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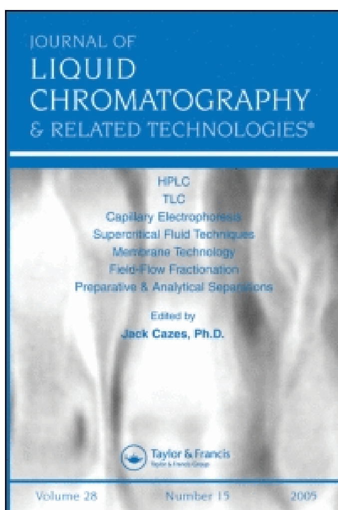

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Development of an RP-HPLC Method for the Analysis of Phenolic Compounds in *Achillea millefolium* L.

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Abstract: Several major phenolic constituents present in yarrow (*Achillea millefolium* L. s.l.) were determined for a homogenized plant sample. In order to optimize the conditions for sample preparation of the botanical matrix two different solvent extraction methods (maceration and ultrasonic agitation) were assayed. The preliminary maceration studies were performed to determine the influence of extracting solvents on the recovery of phenolics by using a different concentration of aqueous ethanol (40–96%, v/v) as extractant. On the basis of these results, sonication extractions were carried out at different time intervals (5, 10, 20, 30, and 60 min) using optimum extractant concentration. Levels of seven constituents (chlorogenic acid, vicenin-2, luteolin-7-*O*-glucoside, rutin, apigenin-7-*O*-glucoside, luteolin, and apigenin) were measured by means of high performance liquid chromatography (HPLC) with ultraviolet and photodiode array detection systems. The main validation steps of the HPLC method were evaluated to demonstrate its selectivity, linearity, and precision. In addition, the method was applied to characterization of the flavonoid and phenolcarbonic acid complex in different samples of yarrow.

Keywords: *Achillea millefolium*, Phenolics, HPLC, Extraction

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INTRODUCTION

Achillea L. is a genus of the well known medicinal plant family of *Asteraceae* that comprises numerous species and wild-growing plants widely spread over the Northern Hemisphere.[1] Several *Achillea* plants have been found to possess a wide spectrum of biological effects that include antispasmodic,[2] anti-inflammatory,[3] antimicrobial,[4] analgesic, antipyretic,[5] choleretic,[6] cytotoxic,[7,8] and estrogenic.[9] It is believed that these pharmacological actions are mainly attributed to the flavonoid and phenolcarbonic acid complex. Flavonoids are a group of natural benzo-γ-pyrone derivatives that are ubiquitous in a wide range of vascular plants,[10] whereas phenolcarbonic acids constitute one of the most abundant phenolic compounds in the plant kingdom.[11] These compounds represent a diverse group of plant secondary metabolites and share a common origin in the highly branched phenylpropanoid biosynthetic pathway.[12] Thus, there is a considerable research interest towards the assay of the flavonoid and phenolcarbonic acid complex composition of various *Achillea* species. Various analytical approaches have been used for the determination of phenolics in the plants of this genus. Total phenolics and tartaric esters as well as total flavonoid content were measured using spectrophotometric techniques.[13–15] For the identification and determination of individual flavonoids, TLC[16] and HPTLC[17] methods have been reported. The separation, identification, and quantitation of flavonoids in several *Achillea* plants have been achieved by capillary zone electrophoresis.[18,19] To our knowledge, there is only one report dealing with the determination of the qualitative and quantitative composition of phenolic compounds by means of HPLC with UV detection.[20] However, no data have been provided about main validation characteristics of the analytical method used.

From the bibliography, it can be concluded that, for the extraction of phenolic compounds from plant materials, commonly alcohols or aqueous mixtures with alcohols, namely methanol[16–20] or ethanol,[13–15] were used. However, there is a lack of information in the literature concerning the influence of solvent polarity on the recovery of phenolics.

The aim of this investigation was to develop an RP-HPLC method with UV detection for analysis of the flavonoid and phenolcarbonic acid complex in yarrow extracts, and to estimate the effects of sample preparation on the flavonoids and phenolcarbonic acid composition in the extracts. Moreover, the optimized method was applied to determine contents of phenolic constituents in herbs of *A. millefolium* s.l., collected in different habitats in Lithuania.

EXPERIMENTAL

Reagents

The reference compounds apigenin, apigenin-7-*O*-glucoside, luteolin, luteolin-7-*O*-glucoside, rutin, chlorogenic acid, were purchased from Fluka (Buchs,

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Switzerland) and Roth (Karlsruhe, Germany). The analyte standards were of HPLC-grade. Vicenin-2 was previously isolated in this laboratory. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (Seelze, Germany). The solvent of acetonitrile, labelled as HPLC gradient grade, accompanied with methanol gradient HPLC grade were, respectively, purchased from Sigma-Aldrich (Buchs, Switzerland) and Scharlau Chemie (Sentmenat, Spain). Ethanol 96% (v/v) was obtained from Stumbras AB (Kaunas, Lithuania). The standard solutions were prepared by dissolving standards in methanol. Ultra pure water from Simplicity™ Water Purification System (Millipore, Bedford, USA) was used throughout the HPLC experiment and for preparation of extraction solvents.

