Determination of Flavonoids and Phenolic Acids in Plant Materials Using SLE-SPE-UHPLC-MS/MS Method

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Abstract

Today, great emphasis is placed on the search for and dissemination of native plant materials in various industries, with particular emphasis on food, cosmetics, dietary supplements, and as livestock feed and a source of biomass. Solid-liquid extraction (SLE), solid phase extraction (SPE), and ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) methods were developed for the simultaneous determination of 30 flavonoids and phenolic acids in plant materials (lucerne (*Medicago sativa* L.), goldenrod (*Solidago virgaurea* L.), phacelia (*Phacelia tanacetifolia* Benth.), buckwheat (*Fagopyrum esculentum*), licorice (*Glycyrrhiza glabra*), and lavender (*Lavandula spica* L.)). Different SLE methods were tested to evaluate their applicability for the isolation of polyphenols from plants. Then extracts were purified with a C18 reversed-phase SPE cartridge. After extraction, samples were analyzed by UHPLC-MS/MS. The SLE-SPE-UHPLC-MS/MS assay method has been fully validated. For the most compounds, good recoveries and RSDs (< 10%) were obtained. Limits of quantification range from 0.4 to 20 ng/mL. The main phenolic acids in the studied plants have been found to be 3-(4-hydroxyphenyl)propionic acid, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid. Quercetin, rutin, glabridin, and naringenin are the major flavonoids detected in the analyzed samples. The obtained scientific data can be useful to select plant materials with high nutritional value and the presence of many biologically active ingredients, as well as selecting samples used as a plant biomass, as food, and as a component of dietary supplements.

Keywords Flavonoids · Phenolic acids · Plant · UHPLC-MS/MS · Extraction

Introduction

Phenolic compounds, ubiquitous in plants, are an essential part of the human diet and animal feed and are of considerable interest due to their antioxidant properties. These compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plants. In plant, phenolics may act as phytoalexins,

antifeedants, attractants for pollinators, contributors to the plant pigmentation, antioxidants, and protective agents against UV light (Treuter 2006).

In food, phenolics may contribute to the bitterness, astringency, color, flavor, odor, and oxidative stability of products. In addition, the health-protecting capacity of some phenolics and the wide range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective, and vasodilatory effects, of other plant phenolics are of great importance to producers, processors, and consumers. Fruits, vegetables, nuts, seeds, flowers, bark, and beverages are common sources for polyphenols in human diets and in animal nutrition (Shahidi and Ambigaipalan 2015).

Today, great emphasis is placed on the search for and dissemination of native plant materials in various industries, with particular emphasis on cosmetics, dietary supplements, and as livestock feed and a source of biomass. Selected plant materials should have both nutritional value and the presence of biologically active ingredients, especially antioxidants. For this purpose, it is necessary to determine biologically active

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compounds (e.g., polyphenols) in raw plant materials. Those plant materials which meet the aforementioned criteria and which also contain important active ingredients are lucerne (*Medicago sativa* L.), licorice (*Glycyrrhiza glabra*), goldenrod (*Solidago virgaurea* L.), buckwheat (*Fagopyrum esculentum*), phacelia (*Phacelia tanacetifolia* Benth.) and lavender (*Lavandula spica* L.). In the literature, there is information about the potential antioxidant activity of these plants; however, only little information is available about the content of polyphenols (Velioglu et al. 1998).

Chromatographic techniques combined with different detectors are preferred to individually identify and quantify phenolic compounds in the above plants. Detection of polyphenols in plant material (buckwheat (Kreft et al. 2006), lucerne (Martin et al. 2006), licorice (Wang and Yang 2007), goldenrod (Apáti et al. 2002) has been performed with a UV-Vis detector, because the conjugated double and aromatic bonds allow phenolics to absorb in UV or UV-Vis regions. A diode array detector (DAD) is also often used, because it gives more information when complex mixtures are present in the plant extracts of lavender (Areias et al. 2000), lucerne (Goławska et al. 2010a, b), licorice (Siracusa et al. 2011), and goldenrod (Sabir et al. 2012). Other methods used for the detection of rutin and catechins in buckwheat include an electrochemical coulometric array detector (EC) (Danila et al. 2007). High-performance liquid chromatograph coupled with mass spectrometry has commonly been used for structural characterization of phenolics (Marczak et al. 2010). Electrospray ionization mass spectrometry (ESI-MS or ESI-MS/MS) has been employed for structural confirmation of phenolics in *Medicago truncatula* (Farag et al. 2007), *Glycyrrhiza glabra* (Montoro et al. 2011), and *Glycyrrhiza uralensis* and *Glycyrrhiza glabra* (Liao et al. 2012). When qualitative analysis has to be performed, the highest resolution for identification purposes is provided by time-of-flight (TOF) mass spectrometers (Verardo et al. 2010).

