Optimization of extraction conditions for secondary biomolecules from various plant species

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Abstract

Extraction of plant secondary metabolites is an essential step in isolation of natural products. Non-optimized extraction conditions can lead to losses, degradation and modification of the biomolecules. In this paper, the influence of different solvent mixtures, solvent amounts, temperature, extraction time, and procedures for defatting on yield and profile of various classes of secondary metabolites was investigated. *Rumex alpinus* was used for the extraction of anthraquinones, *Glycine max* for isoflavonoids, *Chaerophyllum bulbosum* for flavonoids and phenolic acids, *Anthriscus sylvestris* for lignans and coumarins, alkaloids were extracted from *Lupinus albus* and sesquiterpene lactones from *Artemisia absinthium*. Extraction efficiency was evaluated by use of LC-DAD-ESI-MS/MS. The compromise extraction solvent for all of the examined compounds is 80% methanol, mixed in ratio 13:1 with plant material. Maceration should last for six hours, repeated four times with fresh solvent. Defatting of the extracts does not lead to significant losses of the compounds of interest. It is acceptable to use extraction and evaporation temperature of 60 °C, while the extracts should be stored in the dark, on –20 °C.

Keywords: extraction, secondary biomolecules, plant phenolics, flavonoids, phenolic acids.

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For centuries, Chinese traditional medicine has been using nature as a source of compounds which can be used in treatment of various diseases. Today, major attention of pharmaceutical industry is focused on natural products as possible sources of natural remedies. Therefore, there is a growing interest for chemical characterization of medicinal plants [1].

Plants synthesize a wide range of secondary metabolites, as a specific mechanism of their defense from herbivores, bacteria, viruses, fungi and other organisms. By targeting different receptors and enzymes in predators and parasites, they have developed different mechanisms of action. Alkaloids can have agonistic or antagonistic activity towards neurotransmitters, while isoflavonoids have phytoestrogen abilities, due to their structural similarity with estrogen hormones in animals [2,3].

Plant secondary metabolites possess various biological activities. Anthraquinones, major components extracted from *Rumex alpinus* (Polygonaceae), are reported to have anti-inflammatory, antifungal and antibacterial activity. Isoflavonoids extracted from *Glycine max* (Fabaceae) are phytoestrogens, while flavonoids and phenolic acids from *Chaerophyllum bulbosum* (Apiaceae) exhibit immunomodulating, anticancer and hepatoprotective effects. *Anthriscus sylvestris* (Apiaceae) is rich in lignans and coumarins, known for their anticancer, antifungal and anti-inflammatory activities. Alkaloids extracted from *Lupinus albus* (Fabaceae) and sesquiterpene lactones from *Artemisia absinthium* (Asteraceae) are responsible for their bitter taste, as well as their antimicrobial activity [2,4].

Extraction of the active principles is an essential step in evaluation of their bioactivity and chemical characterization. Maceration is widely used technique suitable for extractions of small amounts of plant material in laboratory. However, non-optimized extraction conditions can lead to losses, degradation and modification of the biomolecules. Inaccurate analysis can result in invalid conclusions about the chemical composition and the amount of secondary metabolites present in plant. Therefore, optimization of extraction conditions is important for maximizing yields of the compounds of interest, while minimizing the extraction of unwanted compounds. In this paper, we investigated the influence of different solvent mixtures, solvent amounts, temperature, extraction time, techniques and procedures for fatty acid and chlorophyll removal on yield and profile of various classes of secondary metabolites from the selected plant species, during maceration. Extraction efficiency was evaluated by using several LC-DAD-ESI–MS/MS methods developed to monitor the selected compounds and compound classes.

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EXPERIMENTAL

Chemicals and reagents. HPLC gradient grade methanol, p.a. ethanol and methanol were purchased from J. T. Baker (Deventer, The Netherlands), and p.a. DMSO from Merck (Darmstadt, Germany).

Plant materials. The plant material used for extraction and analysis was collected from different locations in Serbia – *Chaerophyllum bulbosum* (CB) from Vlasinsko Jezero lake and *Anthriscus sylvestris* (AS) from Fruska Gora mountain in 2009, *Artemisia absinthium* (AA) from Stara Planina mountain in 2011, *Rumex alpinus* (RA) from Kopaonik mountain in 2012, while *Glycine max* (GM) and *Lupinus albus* (LA) were collected from Rimski Sancevi, agrarian area in the Pannonian basin, in 2013. Voucher specimens were prepared, identified and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), University of Novi Sad, Faculty of Sciences.

The selected plant species were used for extraction of different secondary metabolites present in their aerial parts, as determined by our preliminary studies. Herb of *Rumex alpinus* (monk's rhubarb, Alpine dock) was used for extraction of anthraquinones, *Glycine max* (soy) for isoflavonoids, and *Chaerophyllum bulbosum* (turnip-rooted chervil) for flavonoids and phenolic acids. Aerial parts of *Anthriscus sylvestris* (wild chervil) were used as a source of lignans, *Lupinus albus* (white lupin) contains alkaloids, while the herb of *Artemisia absinthium* (absinthium, wormwood) is rich in sesquiterpene lactones.

Extracts preparation

Plant material was air-dried at room temperature, and aerial parts were separated from roots and powdered afterwards. All extracts were prepared by maceration. During the extraction process, aerial parts were constantly shaken at three different temperatures, during various periods of time, and using several solvent mixtures (as described in the following subsections). The composition of the extraction medium, as well as the volume of the solvent, extraction time and temperature were optimized during the experiment to obtain the highest content of extractables (monitored by LC-ESI–MS/MS in MS2Scan mode or by LC-DAD, depending on the compound class). Plant material was removed by filtration, raw extracts were evaporated under the reduced pressure and reconstituted in DMSO to the final concentration of 50 mg/mL.

Solvent mixture selection. For the purpose of testing the influence of different solvent mixtures on secondary metabolites extraction, 100 mg portions of each dried plant material were mixed in 1.8 mL HPLC vial with 1 mL of the following solvents: water, 60% methanol, 80% methanol, methanol, 60% ethanol, 80% ethanol and ethanol. The mixtures were shaken for 60 min at room temperature on vortex mixer, and filtered through millipore filters (0.45 μm) before analysis by LC-DAD-ESI–MS/MS in MS2Scan mode. Peaks of the targeted compounds were identified by comparing their molecular weights, mass spectra and UV/Vis spectra with the literature (refer to the Section "Identification of compounds present in plant extracts"). Further on, their abundance profiles obtained from extraction by different solvents were compared. The goal was to achieve the maximum possible yield of a wide range of biomolecules identified in the selected plants. Afterwards, for each of the extracts, LC-MS/MS or LC-DAD method was created to monitor the yield of the compounds of interest.

