding HAE, the explanation could be also supported by the fact that high temperature decreases solvent viscosity and improves diffusion and consequently mass transfer of the molecule, which allowed faster extraction and intensification of the release of

Table 1
The factor levels used in 2^4 full factorial design.

Factor	Notation	Factor levels	
		Low (−)	High (+)
Particle size [mm]	1	0.7	0.3
Solid-to-solvent ratio	2	1:20	1:30
Solvent type [% EtOH]	3	30	50
Time [min]	4	30	15

Table 2
Preliminary screening of each factor level's influence on total polyphenols (TP) in *T. serpyllum* extracts.

Factor	Level	TP [mg GAE/L] Extraction techniques					
		Maceration		HAE		UAE	
Particle size [mm]	0.3	18.12 ± 4.64	a	22.06 ± 4.85	a	22.81 ± 6.23	a
	0.7	17.15 ± 4.52	b	18.97 ± 4.04	b	21.48 ± 4.46	a
	1.5	14.98 ± 3.18	c	18.33 ± 4.58	b	21.23 ± 6.42	a
Solid-to-solvent ratio	1:10	13.68 ± 2.93	c	15.14 ± 2.28	c	16.87 ± 2.84	c
	1:20	17.72 ± 3.81	b	20.63 ± 3.10	b	23.15 ± 4.36	b
	1:30	18.85 ± 4.39	a	23.59 ± 4.11	a	27.50 ± 4.12	a
Solvent type [% EtOH]	30% EtOH	17.30 ± 3.57	b	19.31 ± 4.00	b	22.50 ± 5.39	b
	50% EtOH	19.56 ± 4.77	a	22.60 ± 5.45	a	24.94 ± 4.81	a
	70% EtOH	15.35 ± 3.96	c	19.21 ± 4.12	b	22.20 ± 4.39	b
	Water	14.79 ± 3.34	c	18.20 ± 4.24	c	18.58 ± 4.23	c
Time [min]	5	13.14 ± 2.43	b	18.41 ± 4.21	b	21.59 ± 5.38	a
	15	17.01 ± 4.35	a	20.62 ± 4.63	a	23.09 ± 6.25	a
	30	17.82 ± 4.23	a	20.34 ± 5.16	a	22.84 ± 5.70	a
	60	18.13 ± 4.10	a	–	–	–	–
	90	17.65 ± 4.37	a	–	–	–	–

Values with the same letter in each column showed no statistically significant difference ($p > 0.05$; n = 3).
TP, total polyphenols; GAE, gallic acid equivalents; HAE, heat-assisted extraction; UAE, ultrasound-assisted extraction.

Table 3
Full factorial 2^3 experimental design for screening of factor influence on total polyphenols content with the observed and predicted values.

#	Particle size Design	Solid-to-solvent ratio	Solvent type	Particle size [mm] Factor levels	Solid-to-solvent ratio	Solvent type [% EtOH]	TP [mg GAE/L] Maceration		HAE		UAE	
							Observed	Predicted	Observed	Predicted	Observed	Predicted
1	−1	−1	−1	0.3	1:20	30	18.76	18.86	23.09	22.81	23.62	24.02
2	−1	−1	1	0.3	1:20	50	23.04	22.94	26.84	27.12	27.60	27.19
3	−1	1	−1	0.3	1:30	30	24.10	24.00	25.88	26.16	31.16	30.76
4	−1	1	1	0.3	1:30	50	26.58	26.68	29.80	29.52	32.68	33.08
5	1	−1	−1	0.7	1:20	30	18.60	18.50	19.82	20.10	23.55	23.15
6	1	−1	1	0.7	1:20	50	21.44	21.53	25.82	25.54	24.04	24.44
7	1	1	−1	0.7	1:30	30	22.61	22.70	20.50	20.22	26.96	27.36
8	1	1	1	0.7	1:30	50	24.42	24.32	24.44	24.72	28.21	27.80

TP, total polyphenols; GAE, gallic acid equivalents; HAE, heat-assisted extraction; UAE, ultrasound-assisted extraction.

polyphenols into extraction medium, therefore rapidly reaching saturation of solvent [22]. Furthermore, statistical differences among ratios in UAE were the most probably due to the tissue destroying mechanism produced by the ultrasound waves on the plant material. According to Paz et al. [41] when the ultrasound generates changes in the plant material (disruption of cell walls, reduction of particle size and the increase of contact surface) at the same time it enhances the mass transfer rate, solvent permeability in cells, secondary metabolites diffusion, and thus faster saturation of the liquid. On the contrary, the presence of a large amount of plant material (1:10 ratio) in our case, contributed to the attenuation of the ultrasonic waves and the active part was restricted to a zone located in the vicinity of the ultrasound probe [26]. Consequently, in all three methods the increase in solid-to-solvent ratio was resulted in the prevention of saturation of the extraction medium and the increment of the extraction yield. Several studies have found that the TPC increased quickly with the increase of ratio, at room temperature, as well as by use of high temperature or ultrasound waves [17,38,41–43].