Besides liquid chromatography, nuclear magnetic resonance spectroscopy (NMR) has also been used due to its being another technique that allows unequivocal information on phenolics. ^1H NMR and ^{13}C NMR were used by Farag et al. (2012) to identify the major phenolic compounds in *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and *Glycyrrhiza echinata* roots. In some cases, gas chromatographic and capillary electrophoresis techniques was employed for separation and quantitation of flavonoids and isoflavonoids in roots and cell suspension cultures of *Medicago truncatula* (Farag et al. 2007), rutin in *Fagopyrum esculentum* (Kreft et al. 1999), and five flavonoids in licorice (Chen et al. 2009).

Simultaneous quantification of flavonoids and phenolic acids in plant materials in a single operation is much more convenient than using several separate procedures, especially for routine analysis or for a large number of samples. One of the greatest advantages is the simplification of the overall

analytical process, for example, by reducing the frequency of changing the separation system and the number of mobile phase preparations. Moreover, most of the literature-revealed analytical methods do not appear to have validated the analytical methodology involved or have been applied to determination of polyphenols only in one plant.

Based on the aim of the current study, a reversed-phase ultra-high-performance liquid chromatography coupled with a tandem mass spectrometry (UHPLC-MS/MS) method for simultaneous quantification of 30 polyphenols (flavonoids and phenolic acids) in six different plant materials (lucerne, goldenrod, phacelia, buckwheat, licorice, and lavender) was developed.

The developed method, which allows to simultaneous determine a wide spectrum of flavonoids from various chemical/structural groups, can be certainly useful for the analysis of plant materials, used to a small extent for human consumption. Lucerne, goldenrod, phacelia, buckwheat, licorice, and lavender, known primarily as feed components, could be also widely used as food supplements or ingredients of other food products, due to the content of antioxidants, which are important for health. Moreover, it should be noted that these materials are readily available and cheap.

Experimental

Chemicals and Reagents

Chrysin (CHS) (used as an internal standard; IS), rutin (RUT), hesperetin (HST), quercetin (QUE), naringenin (NAR), naringin (NARG), narirutin (NRT), hesperidin (HSD), neohesperidin (NHSD), pinocembrin (PIN), taxifolin (TAX), fisetin (FIS), glabridin (GLB), eriocitrin (ERC), eriodictyol (ERI), formononetin (FOR), liquiritin (LIQ), liquiritigenin (LQG), 3-hydroxybenzoic acid (3-HBA), benzoic acid (BA), caffeic acid (CA), 3,4-dihydroxybenzoic acid (3,4-DHBA), hippuric acid (HA), α -hydroxyhippuric acid (α -HHA), 3-(4-hydroxyphenyl)propionic acid (3,4-HPPA), 4-hydroxybenzoic acid (4-HBA), 3,4-dihydroxy-phenylacetic acid (DOPAC), 3-hydroxyphenylacetic acid (3-HPA), *p*-coumaric acid (*p*-COA), ferulic acid (FA), and 4-hydroxy-3-methoxyphenylacetic acid (HVA) were purchased from Sigma-Aldrich (Saint Louis, USA). Formic acid (HCOOH) and acetonitrile (ACN) for LC-MS from Merck (Darmstadt, Germany) were used for mobile phase preparation. Ethanol (EtOH) and methanol (MeOH), both from Chempur (Piekary Slaskie, Poland), and hydrochloric acid (HCl) from Stanlab (Lublin, Poland) were used for sample treatment optimization. Purified water from a Milli-Q Element A10 System (Millipore, Milford, MA, USA) was used in the preparation of the mobile phase, for sample preparation and reagent solutions.

Stock Solutions, Calibration Standards, and Quality Control Samples

Standard stock solutions of flavonoids and phenolic acids were prepared by dissolving the analytes in methanol, obtaining concentrations of 1.0 mg/mL. Working solutions for calibration standards (CS) and quality control (QC) samples were prepared by diluting stock solutions of analytes with methanol, which resulted in the polyphenols working solution with a concentration of 1000, 500, 250, 100, and 20 ng/mL. Stock solutions and working solutions were stored in a refrigerator at 4 °C.

Calibration standards (CSs) were prepared at ten levels, ranging from 1 to 1000 ng/mL, by dilution of the polyphenol working solutions with methanol. Quality control (QC) samples for method validation were prepared in the same way as calibration standards and at three concentration levels of polyphenols: 1 ng/mL (low quality control, LQC), 40–100 ng/mL (medium quality control, MQC), and 80–800 ng/mL (high quality control, HQC). Additionally, dilution integrity was studied in a sample spiked at 80–800 ng/mL (depending on compounds). Aliquots of all standards were stored in a refrigerator at 4 °C until further use.

Plant Material

Lucerne (*M. sativa* L.), plants of a species belonging to the bean family, were harvested in the flowering season from plantations located (without the use of agrotechnical) in Kołuda Wielka (Kuyavian-Pomeranian Province) in June 2016. Goldenrod (*S. virgaurea* L.), wild plants, were collected near in the vicinity of Toruń (Kuyavian-Pomeranian Province) in August–September 2016. Phacelia (*P. tanacetifolia* Benth.), agricultural crops, without agronomic treatments, were collected near Bobrowniki (Kuyavian-Pomeranian Province) in August 2016. Raw materials were divided into morphological parts: flowers, leaves, and stalks (lucerne and goldenrod) and flowers, leaves, stalks, and roots (phacelia). The segregated material was dried at ambient temperature, without light, and then ground in a ball mill. Powdered material was stored in glassware without light.