Optimal extraction volume selection. To find the optimal extraction medium volume, 1 mL of the optimal solvent was mixed with 50 mg (solvent mixture to plant material ratio of 1:20), 75 mg (1:13.3), 100 mg (1:10), and 125 mg (1:8) of the dry plant material. After shaking for 60 min on vortex mixer and filtering through millipore filters, all mixtures were diluted to the concentration of 50 mg/mL, for analysis with LC--ESI–MS/MS. Plots showing peak areas versus extraction solvent volume were constructed (Fig. 1).

*Extraction kinetics***.** For the purpose of extraction kinetics evaluation, 5 g of the drug was mixed with the optimal volume of extraction solvent in 250 mL Erlenmeyer flask, closed to avoid evaporation of solvents, and shaken at room temperature for 73 h. Sample aliquots of 500 μL were taken out at 0, 15 and 30 min, and at 1, 2, 6, 10, 24, 49 and 73 h after adding the extraction medium, filtered and analyzed by LC-DAD--ESI–MS technique. The yield was plotted against the extraction time (Fig. 2).

Multiple extractions. 500 mg of the plant material was repeatedly extracted for 5 times with an optimal solvent volume. Each of the five extractions was performed by shaking the mixture for 1 h at room temperature, filtering out the extract using vacuum, and re-extracting the remaining plant material with the same amount of fresh extraction medium. 500 μL of each portion is analyzed separately by LC-DAD-ESI–MS/MS. Cumulative yield of each extraction cycle was calculated, assuming approximately 100 % recovery after the fifth extraction, and was plotted against the number of extraction cycles (Fig. 3).

Optimal temperature selection. Optimization of extraction temperature was performed by shaking 100 mg of the plant material with the optimal extraction medium for 60 min at room temperature (25 °C), and at 60 and 100 °C in water bath. After filtering, the extracts were analyzed by LC-DAD-ESI–MS/MS and compound profiles were compared (Fig. 4). In addition, MS2Scan chromatograms were recorded to monitor possible degradation products.

Evaporation under reduced pressure. To increase the throughput, the maximum acceptable evaporation temperature was assayed. Extracts prepared in the experiment evaluating the extraction kinetics (after 73 h) were evaporated under the reduced pressure, using rotary evaporator with bath temperature set on 30, 45 and 60 °C. Dry residues were reconstituted to the concentration of 50 mg/mL and analyzed by LC-DAD-ESI––MS/MS after filtration. Additionally, MS2Scan chromatograms were acquired and evaluated for identification of possible degradation products.

Unwanted compounds removal. Examination of losses during removal of fats and pigments (chlorophylls and carotenoids) was performed by liquid-liquid extraction with hexane. 1 mL of extracts, prepared in the experiment evaluating extraction kinetics, was evaporated *in vacuo*. Dry residue was suspended in 1 mL of warm water (~60 °C), additional 1 mL of water was then added to wash the evaporation flask, and the water solutions were mixed. To 2 mL of this solution, 2 mL of hexane was added, the content was shaken on vortex mixer and then frozen using dry ice. Hexane layer was decanted from the frozen aqueous layer, and then new portion of hexane (2 mL) was added to the thawed aqueous layer. This procedure was repeated for five times, resulting in 2 mL of aqueous phase and 10 mL of pooled hexane extracts. Both phases were evaporated separately *in vacuo*, dry residues were reconstituted to the concentration of 50 mg/mL, and analyzed by LC-DAD-ESI–MS/MS in MS2Scan mode. The obtained chromatograms were compared to evaluate the compound distribution between the two phases.

Effects of storage conditions. Extracts prepared using previously optimized extraction conditions were stored for 6 months under different conditions: at room temperature exposed to sunlight, at room temperature in the darkness, at 4 °C in the darkness, and at –20 °C in the darkness. In addition, one portion was diluted in typical HPLC mobile phase (0.05% aqueous formic acid:methanol = 1:1) and kept at 4 °C in the darkness. After six months, LC-DAD-ESI–MS/MS analysis of all samples was performed, and profiles of the compounds of interest were compared for the purpose of evaluating possible losses and degradation.

LC-ESI–MS analysis

Extracts were diluted with mobile phase solvents A (0.05% aqueous formic acid) and B (methanol), premixed in 1:1 ratio, to obtain a final concentration of 50 mg/mL. Extracts were analysed using Agilent Technologies series 1200 HPLC instrument coupled with Agilent Technologies 6410A Triple Quad tandem mass spectrometer with electrospray ion source, and controlled by Agilent Technologies MassHunter Workstation software – Data Acquisition (ver. B.03.01). Five μL were injected into the system, and compounds were separated on Zorbax Eclipse XDB-C18 (50 mm×4.6 mm, 1.8 μm) rapid resolution column held at 50 °C. Mobile phase was delivered at flow rate of 1 mL/min in gradient mode (0 min, 30% B, 6 min, 70% B, 9 min, 100% B, 12 min, 100% B, re-equilibration time 3 min). Eluted components were detected by DAD detector and sent to the MS detector.

MS(–) experiments. For detection of flavonoids and phenolic acids from *Chaerophyllum bulbosum* and isoflavonoids from *Glycine max*, MS–ESI ion source parameters were as follows: nebulization gas (N_2) pressure 50 psi, drying gas (N_2) flow 9 L/min and temperature 350 °C, capillary voltage 4 kV, negative polarity (NI). For general screening, data were acquired in MS2Scan mode, using *m/z* range from 120 to 1000 and fragmentor voltage of 100 V. For targeting compounds of interest, MS2SIM mode was used, with specific *m/z* values: 643, 601, 515 and 353 for acetylmalonyl-dicaffeoylquinic acid ($AcMalC_2QA$), malonyl-dicaffeoylquinic acid ($MalC_2QA$), dicaffeoylquinic acid (C_2QA) and caffeoylquinic acid (CQA); 489, 463, 447, 431, and 285 for luteolin/kaempferol acetylhexosides (Lut/Kaempf--AcHex), quercetin-3-O-glucoside (Quer-3-Glc), luteolin/kaempferol-7-O-glucoside (Lut/Kaempf-7-Glc), apigenin-7-O-glucoside and luteolin/kaempferol deoxyhexoside (Api-7-Glc, Lut/Kaempf-dHex) and luteolin/ /kaempferol (Lut/Kaempf), respectively, all detected in *Chaerophyllum bulbosum*. In *Glycine max*, *m/z* 269 and 253 were targeted for genistein and daidzein, respectively.