Since the criteria for selecting the factor levels for further analysis was maximum of TPC, 1:20 and 1:30 ratios were selected to be included in full factorial design.

3.1.3. Effect of solvent type on total polyphenols

The effect of four solvents with different polarities (30%, 50% and 70% ethanol and water) on TPC was tested (Table 2). Initially one more level of solvent type factor was included in analysis (96% ethanol), but TPC yields for this factor level were very low in comparison with other factor levels and consequently its variance contribution violated assumptions for further parametric statistics, so data for this level have been removed from further analysis (Supplement 1). The TP yield was maximized at 50% ethanol, in all extraction procedures (19.6, 22.6 and 24.9 mg GAE/L, for maceration, HAE and UAE, respectively) and this level was significantly different from closest two levels (30% and 70% ethanol), between which was no statistical difference (except in maceration). Water extraction had the lowest TPC yield in HAE and UAE procedures, while in the case of maceration, although the lowest, no statistical significance between water and 70% ethanol levels has been recorded. Generally, in a binary solvent system, one solvent could enhance the solubility of the polyphenols, while the other could improve desorption [22]. Also, the amount of water in these mixtures had significant impact on the polyphenols extraction. Increasing ethanol concentration from 30% to 50% resulted in a decrease in the dielectric constant of the extraction medium and therefore enabled easier separation of the solvent molecules and the entry of the solute molecules between them [44]. These results suggested that most of the polyphenols from wild thyme had a relatively high polarity and they were preferentially extracted with mixture of ethanol and water, especially when they were in a glycoside form. Moreover, addition of small quantity of water to organic solvent has created a more polar medium and

Table 4
Statistical analysis of extraction optimization using 2^3 factorial design.

	Effect	Std. Err.	Effect estimates	Coeff.	Std. Err. Coeff.	*p*
Maceration						
TPC (mg GAE/L)						
Constant				22.442	0.253	0.000
Main factors						
Particle size [mm] (1)	−1.354	0.507	−2.672	−0.677	0.253	0.016
Solid-to-solvent ratio (2)	3.967	0.507	7.826	1.984	0.253	0.000
Solvent type [% EtOH] (3)	2.850	0.507	5.622	1.425	0.253	0.000
Interaction of two factors						
1 by 2	−0.475	0.507	−0.937	−0.238	0.253	0.362
1 by 3	−0.528	0.507	−1.042	−0.264	0.253	0.312
2 by 3	−0.707	0.507	−1.395	−0.354	0.253	0.181
HAE						
TPC (mg GAE/L)						
Constant				24.525	0.215	0.000
Main factors						
Particle size [mm] (1)	−3.755	0.429	−8.744	−1.877	0.215	0.000
Solid-to-solvent ratio (2)	1.261	0.429	2.938	0.631	0.215	0.009
Solvent type [% EtOH] (3)	4.400	0.429	10.247	2.200	0.215	0.000
Interaction of two factors						
1 by 2	−1.613	0.429	−3.757	−0.807	0.215	0.002
1 by 3	0.567	0.429	1.321	0.284	0.215	0.204
2 by 3	−0.470	0.429	−1.094	−0.235	0.215	0.289
UAE						
TPC (mg GAE/L)						
Constant				27.226	0.260	0.000
Main factors						
Particle size [mm] (1)	−3.077	0.521	−5.910	−1.539	0.260	0.000
Solid-to-solvent ratio (2)	5.050	0.521	9.699	2.525	0.260	0.000
Solvent type [% EtOH] (3)	1.805	0.521	3.467	0.903	0.260	0.003
Interaction of two factors						
1 by 2	−1.263	0.521	−2.425	−0.631	0.260	0.027
1 by 3	−0.941	0.521	−1.806	−0.470	0.260	0.089
2 by 3	−0.424	0.521	−0.815	−0.212	0.260	0.426

TPC, total polyphenols content; GAE, gallic acid equivalents; HAE, heat-assisted extraction; UAE, ultrasound-assisted extraction.

breaking hydrogen bonding has facilitated the extraction of polyphenols. Subsequently, solvent mixture could extract polyphenols from both ends of the polarity (highest and low polarity compounds) [29]. Also, the increase in effective swelling of the plant material by water has helped enhancement the surface area for solvent-solid contact [43]. Our results are in accordance with the observations of Costa et al. [45], where 50% ethanol was the most efficient solvent for the extraction of antioxidant polyphenols from *Thymus lotocephalus*. Miron et al. [25] have reported that maximum amount of TP was obtained using a mixture of ethanol:water (1:1) for wild thyme at 100 °C. Moreover, Mustafa and Turner [22] have reported that the polarity of solvent decreased substantially by applying high temperature during the extraction, which made water and ethanol suitable solvents to extract polar, moderately polar and non-polar organic compounds at increased temperature. Chizzola et al. [46] have established that the best result of TP from *Thymus* species was obtained with 60% ethanol compared to other ratios of the ethanol/water mixture, while 96% ethanol has extracted the minor yield of TP by extracting in water and ultrasound bath. Fecka and Turek [24] have pointed that the most suitable medium for extracting polyphenols from thyme and wild thyme was 50% alcohol/water in UAE procedure, while neither water nor alcohol could completely extract polyphenol compounds. Due to very low polyphenol extraction yields proven in our and in several previous studies, mono-solvent system (pure ethanol or water) is not recommended as the most convenient solvent for extraction of polyphenolic compounds [3,25,46].