Buckwheat (*F. esculentum*), licorice (*G. glabra*), and lavender (*L. spica* L.) were obtained from various local herbal markets.

Sample Preparation

Sample treatment was studied using six different plant materials: lucerne, goldenrod, phacelia, buckwheat, licorice, and lavender. Eight different protocols were studied to obtain the maximum recovery of flavonoids and phenolic acids from plants. The following were tested: solid-liquid extraction (SLE) using H₂O, shaking for 5 h (ST1); mixture H₂O/

EtOH (1:1; v/v), shaking for 2 h (ST2); mixture H₂O/EtOH (1:1; v/v), shaking for 5 h (ST3); EtOH, shaking for 5 h (ST4); mixture H₂O/MeOH (1:1; v/v), shaking for 2 h (ST5); mixture H₂O/MeOH (1:1; v/v), shaking for 2 h two times (ST6); mixture H₂O/MeOH (1:1; v/v), shaking for 5 h (ST7); and MeOH, shaking for 5 h (ST8).

For protocols S1–S8, each plant material was previously crushed and homogenized and approximately 2.5 g was accurately weighed. Samples were extracted with 10 mL (for phacelia, lucerne, and licorice) or 20 mL (for lavender, goldenrod, and buckwheat) of MeOH for 5 h at 900 rpm, using an automatic shaker (SLE) (Vibramax 100, Heidolph Instruments GmbH & Co.). The extract was filtered and evaporated to dryness, then the residues were dissolved with 1 mL of MeOH and 14 mL of H₂O, adjusted to pH 3.5 by HCl.

The obtained extract was then purified by SPE using a solid phase extraction vacuum station and a reverse-phase octadecyl (C18, 6 mL, 500 mg) column (BAKERBOND spe-12G system, J.T. Baker Inc., Deventer, Netherlands). A C18 column was conditioned with 6 mL MeOH, followed by 6 mL of H₂O (pH 3.5) acidified by HCl. Then, sample extract was passed through the sorbent at a flow rate of approximately 1 mL/min and the solid phase was air-dried for 2 min. The analytes were eluted with 6 mL MeOH and the eluates were evaporated to dryness. Then, the residues were dissolved with 1 mL of MeOH and samples were filtered through a 0.45-μm PES membrane. Finally, 5 μL of the solution was injected into the UHPLC-MS/MS system. The extraction procedure was performed in triplicate for each sample.

In addition to the tested SLE-SPE procedures, polyphenol extraction efficiency was evaluated using the QuEChERS technique. For this purpose, a salt mixture, 4 g MgSO₄, 1 g NaCl, 1 g trisodium citrate dihydrate, and 0.5 g disodium hydrogen citrate sesquihydrate were filled in a 50-mL PTFE centrifuge tube. Then, 10 mL or 20 mL of 1% FA in ACN and homogenized plant sample (2.5–5.0 g) were added into the tube. The mixture was shaken vigorously for 10 s, vortexed for 1 min, and then centrifuged for 5 min at 25.2×g. A 2–3-mL aliquot from the upper part of the extract was transferred into a 15-mL microcentrifuge tube containing 150 mg PSA, 45 mg GCB, and 855 mg MgSO₄ (for highly pigmented fruits and vegetables). The mixture was then shaken, vortexed, and centrifuged for 5 min at 15.4×g. The harvested ACN extract was then filtered using a 0.45-μm PES filter before UHPLC-MS/MS analysis.

Instrumentation and UHPLC-MS/MS Conditions

The UHPLC-MS/MS analyzes were performed by using a triple quadrupole mass spectrometer with a turbo ion spray interface (API 4500, Applied Biosystems, USA) coupled to a Dionex UHPLC liquid chromatography system (Dionex Corporation, Sunnyvale, CA, USA) equipped with an UltiMate 3000 RS

(Rapid Separation) pump, an UltiMate 3000 autosampler, an UltiMate 3000 column compartment with a thermostable column area, and an UltiMate 3000 variable wavelength detector. Both systems and data treatment were controlled by the Analyst 1.5.1 software (Applied Biosystems, USA).

The optimum condition for the separation of polyphenols using a Zorbax Eclipse XDB-C18 column (50×2.1 mm, $1.8 \mu\text{m}$, Agilent Technologies, USA) was obtained with the mobile phase: 0.1% v/v FA in H_2O (solvent A) and ACN (solvent B). The UHPLC gradient program used was as follows: (1) mobile phase A was set to 95% at 0 min, (2) a linear gradient was dropped to 40% A in 8.0 min, (3) mobile phase A was ramped to 95% again in 0.1 min, and (4) from 8.1 to 10 min, mobile phase A was maintained at 95%. The mobile phase flow rate was 0.5 mL/min, the injection volume was $5 \mu\text{L}$, and the column temperature was maintained at 30°C . The total run time was 10 min (Fig. 1).