MS(+) experiments. Ion source parameters for screening of alkaloids from *Lupinus albus* were identical to those described in previous section, except for the use of positive polarity (PI). For targeting the compounds of interest, MS2SIM in positive mode was used, with specific *m/z* values: 347, 265 and 249 for angeloyloxy/tigloyloxy-lupanin, hydroxylupanin and lupanin, respectively.

The above said parameters were not suitable for analysis of sesquiterpene lactones from *Artemisia absinthium*, due to inefficient ionization by ESI source. Therefore, the analysis of this plant's extracts was performed using an instrument with multimode ion source. Instrument configuration was identical to the one above, with a difference in MMI source parameters: nebulization gas (N_2) pressure 50 psi, vaporizer temperature (APCI heater) 200 °C, drying gas (N_2) flow 5 L/min and temperature 325 °C, capillary voltage 2.5 kV, positive polarity (PI). In MS2SIM mode, compounds of interest were targeted on *m/z* values: 513, 497 and 249 for anabsin, absinthin and artabsin, respectively.

UV/Vis Detection. For analysis of lignans present in *Anthriscus sylvestris* herb extract, UV signals at 280 nm and 330 nm were monitored, with bandwidth of 16 nm. Anthraquinones from extract of *Rumex alpinus* were

monitored in visible area at 430 nm, with bandwidth of 16 nm. For both classes, UV/Vis was found to be more practical than MS in detecting all compounds of the same class.

Additionally, to confirm the identity of peaks, continuous spectra were obtained in range from 190 to 700 nm.

RESULTS AND DISSCUSION

Identification of compounds present in plant extracts

Phenolic acids and flavonoids from Chaerophyllum bulbosum. Phenolic acids in *Chaerophyllum bulbosum* extracts were identified by MS^1 and MS^2 analysis in negative ionization (NI) mode, according to the rules set by Clifford *et al*., 2003 [5]. Examination of the total ion chromatograms (TIC) resulted in four signals: *m/z* 353 for caffeolyquinic acids (CQA), *m/z* 515 for dicaffeoylquinic acids (C_2QA), *m/z* 601 for malonyl-dicaffeoylquinic acids ($MalC_2$QA), and *m/z* 643 for acetyl-malonyl-dicaffeoylquinic acids ($AcMalC_2$QA). CQAs were identified at retention times (t_R) of 0.7, 0.8 and 1.4 min. C_2QAs eluted at t_R 1.7, 1.8, 1.9 and 2.35 min, $MalC_2$QAs at 1.6 and 1.85 min, and $AcMalC_2$QAs at 2.65 and 2.8 min.

Flavonoids were also identified in *Chaerophyllum bulbosum* extracts after MS^1 and MS^2 analysis in negative ionization (NI) mode, according to the rules set by Cuyckens *et al*. (2000) [6]. Examination of the total ion chromatogram (TIC) resulted in five signals: *m/z* 285 for luteolin and kaempferol (Lut/Kaempf), *m/z* 431 for apigenin hexosides and luteolin/kaempferol deoxyhexosides (Api-Hex, Lut/Kaempf-dHex), *m/z* 447 for luteolin and kaempferol hexosides (Lut/Kaempf-Hex), 463 for quercetine hexosides (Quer-Hex), and *m/z* 489 for luteolin and kaempferol acetyl hexoside (Lut/Kaempf--AcHex). The only peak in the EIC signal at *m/z* 285 was identified as luteolin (Lut) by comparison of the retention time (t_R = 3.8 min) with the reference standard. The EIC signal at *m/z* 431 contained two major peaks. The first peak, at t_R 2.6 min, was confirmed to be apigenin-7-*O*-glucoside (Api-7-Glc) by comparison with the reference standard. The second peak, with the retention time of 3.32 min, was identified as kaempferol-3-*O*--deoxyhexoside (Kaempf-3-dHex), according to molecular weight and characteristic UV spectra. Two compounds were detected in EIC chromatogram at *m/z* 447. By comparing the retention times with the reference standards, luteolin-7-*O*-glucoside (Lut-7-Glc, t_R = = 2.09 min) and kaempferol-7-*O*-glucoside (Kaempf-7--Glc, t_R = 2.7 min) were identified. The signal at *m/z* 463 exhibited one major and one minor peak, both corresponding to quercetin hexosides. By comparing the retention times with the reference standards, it was confirmed that the first peak at t_R 2.04 min was quercetin-3-*O*-galactoside (hiperoside), while the dominant peak at 2.15 min was quercetin-3-*O*-glucoside (isoquercitrin). Finally, Lut/Kaempf-AcHexs were identified at t_R 2.4, 2.74 and 2.9 min.

Isoflavonoids from Glycine max. Isoflavonoids in *Glycine max* extracts were identified after MS2Scan analysis in negative ionization (NI) mode. Examination of the total ion chromatogram (TIC) and the extracted ion chromatograms (EIC) resulted in two signals: *m/z* 253 for daidzein (Da) and *m/z* 269 for genistein (Gen). These compounds represent the two major isoflavonoids present in soy, and were identified by their molecular masses, as well as by comparing their MS^2 and UV spectra with the reference data [7].

*Alkaloids from Lupinus albus***.** Alkaloids in *Lupinus albus* extracts were detected by MS^1 and MS^2 analysis in positive ionization (PI) mode. Spectral data from the literature helped in identification of the major compounds present in this plant [8,9]. Peak at 0.57 min belongs to lupanin (*m/z* 249). Overlapping peaks with t_R 0.54 and 0.69 min are identified as two isomeric hydroxylupanins (*m/z* 265). Peak at 1.17 min corresponds to one of the two isomeric hydroxylupanin esters: tigloyloxy- or angeloyloxy-lupanin (*m/z* 347).

*Sesquiterpene lactones from Artemisia absinthium***.** Analysis of sesquiterpene lactones present in *Artemisia absinthium* was performed using APCI MS in MS2SIM mode. The compounds of interest were identified by comparison with the literature spectral data [8,10]. According to the data, peak at 4.9 min represents artabsin (*m/z* 249), anabsin eluted at t_R 5.25 min (*m/z* 513), and peak at 6.93 min is absinthin (*m/z* 497).