Regarding TP content, the highest extraction rate was achieved by 50% ethanol, then by 30 and 70% ethanol in all extraction techniques. Since the screening data showed that there was no significant difference among 30 and 70% ethanol extracts and because of the cost of solvent, it was decided to choose 30 and 50% ethanol for further factorial design.

3.1.4. Effect of extraction time on total polyphenols

The impact of time on the extraction of polyphenolic compounds from *T. serpyllum* was conducted in maceration at five different extraction times (5, 15, 30, 60 and 90 min) and in HAE and UAE at three extraction times (5, 15 and 30 min). The obtained results are presented in Table 2. The maximum TP yield was achieved at 60 min in maceration (18.1 mg GAE/L) and at 15 min in HAE and UAE (20.6 and 23.1 mg GAE/L, respectively). According to our extracts, all ethanol and water extracts at 5 min are poor source of polyphenols (13.1, 18.4 and 21.6 mg GAE/L). In general, according to Fick's second law of diffusion, by extension of the extraction time, the quantity of extracted polyphenols compound is enhanced [47]. However, our results indicated that there was no statistically significant difference between exposure time, except in maceration and HAE, where at extraction time of 5 min TPC was significantly different compared to other extraction times. The reason could be in occurrence of two stages in polyphenol extraction: an initial increase of the concentration of polyphenols in the beginning of the process (for the first 15 min), followed by slow extraction (after 60 min) [42]. Our results are comparable to the previous findings of Fecka and Turek [24], who have reported that there wasn't any significant difference between time of extraction (15 and 30 min) in hot aqueous and methanol extracts from thyme and wild thyme. In addition, Dent et al. [20] reported that

the exposure time in extraction from wild sage did not affect the TPC significantly. Vergara-Salinas et al. [48] have also pointed that time (5–30 min) had no statistically significant effect on extraction of TP from *Thymus vulgaris* at high temperature. Furthermore, the influence of time wasn't significant in both maceration and UAE, in case of optimization of extraction of polyphenols from *Urtica dioica* [49]. High temperature and long exposure time together could also reduce the TP yield, because of temperature sensitivity and enzymatic degradation and oxidation of polyphenolic compounds in aqueous phase, as well as polymerisation among insoluble compounds [48]. Moreover, the extended sonication time could also damage extracted natural antioxidants and degrade the quality of extracts, due to generation of free radicals by the ultrasound waves [31].

Since extracts obtained by HAE and UAE at 15 and 30 min had the highest TPC, these two extraction times were subjected for further factorial design. Although the highest TPC in maceration was obtained at 60 min, 15 and 30 min have been selected for further analysis, because there was no significant difference between extraction time after 15 min, as well as to minimize energy cost.

3.1.5. Effect of high temperature on total polyphenols

The yield of TP in extracts obtained at 80 °C was higher than in the extracts obtained at room temperature (25 °C), for each observed factor level (Table 2). Even at first 5 min of extraction yield of TP in extracts obtained by HAE was increased for 40% in comparison with the extracts obtained by maceration (18.4 and 13.1 mg GAE/L, respectively). Comparing TPC of the extracts obtained by maceration and HAE it could be concluded that the similar amounts of polyphenols were detected after 60 min of maceration and 5 min of HAE (18.1 and 18.4 mg GAE/L, respectively). According to Mustafa and Turner [22], the use of thermal energy improves the efficiency of the extraction by disruption of cellular structures. This feature leads to increased cell membrane permeability and breakdown of secondary metabolites-herb matrix interactions (polyphenols with lipoproteins), what cause enhancement of polyphenols solubility and mass transfer. The increment of solvent temperature could also decrease surface tension and consequently enhance wetting of the plant material resulting in more efficient extraction [48]. High temperature decreases viscosity of the extraction medium, helping the solvent to penetrate the plant particles and resulting in an improved and accelerated extraction process [25]. Our results are in accordance with the previously published reports, where higher temperature had a positive influence on the extractability of polyphenolic compounds in water-ethanol extracts of herbs from family *Lamiaceae* [20,25]. Moreover, Hossain et al. [50] have pointed that temperature was the dominant factor in maximizing the TPC values of rosemary, marjoram and oregano. Also, box plot in Fig. 1 indicates that TP yield achieved in HAE was significantly higher than in maceration. Comparing to maceration and taking into consideration the industrial requirements such as high extraction yield for a shorter time, HAE could be recommended as convenient technique for polyphenols extraction from wild thyme.