Electrospray ionization (ESI) conditions in negative mode were first tested with direct infusion into the mass spectrometer to select the precursor and the product ions resulting from fragmentation, declustering potential (DP), collision energy (CE), entrance potential (EP), and collision cell exit potential (CXP) for each polyphenol (Table S1, Supplementary Material). This study was conducted by direct infusion of standard solutions at 10 ng/mL of flavonoids and phenolic acids at a flow rate of $10 \mu\text{L}/\text{min}$. Flow injection analysis (FIA) was used to optimize capillary voltage, curtain and nebulizer gas flow rates, and source temperature. The experiments were conducted at a mobile phase flow rate (solvent A/solvent B, 1:1 v/v) of 0.5 mL/min. The following settings were also applied to the turbo ion spray source: capillary voltage (IS), -4500 V; temperature (TEM), 500°C ; nebulizer gas (GS1), 60 psi; turbo-gas (GS2), 50 psi; curtain gas (CUR), 20 psi; and collision activated dissociation gas (CAD), 4 psi. The polyphenols were evaluated employing the selected reaction monitoring (SRM) mode with a dwell time of 50 ms. The most intense transition was used for quantification and the other transition was used for confirmation.

Fig. 1 UHPLC-MS/MS chromatogram of a standard solution containing the analyzed polyphenols and IS

Method Validation

The established chromatographic method was evaluated for calibration curve linearity, limit of quantification (LOQ), intra- and inter-day precision and accuracy, recovery, stability, dilution integrity, and carry-over. The full description was placed in the [Supplementary Material](#).

The linearity of the calibration curve was evaluated by analyzing polyphenol standard solutions in MeOH at ten concentrations ranging from 0.4 to 1000 ng/mL. The LLOQ was defined as the lowest amount of analyte which could be quantified reliably, while complying with the criteria for accuracy and precision. Precision, accuracy, recovery, matrix effect, and stability were determined for the three QC levels (LQC 1 ng/mL, MQC from 40 to 300 ng/mL, and HQC from 80 to 800 ng/mL). According to the guidelines matrix effect was determined by comparing the peak areas obtained from blank extract spiked with the analytes to those of pure standard solutions containing the same amount of the analytes. QC sample stability analyzes were performed at $2\text{--}8^\circ\text{C}$ for the short (7 days) and long term (28 days) and the 24-h stabilities were measured in standard solutions at a UHPLC-MS/MS autosampler temperature of 10°C . Dilution integrity was studied after tenfold dilution of samples spiked with flavonoids and phenolic acids with MeOH. Methanol, which did not contain any analytes or internal standards, was injected after the ULOQ samples to investigate the carry-over of this method in each validation batch.

Results and Discussion

SLE-SPE Method Development

The effects of organic and aqueous solvents on the content of flavonoids and phenolic acids determined in lucerne, goldenrod, buckwheat, lavender, phacelia, and licorice were studied using the SLE extraction method. Table S2 (Supplementary Material) summarizes the content of each of the identified

compounds after eight different extraction procedures (ST1–ST8) and results for selected compounds are shown in Fig. 2.

Fig. 2 Effect of the SLE extraction conditions on the peak area of selected polyphenols determined in four different plant materials (ST1: H₂O, shaking for 5 h; ST2: mixture H₂O/EtOH (1:1; v/v), shaking for 2 h; ST3: mixture H₂O/EtOH (1:1; v/v), shaking for 5 h; ST4: EtOH, shaking for 5 h; ST5: mixture H₂O/MeOH (1:1; v/v), shaking for 2 h; ST6: mixture H₂O/MeOH (1:1; v/v), shaking for 2 h two times; ST7: mixture H₂O/MeOH (1:1; v/v), shaking for 5 h; ST8: MeOH, shaking for 5 h)

The content of polyphenols varied in response to the extraction solvents and extraction times. For most of the studied compounds, MeOH (ST8) and EtOH (ST4) extracts of all plant materials had approximately a few times higher values of polyphenols and phenolic acids compared to H₂O (ST1) and a mixture of H₂O/EtOH (1:1; v/v) (ST5) or H₂O/MeOH (1:1; v/v) (ST7) extracts. The mean content of studied polyphenol content increased from 207 ng/g, when the extractions were performed with H₂O (ST1) to 5566 ng/g (for licorice), when extractions were performed with MeOH (ST8). Also increases in the extraction yields of selected analytes were obtained when EtOH (mean amount for example for licorice was 1581 ng/g) was used as an extraction solvent (ST4). Intermediate mean amounts (2980 ng/g and 2659 ng/g, for licorice) of studied polyphenols were observed after the use of the H₂O/MeOH (1:1; v/v) (ST7) and H₂O/EtOH (1:1; v/v) mixtures (ST5), respectively.

The solubility of polyphenols selected in this study was the highest in MeOH, a little lower in ethanol and the mixture of H₂O/MeOH (1:1; v/v) or H₂O/EtOH (1:1; v/v), and the lowest in H₂O. MeOH showed slightly better characteristics as a solvent for polyphenols and phenolic compounds than EtOH, but the differences were not great, so EtOH is the more appropriate solvent for use in the food industry.