*Lignans from Anthriscus sylvestris***.** The lignans dominant in *Anthriscus sylvestris* extracts were identified by using the literature data about this plant's chemical composition, including UV spectra and retention information [11,12], as well as the fragmentation rules set by Wong *et al*., 2000 [13]. Peaks at 4.47, 5.47 and 5.65 min have UV spectra characteristic for aryltetralin and saturated dibenzobutyrolactone lignans: absorbance maximum below 290 nm and lack of maxima above 300 nm. Based on MS^2 data, these compounds were identified as podophyllotoxin, deoxypodophyllotoxin, and yatein, respectively. Peaks at 5.79, 5.87 and 6.49 min have absorbance maximum between 320 and 330 nm, and have been identified as chaerophyllin, nemerosin and sylvestrin/isochaerophyllin. Additional minor peaks were detected, with retention and UV spectra similar to those of the compounds identified.

*Anthraquinones from Rumex alpinus***.** Peaks of anthraquinone aglycones and glycosides were identified by their characteristic UV/Vis spectra, with a prominent absorbtion maximum at 430 nm and a series of maxima under 300 nm. Compounds eluting between

4.5 and 5.5 min were identified according to their molecular masses and retention times as anthraquinone glycosides, while the compounds eluting later (from 7.9 to 9 min) were identified as anthraquinone aglycones.

Extraction conditions optimization

*Extraction medium optimization***.** Various extraction mediums, differing in polarity, proton-donating and proton-accepting abilities, extract different classes of compounds from plant material with a varying yield. With a goal of maximizing the yield of a wide range of molecules of interest, the optimal extraction medium was selected by comparison of abundance profiles of the selected compounds, obtained by analysis after the extraction by different solvent mixtures. According to literature data, optimal extraction mediums for most of the plant compounds are methanol and ethanol, mixed with water in different ratio [14,15]. For all of the identified chlorogenic acids, 60 and 80% methanol were found to be the most efficient in their extraction from *Chaerophyllum bulbosum*. 60% ethanol provided a high yield for dicaffeoylquinic acids and their malonyl derivatives, but lower for caffeoylquinic and acetyl-malonyl-dicaffeoylquinic acids. Furthermore, it was noticed that water and neat ethanol are not suitable for chlorogenic acids extraction, due to very low yields.

Extraction profiles for flavonoids from *Chaerophyllum bulbosum* depend on the structure. Efficiency of glycosides extraction increased with methanol content, while for the ethanol-based solvents it peaked at 80% ethanol. Generally, the pure methanol was found to be the most efficient, followed by 80% methanol. As for lutheolin and its acetylhexosides, the pattern was reversed, showing that 60 and 80% methanol provide higher yields than pure methanol. Yields of flavonoids, obtained using water extraction, were very low. Surprisingly, isoflavonoid aglycones daidzein and genistein were the most efficiently extracted with solvents of intermediate polarity, while neat water and alcohols provided lower yields. The effect of the solvent on the investigated sesquiterpene lactones extraction from *Artemisia absinthium* was less pronounced. While the extraction efficiency of water was low, 60–100% alcoholic solvents provided high yields. Similarly, the most suitable extraction solvents for hydrophobic, permethylated lignans from *Anthriscus sylvestris* were 60 and 80% ethanol. Methanolic extraction provided a somewhat lower yield of these compounds, while water and ethanol were found to be inefficient. Polar solvents – water, 60–80% methanol and 60% ethanol – were found to be the most efficient for extraction of the selected alkaloids (lupanin, hydroxylupanin and angeloyl/tigloyloxylupanin) from *Lupinus albus*, while pure ethanol gave very low yields. It should be noted that a pH adjustment of the sample may be needed for obtaining the maximum yield of alkaloids. Finally, for anthraquinones, as hydrophobic compounds, water was found to be the unsatisfactory solvent. To obtain the maximum yield of both glycosides and aglycones, 80% methanol or 80% ethanol should be used.

This experiment resulted in conclusion that 80% methanol is an adequate medium for extraction of the majority of compounds present in plants used in this experiment, with 60% ethanol as a viable alternative. This is in accordance with the literature data [14,15].

Extraction efficiency of different solvent mixtures towards various plant pigments, such as chlorophylls and carotenes, was also evaluated. The pigments were identified by their UV/Vis spectra, and their content was estimated from the total signal on λ 400–700 nm in late-eluting region of a chromatogram. Pigments can interfere with the analysis of extracts, especially when using spectrophotometric methods for evaluation of chemical composition and biological activity. Therefore, the optimal sample preparation procedure should minimize co-extraction of pigments with the compounds of interest. It was found that the selected optimal extraction solvent, 80% methanol, also extracts high amounts of pigments (although less than 60% methanol, pure methanol and 80% ethanol), therefore the extract purification may be necessary. It was also observed that 80% methanol extracts the highest amount of fatty acids that can interfere with anthraquinones determination due to similar elution and isobaric molecular weights, although these problems can be alleviated by using MS/MS or UV/Vis detection.

*Optimal extraction medium volume***.** Optimal extraction medium volume represents the ratio of dry plant material weight to volume of the extraction solvent. Volume optimization leads to use of the minimum amount of the extraction medium needed for a high yield of the extracted compounds, thus saving large amounts of toxic and expensive solvents. Furthermore, evaporating smaller amounts of solvent increases the extraction throughput and reduces energy expenditure. As expected, the results show that the extraction yield dramatically increases with the increase of solvent-to-drug ratio, due to increase of the concentration gradient needed for efficient extraction. To obtain a high yield in one-step extraction, the ratio of at least 20:1 should be used (Fig. 1). Since this results in a very high solvent usage, a lower ratio of approx. 13:1, which provides approximately twice the yield of commonly used 8:1 extraction for majority of the compounds investigated, was selected for the following experiments. This result is in correlation with the recommended ratio of between 1:10 and 1:15 [14].