3.1.6. Effect of ultrasound on total polyphenols

The results have showed that TPC was higher in the extracts obtained by UAE compared with extracts obtained by maceration and HAE observed factor levels (Table 2). After 5 min of UAE the amount of TP was 64.3% higher than in the extracts at room temperature and 17.3% higher than in the extracts obtained by HAE. TPC obtained by UAE after 5 min of extraction was higher than by maceration after 60 min. This behavior could be explained through the mechanism of plant tissue destruction by ultrasound waves, which as mechanical vibrations travel inside of plant cells and cause expansion and compression cycles during movement through the extraction medium [26]. The expansion can create bubbles in a liquid and provoke local rise of temperature and negative pressure. The bubbles form, grow and finally occurs the cavity collapse which is asymmetric and produces high-speed jets of liquid, which have strong impact on the solid surface. These mechanical and thermal effects induce destruction of cell walls, release of cell contents, as well as a greater penetration of extraction medium into herb matrix and intensify mass transfer. Consequently, efficient cell disruption and effective mass transfer, as two major factors, lead to the increment of extraction yield in use of ultrasound waves [31,40]. Very high content of bioactive compounds in UAE extracts, as well as less extraction time compared to conventional solid/liquid extraction, were demonstrated in several publications [31,50–52]. UAE has various advantages over conventional extraction techniques (maceration and HEA), such as the increase of extraction yield, improvement of the extract quality and very important property for industrial requirements - faster kinetics. In comparison to other modern extraction methods like microwave- and high hydrostatic pressure-assisted extraction and supercritical fluid extraction, the ultrasound instrument has lower price and it is easier to work on it. Additionally, any solvent could be used for extracting a wide range of pharmacologically and biologically active phytochemicals, as well as thermosensitive compounds [26]. Differences in TPC yield among tested extraction techniques are presented through box plot in Fig. 1, where yield achieved in UAE was significantly higher than in maceration and HAE. Consequently, UAE could be proposed as an efficient technique for extraction of bioactive polyphenolic compounds from *Serpylli herba*.

3.2. Experimental design (total polyphenols)

In this study, a complete factorial design was employed to determine the best combination of extraction variables. Following the results of the preliminary screening study, particle size (0.3 and 0.7 mm), solid-to-solvent ratio (1:20 and 1:30), type of solvent (30% and 50% ethanol) and extraction time (15 and 30 min), each at two levels, were used for the experimental design of the extraction process optimization. In the first factorial design, 2-level screening design (Plackett-Burman) has been conducted. All four observed factors have been represented through two selected levels in 2^4 full factorial design. The purpose of this analysis was to establish which factors had significant effect on dependent variable (TPC). The Pareto charts in Fig. 2 represent all factors that influence the yields of polyphenolic compounds. The lengths of the horizontal bars are proportional to the absolute magnitude of the estimated effects, while the dashed line represents the minimum value of statistically significant effects with respect to TPC (95% of the confidence interval). It can be observed that solvent type (factor 3) and solid-to-solvent ratio (factor 2) were the most significant factors for the TP yield in maceration, whereas particle size (factor 1) and extraction time (factor 4) had lower effects (Fig. 2A). Similar to our findings, in optimization of polyphenol extraction from *Urtica dioica* leaves, type of solvent has influenced TPC significantly, while effect of time was not significant [49]. Furthermore, solid-to-solvent ratio was the second relevant factor for obtaining high yield of TPC from dried chokeberry using maceration, whereas extraction time was non-significant factor [42]. From the Fig. 2B, it can be concluded that solvent type had a dominant effect on the extraction yield in HAE, followed by particle size and solid-to-solvent ratio, whereas time of extraction had no significant influence on extracted TPC. Our results are in accordance with the literature data, where type of extraction solvent was significant in polyphenols extraction from *Rosmarinus officinalis*, *Origanum majorana*, *O. vulgare*, *Inga edulis* and *Lawsonia inermis*, at elevated temperature [29,35,50]. Furthermore, size of plant parti-