The content of polyphenols in all plant material extracts increased with extraction times over 5 h. Moreover, two extraction cycles using the H₂O/MeOH (1:1; v/v) mixture (ST6) improved the extraction efficiency of polyphenol compounds from plant materials compared to one extraction cycle with the same solvent (ST5). On the other hand, the results obtained for the one-step batch extraction mode using H₂O/MeOH (1:1; v/v) for 5 h (ST7) and in the two-step batch extraction mode using the same mixture two times over 2 h (ST6) were very similar.

A significant difference was also observed between the contents obtained by the above methods from each of studied plant materials. The difference between extraction yields obtained by the eight methods depended on the raw material analyzed. This remained true, no matter which solvent was used. As shown above, the maximum contents of all the polyphenols were obtained using methanol as the extraction solvent. But, the correlations obtained for the materials were different. Variation in the contents of various extracts is attributed to polarities of different compounds present in the plants and such differences have been reported in the literature (Abarca-Vargas et al. 2016).

Additionally, to achieve better analyte extraction, QuEChERS methods were tested. PSA and GCB were used to remove interferences and pigments. Trials using QuEChERS proved to be less efficient than SLE-SPE extraction for all of the flavonoids and phenolic acids. Some of the compounds (e.g., 3,4-HPPA, 3-HPA, DOPAC, HVA, α -HPA, and NRI) were not detected after application of QuEChERS

Table 1 Content of phenolic acids and flavonoids determined in plant materials

Compound	Content (ng/g)												
	SD (ng/g)												
	Lucerne			Goldenrod			Phacelia				Buckwheat	Liquorice	Lavender
	Flowers	Leaves	Stalk	Flowers	Leaves	Stalk	Flowers	Leaves	Stalk	Root	Whole plant	Whole plant	Whole plant
Phenolic acids													
3,4-DHBA	1808	569.1	158.1	521.7	1182	743.5	347.5	230.2	212.9	211.9	4241	720.4	300.9
	63.2	15.3	4.5	27.1	36.6	6.6	20.8	9.7	7.9	6.3	265	31.1	6.3
α -HPA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DOPAC	7.0	5.6	0.17	0.22	NQ	NQ	868.9	1956	1199	380.6	7.4	38.8	7.4
	0.1	0.2	0.01	0.01	NQ	NQ	24.9	38.7	65.5	16.5	0.2	0.5	0.2
4-HBA	8313	3307	2163	702.3	1423	115.2	4784	3915	3809	4049	1241	2156	1578
	214	58.6	61.6	29.0	55.0	4.58	137	57.5	92.9	191	47.0	97.4	16.4
CA	1675	178.5	42.8	870.3	1346	557.9	404.1	811.9	873.0	1444	193.0	108.1	584.9
	54.4	3.8	1.3	39.5	37.8	18.8	22.7	29.9	34.5	78.8	2.0	2.9	30.6
HA	4.5	3.8	0.89	0.75	0.42	0.33	0.80	27.1	0.76	0.73	21.0	11.0	22.0
	0.2	0.1	0.03	0.04	0.01	0.01	0.03	0.5	0.03	0.03	0.5	0.6	1.4
3-HBA	64.5	169.4	172.8	1.73	5.1	0.28	21.6	0.34	0.27	2.47	25.1	28.2	18.1
	2.7	9.2	4.7	0.03	0.2	0.01	0.7	0.01	0.01	0.05	1.0	1.5	0.7
3-HPA	3.5	4.42	11.8	0.27	0.24	NQ	7.9	1.37	0.20	0.22	207.8	72.3	24.0
	0.1	0.08	0.4	0.01	0.01	NQ	0.1	0.06	0.01	0.01	14.6	2.0	0.7
HVA	48.0	24.7	34.8	1.53	33.0	17.1	24.1	13.0	25.0	43.5	63.6	821.2	65.1
	0.8	0.7	1.6	0.06	0.8	0.6	0.3	0.3	0.6	0.6	3.8	33.9	1.6
3,4-HPPA	2397	632.8	446.4	265.7	214.7	79.9	77.9	161.5	141.4	237.2	257.1	118,535	558.3
	93.7	36.3	17.2	12.6	6.2	2.0	2.9	2.5	2.0	2.3	10.2	3733	11.3
p-CA	2160	781.7	544.5	365.3	264.7	104.3	173.4	297.9	11,778	324.8	396.1	703.8	520.3
	58.5	7.6	23.7	19.9	4.0	1.1	9.3	8.1	233	8.7	16.5	13.9	26.7
FA	5496	1332	462.1	919.8	519.3	611.1	385.6	610.1	608.1	823.8	158.7	166.2	379.9
	112	41.4	4.0	13.9	12.3	33.0	13.8	23.6	6.5	12.8	6.3	4.7	12.0
BA	7086	10,000	3717	280.6	871.2	195.9	648.5	360.9	170.9	197.3	665.9	3085	687.3
	225	417	47.2	5.5	15.2	3.4	19.3	13.3	4.2	1.9	14.5	33.3	20.4
Flavonoids													
ERC	0.24	0.41	NQ	0.07	NQ	NQ	NQ	3.09	NQ	NQ	9.6	NQ	4.3
	0.01	0.01	NQ	0.01	NQ	NQ	NQ	0.03	NQ	NQ	0.5	NQ	0.1
LIQ	13.5	0.59	1.28	ND	ND	0.47	ND	ND	ND	NQ	13.8	1359	2.21
	0.4	0.01	0.04	ND	ND	0.02	ND	ND	ND	NQ	0.2	21.2	0.02
RUT	147.6	148.4	59.0	17,384	23,523	6394	13,922	10,296	1129	2336	7521	2.19	282.9
	1.8	4.1	2.3	615	597	228	414	351	44.9	78.9	130	0.08	6.1
TAX	101.3	45.5	43.6	0.73	2.62	0.30	0.79	5.9	1.59	4.0	40.4	1.05	3.88
	3.2	0.9	1.6	0.03	0.05	0.01	0.03	0.1	0.04	0.2	1.9	0.03	0.04
NRI	15.4	5.8	8.2	3.9	3.18	3.1	7.8	3.6	7.6	4.75	8.9	30.3	13.9
	0.5	0.1	0.5	0.2	0.08	0.1	0.5	0.2	0.3	0.06	0.2	0.9	0.4
NARG	0.75	0.43	0.30	NQ	ND	0.22	0.78	NQ	0.31	NQ	0.51	2.7	0.87
	0.03	0.01	0.01	NQ	ND	0.01	0.02	NQ	0.02	NQ	0.01	0.1	0.02
HSD	29.9	8.9	19.5	0.26	0.25	63.3	93.1	66.5	57.6	0.97	1227	7.6	23.0
	0.2	0.3	0.3	0.01	0.02	2.6	2.9	1.7	2.8	0.05	29.9	0.3	0.9
NHSD	12.3	11.5	10.5	0.15	0.13	33.1	62.1	22.2	34.1	0.52	38.8	5.5	32.1
	0.1	0.3	0.2	0.01	0.01	1.5	2.4	0.9	0.2	0.01	1.8	0.3	1.6