*Optimal extraction time***.** Extraction yield increases with time until the equilibrium is reached. Prolonging the extraction after this point has little sense, since it

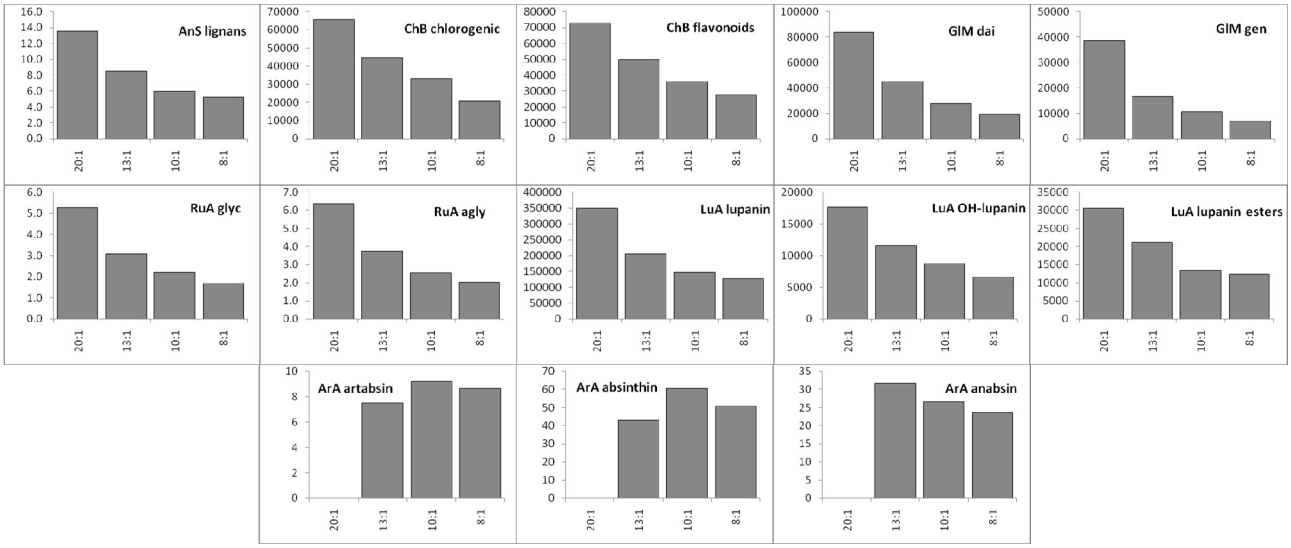

Figure 1. Peak areas of compounds extracted with different solvent volumes.

leads to longer experiment time, and also increases the chance of artefact formation through hydrolysis, oxidation, isomerisation etc. Extraction time is optimized by evaluation of extraction kinetics, *i.e*., by monitoring the change of the investigated substances concentrations in time.

The obtained results show that concentrations of all compounds grow rapidly during the first two hours of extraction, reaching the estimated yields of 70–90%. Afterwards, the yield rises slowly due to solvent saturation by the compounds present in plant material, to reach equilibrium after over 70 h (Fig. 2). By analysing of kinetic plots, we concluded that 6 h extraction provides a satisfactory yield (approx. 84–93%) within a reasonable amount of time, which is in correlation with other studies in this field [15].

Number of solvent portions for extraction. To increase efficiency, extraction should be repeated several times with a fresh portion of an extracting solvent. However, multistep extraction leads to increased solvent usage, prolonged procedure, and a larger volume of extract that needs to be evaporated. Thus, it is essential to optimize the number of solvent portions needed for efficient extraction.

Results show that the yield of one extraction step is between 42 and 68%, depending on the compound class. The average cumulative yield increases at an expected rate in subsequent steps, reaching 66–88%, 84–94 and 95–97% in the second, third and fourth step. Therefore, at least four consecutive extractions are required for obtaining acceptable yields (Fig. 3). Considering the previous results, which showed that most of the components are extracted after 6 h, we concluded that the optimal extraction should be performed 4 times for 90 min in order to get the maximum extraction yield within a reasonable time.

*Extraction temperature***.** With increasing the temperature, solvent viscosity decreases, which enhances a solvent penetration into plant material and increases the rate of extraction process. In addition, solubility of many compounds increases at higher temperatures due to either thermodynamic factors (if dissolution is endothermic process) or to decreased water polarity at higher temperatures. However, high temperature may lead to degradation of the thermolabile compounds and evaporation of the volatile ones. Extraction temperature is thus optimized to provide a maximum yield of the compounds of interest, without their further degradation.

For extraction of lignans from *Anthriscus sylvestris*, that are non-polar due to permethylation, higher temperatures (100 °C) were found to be beneficial, with yield at 100 °C about 33% higher than the one at 25 °C. This can be explained by increased solubility due to a decrease of polarity of water in the extraction solvent [16]. The profile of the dominant lignans was mostly unaffected by temperature. However, it should be noted that abundances of some minor components either decreased or increased at 100 °C, thus indicating interconversion.

A very similar yield increase was observed for flavonoid aglycones and non-conjugated glycosides from *Chaerophyllum bulbosum*. However, upon a detailed chromatogram analysis, changes in acylated glycosides profile were observed at 100 °C as compared to 25 and 60 °C. Notably, peaks of quercetin-, luteolin- and kaempferol-malonylglycosides decreased, while an equal number of peaks of the corresponding acetylhexosides increased. This was expected, since it is well-known that malonyl esters undergo decarboxylation into acetates at elevated temperatures [17].