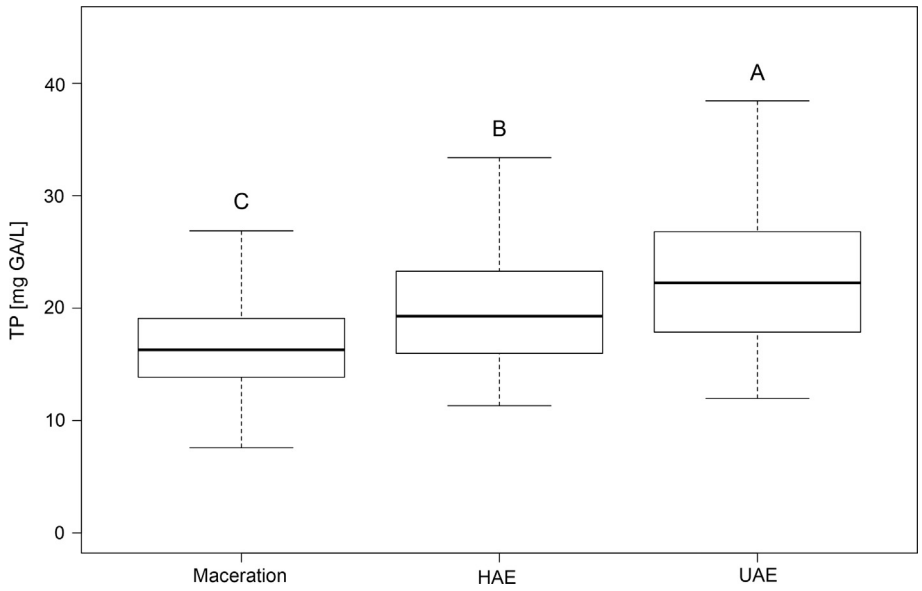

Fig. 1. Effects of extraction procedures on TP yield: maceration, heat-assisted extraction (HAE), ultrasound-assisted extraction (UAE); different letters on box plot error bars represent different population groups based on Duncan's multiple range test ($p < 0.05$).

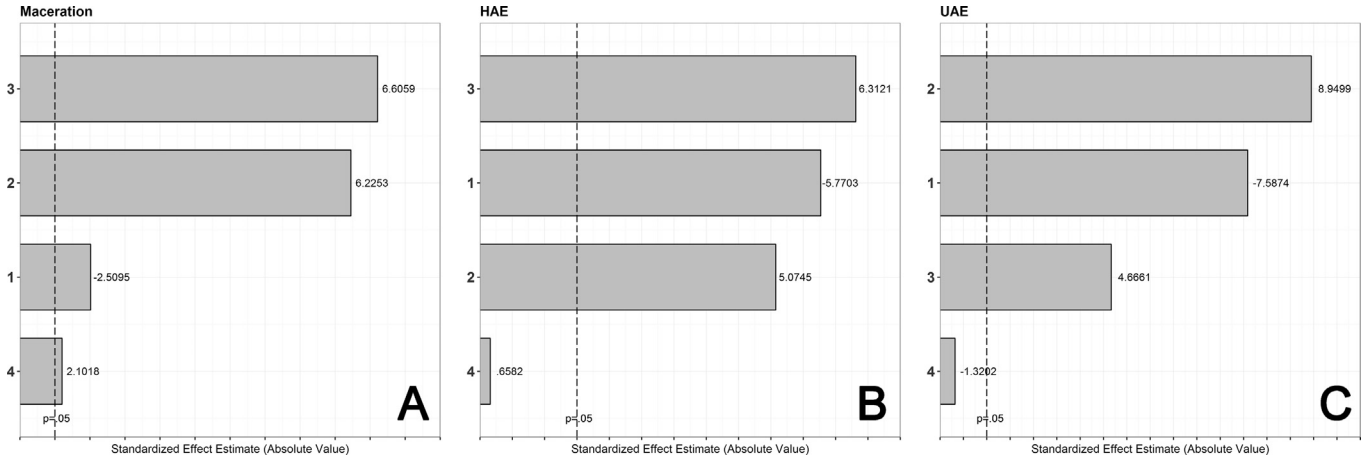

Fig. 2. Pareto charts of factors influence on the yield of TP in maceration (A), heat-assisted extraction - HAE (B) and ultrasound-assisted extraction - UAE (C); factor codes: 1, particle size; 2, type of solvent; 3, solid-to-solvent ratio; 4, extraction time.

cles and solid/liquid ratio have influenced the amount of polyphenols compounds extracted from parsley flakes at high temperature, while no significant changes in the extraction yields were observed at different time levels [39]. In Fig. 2C, influence of observed factor in UAE has been explored, where solid-to-solvent ratio, particle size and solvent type were the variables that influenced the TPC mostly. Duration of the extraction again had no significant effect. These results are comparable with results of Paz et al. [41], who reported that for the *Jatropha dioica* plant, solid/liquid ratio was a crucial factor affecting TPC yield in UAE, while the ethanol concentration is the second important factor. Also, in the same study the extraction time has exhibited a weak effect on the concentration of polyphenols from *Eucalyptus camaldulensis*. Furthermore, in UAE of polyphenols from orange peel, ethanol:water ratio had significant influence [51]. In all three extraction techniques, time of extraction was the least relevant factor for obtaining TP, hence this factor in our experiment was excluded from further optimization of the extraction process and the optimum extraction time was fixed at 15 min. Moreover, shorter extraction time could be accepted as more convenient regarding energy efficiency.