Table 1 (continued)

Compound	Content (ng/g)												
	SD (ng/g)												
	Lucerne			Goldenrod			Phacelia				Buckwheat	Liquorice	Lavender
	Flowers	Leaves	Stalk	Flowers	Leaves	Stalk	Flowers	Leaves	Stalk	Root	Whole plant	Whole plant	Whole plant
FIS	5.4	1.44	0.32	5.9	0.70	0.21	14.7	NQ	NQ	NQ	ND	44.6	0.40
	0.3	0.03	0.02	0.2	0.02	0.01	0.6	NQ	NQ	NQ	ND	0.4	0.01
LQG	38.6	11.4	26.7	ND	NQ	ND	NQ	NQ	ND	ND	12.3	1463	0.41
	1.7	0.3	0.9	ND	NQ	ND	NQ	NQ	ND	ND	0.2	40.0	0.03
ERI	2.9	0.76	2.8	1.14	6.1	0.25	21.3	25.8	2.5	0.19	21.5	29.0	7.4
	0.1	0.03	0.1	0.02	0.1	0.01	0.8	0.7	0.1	0.01	0.3	1.3	0.2
QUE	1168	56.9	NQ	1281	3363	NQ	20.6	58.1	74.3	0.27	908.3	5.4	15.9
	25.3	2.3	NQ	11.1	104	NQ	0.5	2.2	2.0	0.01	32.7	0.1	0.4
NAR	16.3	6.9	0.38	0.45	1.44	0.50	1.60	1.88	0.99	1.00	146.8	474.7	398.1
	0.6	0.3	0.02	0.03	0.01	0.02	0.05	0.07	0.03	0.02	4.2	5.0	15.8
HST	NQ	0.18	NQ	NQ	24.9	NQ	0.16	0.67	NQ	0.42	22.2	1.17	0.81
	NQ	0.01	NQ	NQ	1.5	NQ	0.01	0.02	NQ	0.02	0.8	0.03	0.01
FOR	245.2	74.7	253.7	ND	ND	ND	ND	ND	ND	ND	1.56	751.7	7.0
	15.4	5.2	10.7	ND	ND	ND	ND	ND	ND	ND	0.04	31.3	0.2
PIN	0.63	0.50	NQ	0.45	0.68	NQ	0.24	0.33	NQ	NQ	1.98	7.6	0.91
	0.02	0.03	NQ	0.01	0.02	NQ	0.01	0.01	NQ	NQ	0.06	0.3	0.02
GLB	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	186.8	25,230	ND
	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	5.0	393	ND

Each value is the mean of three replications

SD standard deviation, ND not detectable, NQ not quantifiable

procedures for sample preparation in all studied plants. The mean contents of polyphenols were lower than the mean content obtained after application of the ST8 procedure: 2 times lower for lucerne, 40 times lower for buckwheat, 35 times lower for goldenrod, 5 times lower for lavender and licorice, and 15 times lower for lacy phacelia. Based on these results, it can be concluded that a QuEChERS method could not yield satisfactory extraction of all target analytes.