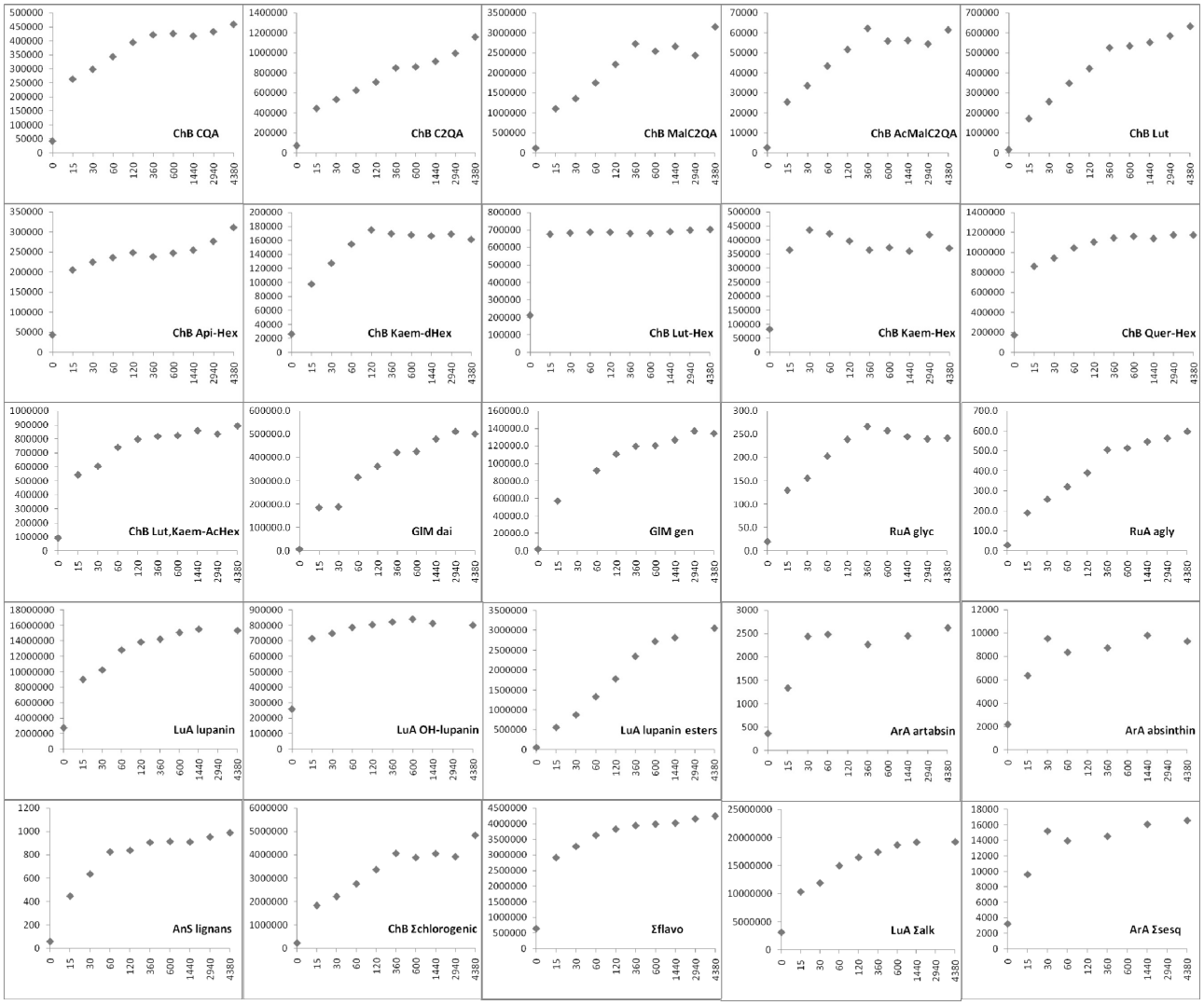

Figure 2. Peak areas of compounds extracted during different periods of time, cumulatively.

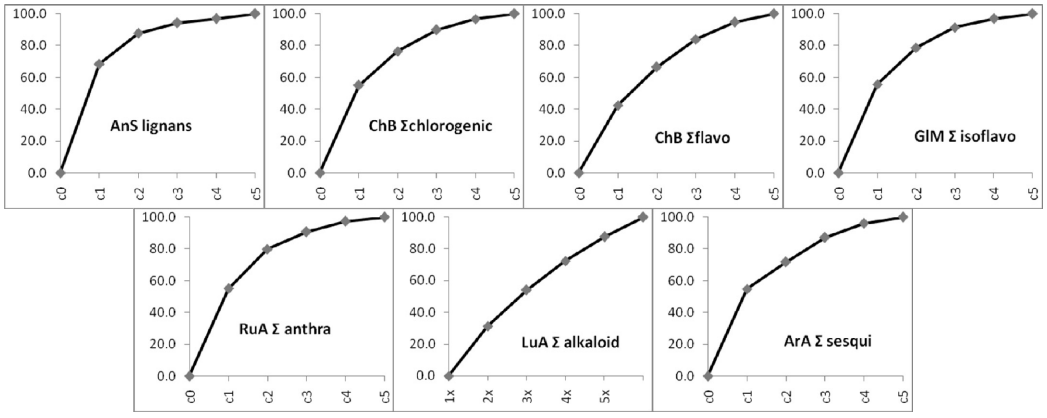

Figure 3. Peak areas of compounds extracted with different number of solvent portions.

Similar effects were observed for chlorogenic acids from *Chaerophyllum bulbosum*. The increase of temperature from 25 to 60 °C provides only a slight increase in yield of these compounds. Further heating to 100 °C resulted in approximately a twofold drop of $MalC_2QA$ levels, and an increase in content of C_2QA, AcC_2QA and $AcMalC_2QA$ (and CQA, to a lesser extent). This indicates that, at elevated temperatures, $MalC_2QA$ undergoes hydrolysis (into C_2QA and CQA) and malonyl moiety decomposition (into AcC_2QA). The conversion

into $AcMalC_2QA$ has not been previously observed, to the best of our knowledge.

Isoflavonoids from *Glycine max*, despite their structural similarity to other flavonoid aglycones, behaved differently. While genistein yield changed only slightly, losses of daidzein were observed at 60 °C and, especially, at 100 °C. The results are in agreement with the previous findings demonstrating thermal lability of isoflavonoids. Studies conducted by Ungar et al. indicated that daidzein is fairly stable while genistein undergoes decomposition, as opposed to our findings. This discrepancy may be attributed to a difference in test systems, *i.e.*, neat compounds in buffer *vs*. plant material in aqueous-methanolic solvent.

In case of *Rumex alpinus*, a temperature increase from 25 to 60 °C was found to be beneficial, increasing the yield of anthraquinone aglycones and glycosides by approximately 30%. Higher temperatures (100 °C) have no further effect on glycosides levels, while aglycones yield drops. It should be noted that glycosides profile changes at 100 °C, indicating possible interconversion.

Yield of alkaloids from *Lupinus albus* exhibited only a small change with a temperature increase from 25 to 60 °C, while heating to 100 °C resulted in a more pronounced yield drop. In the case of esterified hydroxylupanin, the highest yield was observed at 60 °C (Fig. 4).