In the second factorial design, experimental data from the previous investigation (2^4 factorial screening design) have been introduced to 2^3 full factorial design (three independent variables which had significant effect in screening design: particle size, solid-to-solvent ratio and solvent type, each at two levels). A new full factorial design was applied to assay the influence of factors on the TP yield and to select the optimal conditions of the polyphenols extractions from *Serpylli herba* (Table 3). Additionally, this statistical method has allowed the evaluation of the interactions among the factors. The effects and corresponding regression coefficients of factors and interactions of two factors and probability for 2^3 full factorial design are presented in Table 4. As can be seen in Table 4, solid-to-solvent ratio was the most significant factor for polyphenols extraction during maceration, followed by type of solvent and particle size, whereas all interactions between factors were not decisive. In HAE the ethanol concentration was the dominant factor. The second important factor for maximizing polyphenols yield was size of herbal particles, followed by the significant interaction between particle size and solid-to-solvent ratio, which indicated that the effect of the particle size was not

the same at all solid-to-solvent ratios. In this extraction procedure solid-to-solvent ratio has significantly affected TP yield, but its value was lower than solvent type and particle size. Our experimental results regarding UAE indicated that solid-to-solvent ratio had the strongest influence on TPC, followed by particle size and ethanol concentration with lower absolute values (Table 4). Since the interaction of particle size and solid-to-solvent ratio was significant, we can conclude that the impact of the particle size depends on the used ratio levels. In the case of UAE the interaction effect of particle size-solvent type and solid-to-solvent ratio-solvent type was not statistically significant, as in the case of HAE.

Results of the 2^3 full factorial design, the observed and predicted values of TPC are listed in Table 3. The highest polyphenols concentration (measured value) in all extraction techniques was reached under the following parameters: the lowest particle size (0.3 mm), the highest solid-to-solvent ratio (1:30) and 50% ethanol (26.6, 29.8 and 32.7 mg GAE/L for maceration, HAE and UAE, respectively). The model has predicted maximum extraction efficiency under the same conditions: particle size of 0.3 mm, 1:30 ratio and 50% ethanol (26.7, 29.5 and 33.1 mg GAE/L, for maceration, HAE and UAE, respectively). The lowest TPC in all methods was observed in combination of 0.7 mm particle size, 1:20 solid-to-solvent ratio and 30% ethanol solvent (18.6, 19.8 and 23.6 mg GAE/L, for maceration, HAE and UAE, respectively). The model has predicted the minimum TP yield (18.5, 20.1 and 23.2 mg GAE/L, for maceration, HAE and UAE, respectively) under the same operational conditions as for observed values. Differences among the predicted results and the experimental data were minimal (< 2%). Therefore, full factorial design could be suggested as an adequate model for optimization of the extraction process of polyphenolic compounds from *T. serpyllum*.

According to the results of our experiment, it could be concluded that the optimal extraction conditions for obtaining the wild thyme extract most abundant in TP are: particle size, 0.3 mm; solid-to-solvent ratio, 1:30; solvent type, 50% ethanol; time, 15 min; by using ultrasound probe.

3.3. Influence of selected factors on total flavonoids

The influence of different solid-to-solvent ratios (1:10, 1:20 and 1:30), solvent types (30, 50 and 70% ethanol and water) and extraction techniques (maceration, HAE and UAE), on TF yield was tested, while particle size of 0.3 mm and extraction time of 15 min were fixed. The results of effects of solid-to-solvent ratio and solvent type on TPC in selected *T. serpyllum* extracts are shown in Fig. 3. In all extraction procedures, solid-to-solvent ratio had significant influence on TF yield and it was higher as solvent volume increased, as in the case of total polyphenols, which was in accordance with literature data [43]. Further, the highest TF yield was reached with 50% ethanol in all three extraction techniques (14.3, 12.4 and 16.7 mg CE/L, for maceration, HAE and UAE, respectively), while the lowest TFC was determined in water extracts, in the same manner as in the case of TPC. Horžić et al. [31] have reported that the best flavonoids extraction performance was achieved with aqueous ethanol as an extraction medium, in comparison to water, by using conventional extraction and ultrasound probe. Furthermore, catechins have represented a smaller amount of flavonoids in aqueous infusions from wild thyme [3]. Moreover, it was found that TFC increased with 50% of ethanol, because ethanol interacted with the flavonoids through non-covalent interactions and promoted a rapid diffusion into the extraction medium [21,53]. In extracts obtained by UAE the amount of total flavonoids was statistically significant higher than by using maceration and HAE, between which there was no statistically significant difference. Our results are in accordance with the previous findings that the highest content of flavonoids in yellow tea was obtained by ultrasound probe assisted extraction, while lower TFC was detected in conventional extraction [31]. Silva et al. [35] have shown negative influence of high temperature on the response of total flavonoids. Lower value of TFC obtained in HAE could imply that flavonoid compounds are more sensitive to increased temperature than to ultrasound. These results are favorable for further implication of ultrasound waves in wild thyme extraction, especially for flavonoids-rich extracts.