Method Validation

The full results obtained during method validation were placed in the [Supplementary Material](#).

Linearity for flavonoids and phenolic acids were obtained over different concentration range depending on studied compounds (Table S3). Limits of quantification range from 0.4 to 20 ng/mL. The precision of the method presented RSD values was lower than 9.12%. The intra-day accuracy ranged from –7.54 to 6.92% and inter-day accuracy ranged from –9.57 to 8.63% (Table S3). The recoveries of polyphenols were found to be 48.9–97.2% (Table S3). All analytes and IS kept in different conditions showed a variation lower than 8.47% at all concentrations (Table S4).

Analysis of Plant Materials

The SLE-SPE-UHPLC-MS/MS assay method was subsequently applied to the simultaneous determination of the 30 major active polyphenols in the different plants and in different parts of the plants. The results of the quantitative determinations of the flavonoids and phenolic acids are listed in Table 1. The analysis of each sample was replicated three times, i.e., from sample preparation to chromatographic analysis.

Lucerne (Flowers, Leaves, and Stalks) Figure 3 reports MRM chromatograms for selected compounds analyzed in extracts of lucerne flowers, leaves, and stalks. A higher amount of phenolic acids was observed for lucerne leaves (from 3.77 to 10,000 ng/g) than for lucerne flowers and stalks (from 3.52 to 8313 and from 0.17 to 3717 ng/g, respectively). The concentration of flavonoids was higher in flowers (0.24–1168 ng/g) than in leaves and stalks (0.18–148.4 and 0.30–253.7 ng/g). The main phenolic acid of lucerne flowers was a 4-HBA component, which was present at the level of 8313 ng/g dry sample. The content of 4-HBA in the samples of lucerne leaves and stalks was markedly lower (3307 ng/g and 2163 ng/g,

Fig. 3 Representative MRM chromatograms of selected phenolic acids and flavonoids analyzed in extracts of lucerne flowers, leaves, and stalks

respectively). Flowers of lucerne contained a high level of QUE (flavonoid), and these could be used as a new resource of this bioactive compound. α -HPA and GLB were not detected in all analyzed extracts of lucerne; HST was not quantified in extracts of flowers and stalk; and ERC, QUE, and PIN were not quantified in lucerne stalks ($< \text{LOQ}$).

Goldenrod (Flowers, Leaves, and Stalks) UHPLC-MS/MS profiles of the flowers, leaves, and stalk are similar, indicating that they contained the same phenolic acids and flavonoids and only the amounts are slightly different (Fig. 4). Extracts of goldenrod leaves showed the highest concentrations of the determined polyphenols (0.13–23,523 ng/g) compared to flowers (0.07–17,384 ng/g) and stalks (0.21–6394 ng/g). When exploring the phenolic acids present in goldenrod, 4-HBA was the main compound in leaves (1423 ng/g), while FA was most present in the flowers (919.8 ng/g) and stalks (611.1 ng/g).

DOPAC and ERC were missing in leaves and stalks and were present at a low amount in the flowers (0.22 and 0.07 ng/g, respectively). One phenolic acid (α -HPA) and three flavonoids (LQG, FOR, and GLB) were not detected or quantified in the whole extracts of goldenrod.

Phacelia (Flowers, Leaves, Stalks, and Roots) RUT (flavonoid) and 4-HBA (phenolic acid) were predominant in all investigated phacelia samples analyzed using the UHPLC-MS/MS method, but there was no significant difference between their content in flowers and leaves. The highest flavonoid content was found in the phacelia flowers (from 0.16 to 13,922 ng/g). The compounds with the highest concentrations in the flowers samples were RUT, followed by HSD and NHSD. Also, a higher phenolic acid content was observed in the flowers of phacelia (from 0.80 to 4784 ng/g). The data obtained for HA, 3-HBA, and 3-HPA mostly indicated lower concentrations

Fig. 4 Representative chromatograms of all determined polyphenols in an extracts of goldenrod flowers, leaves, and stalks

in comparison with other acids. Extracts of phacelia flowers, leaves, and roots presented similar levels of phenolic acids (0.8–4784 ng/g, 0.34–3915 ng/g, and 0.22–

4049 ng/g, respectively). Figure 5 shows MRM chromatograms for selected polyphenols obtained after analysis of extracts of phacelia flowers, leaves, stalks, and roots.

Fig. 5 Representative MRM chromatograms of selected phenolic acids and flavonoids analyzed in extracts of phacelia flowers, leaves, stalks, and roots

Buckwheat According to UHPLC-MS/MS experiments, 27 polyphenols could be detected in the buckwheat, while α -HPA and FIS were not present in this plant. The most abundant phenolic acids were 3,4-DHBA (4241 ng/g) and 4-HBA (1241 ng/g) and the most abundant flavonoids were RUT (7521 ng/g) and QUE (908.3 ng/g). The amounts of all phenolic acids were in the range from 7.39

to 4241 ng/g. Flavonoids were present at levels from 0.511 to 7521 ng/g.