Based on the results obtained, elevated temperature of 60 °C appears to be relatively safe, not resulting in degradation of majority of the investigated compounds (even malonyl esters, that are commonly claimed to degrade at above 35 °C), while increasing the yield of some compounds. It can also be expected

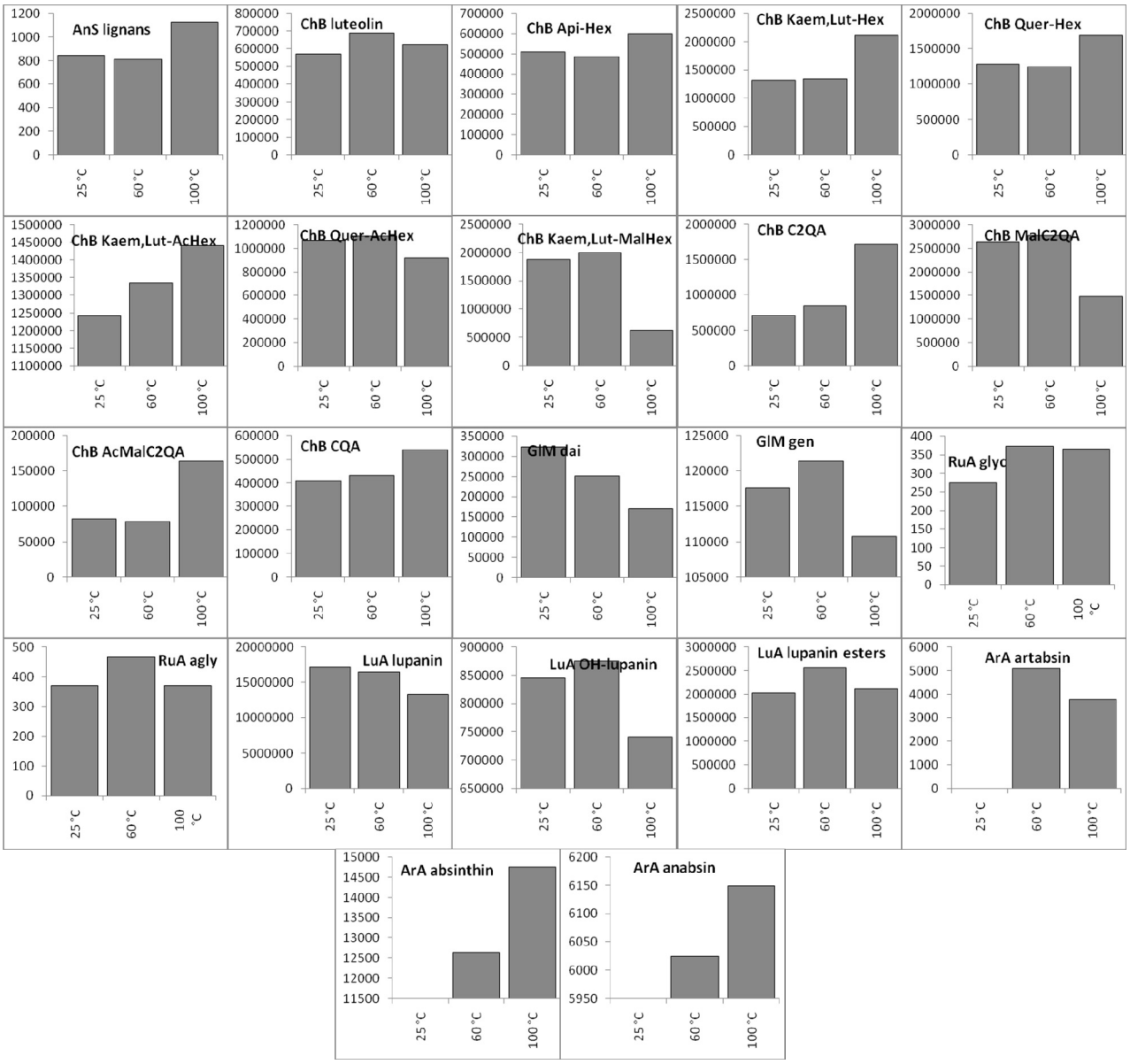

Figure 4. Peak areas of compounds extracted on different temperatures.

that the extraction equilibrium is reached faster due to a decreased solvent viscosity, leading to better penetration. Temperature of 100 °C should be avoided (except when specifically studying decoctions) since it leads to losses in several compounds classes.

Evaporation temperature. After extraction, evaporation is needed to concentrate the extract, usually by using rotary evaporator under reduced pressure and below the solvent boiling point. While elevated temperature enhances the process, it may lead to chemical modification, degradation, or evaporation of the compounds of interest. Thus, it is necessary to find the optimal temperature for evaporation of extracts.

Results show that for the majority of the investigated compound classes there are no differences in abundances of the extracted compounds after evaporation at different temperatures. Thus, the temperatures of up to 60 °C can be used if a high-throughput sample preparation is needed. The notable exceptions are isoflavonoids daidzein and genistein, which exhibit a sharp drop in concentration when evaporated at 60 °C. These compounds have previously been reported to react with proteins at elevated temperatures, which decreases their abundance in solvent [18, 19].

*Losses during the defatting process***.** Extraction of the compounds of interest is usually accompanied by co-extraction of the unwanted matrix compounds, both hydrophilic (sugars, amino acids, metabolic intermediates) and lipophilic (chlorophylls, carotenoids, triacylglycerols and phospholipids). Since these compounds can interfere with compounds of interest in further analyses, they should be removed. Removal of the lipophilic components is performed by liquid-liquid extraction using non-polar solvents like hexane or petroleum ether. However, some compounds of interest can also transfer to organic phase, resulting in a decreased yield or negative systematic error in quantification. Thus, it is of essence to get an insight into the amount of compounds lost during this process.

Results show that defatting does not influence the yield of phenolic acids, flavonoids, isoflavonoids and anthraquinone glycosides, due to their polar nature. Average losses were about 0.8%, and did not exceed 2.2%. Transfer to hexane phase was more pronounced with lupin alkaloids, reaching up to 8.5% for hydroxylupanin. On the other hand, non-polar compounds like sesquiterpene lactones from *Artemisia absinthium* and especially lignans from *Anthriscus sylvestris* are lost to a significant extent during the defatting process (76% for lignanes and 29–57% for sesquiterpenes). The situation is more complex with anthraquinone aglycones. While apparent total loss is about 22%, individual compounds differ in their partitioning behavior, some remaining in water while others predominantly extracting into organic phase.

Consequently, while there are no major losses of polar compounds of interest during the removal of non-polar matrix compounds, there is a need for re-extraction of lipophilic compounds from hexane phase (e.g. with methanol).

Storage conditions. Often, it is not possible to analyze the extracts immediately after extraction, which leads to the need for their storage for a prolonged period. During the storage, partial or complete decomposition of compounds can take place, depending on the storage conditions, such as temperature and illumination.