3.4. LC/MS and HPLC analysis results

Using LC/DAD/MS technique, 9 polyphenolic compounds were found in the studied extract (Table 5). According to their UV spectra, the polyphenolic compounds were classified on two main groups: caffeic acid derivates (CA) and flavonoids (FL). Molecular formula of the analytes was revealed by exact mass measurements of their deprotonated molecular ions, performed using a time-of-flight (TOF) mass spectrometer in negative polarity mode. Molecular formula determinations were performed by Molecular Feature Extractor program, taking into account *m/z* values and isotopic abundance patterns for all ion species noticed for respective compound. The spectral data of the identified compounds were compared to those of literature [54–56]. Further, the quantification of individual phenolic acids and flavonoids in selected wild thyme extracts was carried out using HPLC method, and the results are shown in Table 6. The main phenolic acid was rosmarinic acid, followed by salvianolic acid K isomer and salvianolic acid I. Boros et al. [14] also showed that rosmarinic acid was the dominant

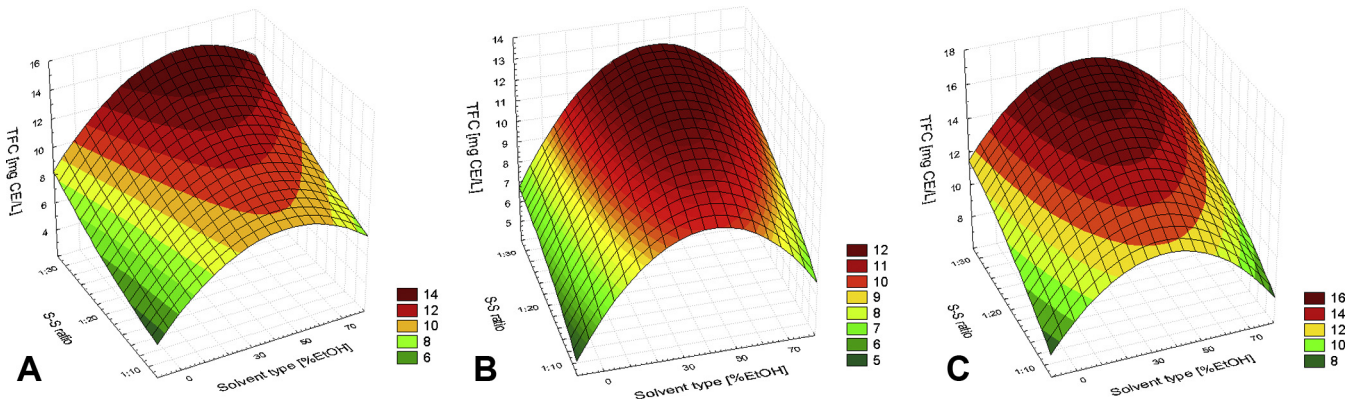

Fig. 3. Surface plots of solid-to-solvent ratio and solvent type effects on TFC (mg CE/L) of selected *T. serpyllum* extracts in maceration (A), heat-assisted extraction - HAE (B) and ultrasound-assisted extraction - UAE (C).

Table 5
Identified polyphenolic compounds in chosen *T. serpyllum* extract (LC/MS).

				DAD		MS	
Peak	Retention time [min]	Compound	Compound class	λ_{max} [nm]	Species	Mass	Molecular formula
1	6.93	6,8-Di-*C*-glucosylapigenin	FL	236, 278, 326	M-H, 2M-H	594.1573	$C_{27}H_{30}O_{15}$
2	6.96	Chlorogenic acid	CA	302 sh, 324	M-H, M+HCOO, 2M-H	354.0949	$C_{16}H_{18}O_9$
3	8.27	6-hydroxyluteolin 7-*O*-glucoside	FL	284, 342	M-H, 2M-H	464.0947	$C_{21}H_{20}O_{12}$
4	8.33	Caffeic acid	CA	300 sh, 328	M-H, 2M-H	180.0421	$C_9H_8O_4$
5	9.57	Luteolin 7-*O*-glucuronide	FL	256, 268 sh, 342	M-H, M+HCOO, M+Cl, 2M-H	462.0788	$C_{21}H_{18}O_{12}$
6	11.12	Apigenin glucuronide	FL	268, 334	M-H, 2M-H	446.0844	$C_{21}H_{18}O_{11}$
7	11.44	Salvianolic acid K isomer	CA	286, 332	M-H, M+HCOO, 2M-H	556.1205	$C_{27}H_{24}O_{13}$
8	11.75	Rosmarinic acid	CA	294, 330	M-H, M+HCOO, M+Cl, 2M-H	360.0846	$C_{18}H_{16}O_8$
9	12.44	Salvianolic acid I	CA	294, 326	M-H	538.1099	$C_{27}H_{22}O_{12}$

FL, flavonoids; CA, caffeic acid derivates.