Licorice The contents of phenolic acids and flavonoids in licorice sample were from 11.04 to 118,535 ng/g and between 1.047 and 25,230 ng/g, respectively. GLB, a major flavonoid, was present at a higher concentration level (25,230 ng/g),

while 3,4-HPPA, another major phenolic acid, was present at the concentration of 118,535 ng/g. ERC and α -HPA were not quantified in the analyzed extracts of licorice.

Lavender The results obtained using the proposed UHPLC-MS/MS method for phenolic acids varied from 7.38 to 1578 ng/g and for flavonoids from 0.401 to 398.1 ng/g. The main phenolic acids and flavonoids found in lavender were 4-HBA (1578 ng/g) and NAR (398.1 ng/g), respectively. In lavender samples, α -HPA and GLB were not detected.

As a summary of the analysis of different plant materials, it may be conducted that the qualitative and especially quantitative polyphenol profiles in lucerne, goldenrod, phacelia, buckwheat, licorice, and lavender are significantly different. It may be supposed that the pharmacological activities of the studied plants are not equal.

The highest total content of the analyzed polyphenols was found in licorice (156 $\mu\text{g/g}$ dry sample). The extracts of licorice contain nearly ten times higher levels of phenolic acids and flavonoids in comparison with those of buckwheat (17.6 $\mu\text{g/g}$), phacelia (21.9 $\mu\text{g/g}$), and lucerne (29.1 $\mu\text{g/g}$). The main phenolic constituents of the mentioned plants are also different, depending on the type of plant (Figure 6). The lowest contents of all the target polyphenols were found in lavender (4.75 $\mu\text{g/g}$). Most of the compounds selected in this study were determined in the extracts of whole plants. Only α -HPA was not detected in all analyzed extracts, while GLB was quantified only in buckwheat and licorice. The content of the acids and flavonoids assayed in selected plants was of the same order of magnitude as that given in the literature (Wang and Yang 2007; Sabir et al. 2012).

Determining the amounts of individual polyphenols is important owing to their specific properties. Different phenolic

Fig. 6 Representative chromatograms obtained for extracts of lucerne, goldenrod, phacelia, buckwheat, licorice, and lavender using the proposed SLE-SPE-UHPLC-MS/MS method

acids and flavonoids have different abilities for scavenging free radicals (Rice-Evans et al. 1996), between them there are differences in stability (Sharma et al. 2015), and they also have different pharmacological activities (Benavente-García et al. 1997). Moreover, identifying individual polyphenols is also important because they can be used as markers to evaluate the authenticity of plant products, even if the composition of cosmetics and dietary supplements is affected by the processing and storage conditions. Since the validated UHPLC-MS/MS method allows the identification and quantification of the main polyphenols present in the six plants, it may find application as an important analytical tool for researchers from various industries, with particular emphasis on quality control of cosmetics and dietary supplements.

According to the literature, this is the first study that combines SLE-SPE and UHPLC-MS/MS for the extraction and determination of flavonoids and phenolic acids in six different plants. The total analysis time of the UHPLC protocol was within 10 min, in contrast to the previous HPLC procedures that involved analysis times of 40–100 min (Farag et al. 2012; Liao et al. 2012; Montoro et al. 2011; Verardo et al. 2010). Other non-negligible advantages to our method are simultaneous determination of 30 main flavonoids and phenolic acids. In addition, the SLE-SPE-UHPLC-MS/MS procedure provided higher sensitivity and selectivity in comparison with the HPLC-DAD method (Kreft et al. 2006; Wang and Yang 2007).

Conclusion

In this paper, an efficient method for simultaneous determination of 30 polyphenols in plants has been developed for the first time using UHPLC-MS/MS based on SLE and SPE extractions.

Based on our results, it can be stated that the extraction solvent significantly affected polyphenol content in extracts. Both phenolic acids and flavonoids were isolated from plants by extraction using methanol and purification with a C18 reversed-phase SPE cartridge. For the most compounds, good recoveries and RSDs (< 10%) were obtained, and only for α -HPA, DOPAC, and LIQ, the recoveries were lower. Limits of quantification range from 0.4 to 20 ng/mL.

The developed procedure demonstrated to be effective for quantifying polyphenols in very complex matrixes such as lucerne, licorice, goldenrod, buckwheat, phacelia, and lavender. The main phenolic acids in the studied plants have been found to be 3,4-HPPA, 4-HBA, and 3,4-DHBA. QUE, RUT, GLB, and NAR are the major flavonoids detected in the analyzed samples.

This method has already been successfully applied to determine those active components in different parts of the plants or in whole plants. The obtained scientific data can be useful to select plant materials with high nutritional value and

the presence of many biologically active ingredients, as well as selecting samples used as a plant biomass and as a components of dietary supplements.

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Compliance with Ethical Standards

Conflict of Interest Sylwia Bajkacz declares that she has no conflict of interest. Irena Baranowska declares that she has no conflict of interest. Bogusław Buszewski declares that he has no conflict of interest. Bartosz Kowalski declares that he has no conflict of interest. Magdalena Ligor declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human or animal subjects.

Informed Consent Informed consent was not applicable.

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