Results show that after a period of six months, CQA, C_2QA, MalC_2QA and AcMalC_2QA are almost completely degraded at room temperature in the presence of light, with their content dropping to 0.1–13% of the one observed in samples stored at –20 °C. While the abundance of free quinic acid was slightly elevated, it was not possible to identify any other degradation products, implying that chlorogenic acids decomposed mainly to low molecular weight compounds (below used *m*/*z* threshold of 120). Caffeoyilquinic acid was stable at room temperature in the dark. Under the same conditions, malonylated chlorogenic acids – MalC_2QA and AcMalC_2QA – decomposed almost completely, but this time clearly giving rise to mono- and dicaffeoylquinic acids as the main products. At 4 °C, only MalC_2QA degradation was observed, although to a lesser extent – about 30% drop, accompanied by a proportional rise in C_2QA content. It can be concluded that, due to instability of chlorogenic acids, samples should be kept in dark at –20 °C. The solvent also influences the stability of chlorogenic acids – the sample kept at 4 °C in solvent containing 0.01% formic acid and methanol (1:1) exhibited a 25–45% drop in concentration of all chlorogenic acids, accompanied by a rise of quinic acid content, indicating hydrolysis.

Flavonoid aglycones were significantly (about 80%) degraded during storage at room temperature while being exposed to sunlight. The behaviour of flavonoid glycosides in the absence of light depended on the structure. Levels of malonylhexosides decreased 1.6–2.6 times during storage at 4 °C, while decomposition at room temperature was practically complete, independent of insolation. Levels of acetylhexosides also dropped when exposed to light and room temperature. Kaempferol acetylhexosides appeared to be the most stable, while quercetin acetylhexosides decomposed almost completely upon storage at room temperature. Finally, levels of hexosides increased with the temperature in the absence of illumination, indicating a possible conversion of malonylhexosyl and acetylhexosyl moiety into hexosyl, while exposure to sunlight resulted in degradation, especially of quercetin derivative. Thus, it can be concluded that, if flavonoid glycosides spe-

ciation is to be done, samples must be stored at –20 °C, protected from light. Due to lower solubility of flavonoid aglycones in water, a less polar solvent (*e.g.*, methanol) should be present in sufficient amount (*e.g.*, 80%), otherwise precipitation will occur, resulting in losses during filtration prior to HPLC analysis.

Genistein decomposed to a great extent in samples kept at room temperature (especially under insolation), while daidzein was stable under all examined conditions. In samples diluted in mobile phase and kept at 4 °C, both of the examined isoflavonoids partially precipitated were lost during filtration process.

The obtained results show that light causes only partial degradation of lignans, less pronounced than with chlorogenic acids and flavonoids. However, due to low polarity, aqueous-alcoholic mixtures with high alcohol content should be used for storage if losses due to precipitation are to be avoided.

Anthraquinones from *Rumex alpinus* are known to be photosensitizers. Their aglycones were significantly degraded (approx. 90%) upon storage at room temperature in the presence of light, while glycosides seem to be more resistant (only about 15% was lost, when compared to content in non-illuminated sample stored at room temperature).

Decrease of angeloyl/tigloyloxy lupanin amount with simultaneous increase of hydroxylupanin content, points out their gradual hydrolysis at room temperature in the presence of light. In the non-illuminated sample stored at room temperature, as well as in the sample stored in acidic solvent (premixed mobile phase), the content of esters dropped to a similar level, but without the accompanying rise in free alcohol, indicating different degradation mechanism.

Based on the results obtained, it can be concluded that, due to instability of the majority of the compounds investigated, samples should be stored in the freezer (–20 °C) and protected from light.

CONCLUSION

The proposed optimized method is simple, and can be used for extraction of a wide range of plant secondary biomolecules, namely anthraquinones, isoflavonoids, flavonoids, phenolic acids, lignans, coumarins, and sesquiterpene lactones. 80% methanol provides acceptable extraction yield for all examined compounds, mixed in ratio 13:1 with dry plant material. Six-hour maceration, repeated for four times with fresh extraction solvent, provides a satisfactory yield of about 90%, within a reasonable amount of time. Temperature of 60 °C provides highly efficient extraction. Since it does not lead to compound degradation, it can be also used for crude extract evaporation. Defatting of extracts with hexane does not lead to major losses of compounds of interest. Thus, it can be used for removal of fats and pigments. Finally, results show that prepared extracts should be stored away from sunlight, on –20 °C.

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IZVOD

OPTIMIZACIJA USLOVA EKSTRAKCIJE SEKUNDARNIH METABOLITA IZ RAZLIČITIH BILJNIH VRSTA

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Izolacija sekundarnih metabolita iz biljaka je veoma važna za njihovu identifikaciju, karakterizaciju i ispitivanje biološke aktivnosti. Prvi korak u izolaciji biomolekula je njihova ekstrakcija iz biljnog materijala. Neoptimizovani uslovi ekstrakcije mogu dovesti do gubitaka, degradacije i modifikacije jedinjenja od interesa i rezultovati u pogrešnim zaključcima vezanim za hemijski sastav biljke. Usled toga, cilj ovog rada bio je ispitivanje uticaja raz ekstrakcionih smeša, količina ekstragensa, temperature ekstrakcije i uparavanja, vremena ekstrakcije, odmašćivanja i načina čuvanja na prinos i profil različitih klasa sekundarnih metabolita. *Rumex alpinus* je korišćen za ekstrakciju antrahinona, *Glycine max* za izoflavonoide, *Chaerophyllum bulbosum* za flavonoide i fenolne kiseline, *Anthriscus sylvestris* za lignane i kumarine, *Lupinus albus* za alkaloide i *Artemisia absinthium* za ekstrakciju seskviterpenskih laktona. Efikasnost ekstrakcije je ispitivana korišćenjem tečne hromatografije visokih performansi sa detektorom sa nizom dioda i trostrukim kvadrupolnim masenim detektorom sa elektrosprej jonizacijom (HPLC--DAD-ESI–MS/MS). Kao najpogodniji ekstragens za većinu ispitanih jedinjenja je 80% metanol, pomešan u odnosu 13:1 sa suvim, usitnjenim biljnim materijalom. Maceracija od šest sati je dovoljna za ekstrakciju oko 90% biljnih komponenti. Ekstrakciju je najbolje izvršiti u 4 ponavljanja (po 90 min), sa dodatkom svežeg ekstragensa. Odmašćivanje ekstrakata, vršeno u cilju uklanjanja hlorofila i prisutnih masnih kiselina, ne dovodi do velikih gubitaka jedinjenja od interesa. Ekstrakciju i uparavanje je prihvatljivo vršiti na temperaturi od 60 °C. Ekstrakte je najbolje čuvati zaštićene od svetlosti, na –20 °C.

Ključne reči: Ekstrakcija • Sekundarni metaboliti • Biljni polifenoli • Flavonoidi • Fenolne kiseline