Table 6
Content of phenolic acids and flavonoids in selected *T. serpyllum* extracts determined by HPLC.

	S-S ratio	Solvent type [% EtOH]	6,8-Di-*C*-glucosyl apigenin (μg/mL)	ChA (μg/mL)	6-hydroxy luteolin 7-*O*-glucoside (μg/mL)	Caffeic acid (μg/mL)	Luteolin 7-*O*-glucuronide (μg/mL)	Apigenin glucuronide (μg/mL)	SA K isomer (μg/mL)	RA (μg/mL)	SA I (μg/mL)
	1:20	30	0.09	tr	0.39	0.07	1.56	0.35	0.73	3.22	/
M	1:20	50	0.11	tr	0.71	0.04	1.63	0.39	0.82	3.68	0.61
	1:30	30	0.13	tr	0.51	0.12	1.55	0.37	0.83	3.50	0.07
	1:30	50	0.12	tr	0.53	0.14	1.48	0.34	0.77	3.73	0.97
	1:20	30	0.08	tr	0.35	0.12	1.36	0.30	0.68	3.41	0.43
HAE	1:20	50	0.06	tr	0.28	0.18	1.48	0.39	0.80	3.62	0.51
	1:30	30	0.11	tr	0.41	0.12	1.50	0.29	0.89	3.59	0.47
	1:30	50	0.16	tr	0.66	0.13	1.89	0.38	0.93	4.06	0.94
	1:20	30	0.06	tr	0.21	0.16	0.88	0.13	0.49	1.69	/
UAE	1:20	50	0.12	tr	0.48	0.20	1.63	0.42	0.95	4.12	0.72
	1:30	30	0.09	tr	0.26	0.07	1.23	0.31	0.78	3.37	0.34
	1:30	50	0.17	tr	0.78	0.16	2.04	0.29	0.97	4.18	1.21

S, solid-to-solvent; M, maceration; HAE, heat-assisted extraction; UAE, ultrasound-assisted extraction; ChA, chlorogenic acid; SA, salvianolic acid; RA, rosmarinic acid; tr, traces.

compound in *Thymus* species. Among flavonoids, the dominant were luteolin derivates, such as luteolin 7-*O*-glucuronide and 6-hydroxyluteolin 7-*O*-glucoside, followed by apigenin glucuronide which was in accordance with the literature data [14,24,25]. The highest content of rosmarinic acid, salvianolic acid K isomer, salvianolic acid I, 6,8-Di-*C*-glucosylapigenin, 6-hydroxyluteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide was achieved with extraction parameters of 50% ethanol and 1:30 ratio, in UAE, which was in accordance with previously obtained results of TPC and TFC. On the other hand, caffeic acid and apigenin glucuronide were obtained in the highest amount under experimental conditions of 50% ethanol and 1:20 ratio by using ultrasound probe.

4. Conclusion

This study was the first attempt to evaluate the effects of different particle size, solid-to-solvent ratio, type of solvent and time on the extraction of polyphenols from *Serpylli herba* using various extraction techniques (maceration, HAE and UAE). The results indicated that particle size, solid-to-solvent ratio, solvent type, and extraction procedure have significantly affected the content of total polyphenols, while the extraction time has not been relevant factor. According to TPC, the efficiency of the extraction procedures for all variables is ranked by significance in the following order: UAE > HAE > maceration, whereas total flavonoids yield was significantly higher in UAE, while between maceration and HAE there was no statistically significant difference. With regard to the experimental design, the best extraction performance was achieved with the use of ultrasound probe, particularly in combination of particle size of 0.3 mm, 1:30 ratio and 50% ethanol. Considering that the predicted and experimental values were approximately the same, full factorial design can be assumed as an adequate model to optimize the process of polyphenols extraction from *Serpylli herba*. UAE could be used for future polyphenols extraction, due to obtained high TP and TF yield, short extraction time, and low degradation of the extracted compounds, compared to other tested extraction methods. Furthermore, LC-MS analysis detected presence of two classes of polyphenols compounds: phenolic acid (chlorogenic acid, caffeic acid, salvianolic acid K isomer, rosmarinic acid and salvianolic acid I) and flavonoids (6,8-Di-*C*-glucosylapigenin, 6-hydroxyluteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide and apigenin glucuronide). According to HPLC, the dominant compound was rosmarinic acid, followed by salvianolic acid K isomer, salvianolic acid I and luteolin 7-*O*-glucuronide. Moreover, this study was an initial step in production of polyphenols-rich wild thyme extracts aimed to be used for formulation of foodstuffs and medicines. Further research concerning the antioxidant and other biological and pharmacological activities of these extracts, as well as encapsulation of selected extracts are needed.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2017.01.055.

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