Instrumentation

A liquid chromatographic Waters 2690 Alliance HPLC system (Waters Corporation, Milford, MA, USA) was used throughout this work. The HPLC apparatus consisted of two independently driven plungers, an in-line vacuum degasser, an auto sampler, a Waters 2487 Dual λ Absorbance Detector (UV/Vis), and a Waters 996 Photodiode Array (PDA) Detector. The system was interfaced with a personal computer utilising the Waters Millennium 2000® chromatographic manager system (Waters Corporation, Milford, MA, USA) for control and data collection. Separations were carried out using a 5 μm Ascentis™ RP-Amide analytical column (150 × 4.6 mm), guarded with a guard column 5 μm Supelguard™ Ascentis™ RP-Amide (20 × 4.00 mm) (SUPELCO, Bellefonte, PA, USA).

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Chromatographic Conditions

The chromatographic separation was carried out using as mobile phase 0.1% trifluoroacetic acid solution in water as solvent A and 0.1% trifluoroacetic acid solution in acetonitrile as solvent B, with the following gradient elution program (Table 1). The signal was monitored at 360 nm with a UV/Vis

Table 1. Gradient elution method performed with binary solvent system using as mobile phase 0.1% trifluoroacetic acid solution in water as solvent A and 0.1% trifluoroacetic acid solution in acetonitrile as solvent B

Time (min)	A (%)	B (%)
0–25.5	90 → 76	10 → 24
25.5–27	76 → 72	24 → 28
27–45	72 → 45	28 → 55
45–48	45	55
48–52	45 → 90	55 → 10
52–55	90	10

detector. This LC method was used at a flow rate of 1.5 mL/min at an ambient temperature. The sample injection volume was 10 μL. Three injections were performed for each sample.

Chromatographic peaks were identified by comparing retention times of samples with those of standard compounds. Furthermore, in order to confirm the identity of the eluted constituents', spectral characteristics of the eluting peaks were recorded with diode-array detector, from 200 to 400 nm, and compared with UV spectra of authentic standards. The external standard approach was chosen for quantitation. The content of the bioactive compounds was determined using a calibration curve established with five dilutions of each standard. Each concentration was measured in triplicate. The calibration plots were produced by plotting the corresponding peak areas vs. concentration. Standard solutions were prepared at the approximate concentration of constituent levels in the extracts of the samples.

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Plant Material

The plant material represents randomly gathered tops of plants of 10 cm length, which were collected from wild populations at the full flowering stage in 2004 (Table 2). The material was dried at room temperature (20–25°C), in the ventilated lodge, avoiding direct solar radiation for two weeks. Dry material was packed into multilayer paper bags and stored in the dark room at an ambient temperature.

The loss on drying of dried material was 8.42–9.53% (Table 2). All obtained results were recalculated for absolutely dried material.

Sample Extraction

Sample Pretreatment

To obtain a more homogeneous plant matrix for the solvent extraction studies, dried plant material was milled at room temperature. Because the ground plant material appeared inhomogeneous, the material was sieved in order to obtain the fraction with the particle size of 0.10–0.30 cm and this material was retained for the extraction studies.

Extraction Parameters

In order to optimize the conditions for the whole extraction procedure, two different solvent extraction methods (maceration and ultrasonic agitation) were assayed. The preliminary maceration studies were performed to determine the influence of extracting solvents on the recovery of phenolics by using aqueous ethanol as extractant. The concentration of ethanol in extractant was varied in the range from 40 to 96% (v/v). On the basis of these

Table 2. Characteristic of *Achillea millefolium* habitats and loss on drying

No.	Collecting site location	Total N (%)	Humus (%)	P (mg/kg)	K (mg/kg)	pH_{KCI}	Loss and drying (%)
1	Zuikine (Kaunas city)	0.28	3.42	55.90	151.60	7.49	9.25
2	Viciunai (Kaunas city)	0.23	3.09	442.00	55.30	6.43	9.00
3	Petrasiunai (Kaunas city)	0.33	4.43	133.10	20.90	7.03	8.89
4	Panemune (Kaunas city)	0.33	3.75	86.90	46.40	7.40	9.33
5	6 Fortas (Kaunas city)	0.49	7.58	991.70	255.60	7.08	8.98
6	Domeikava (Kaunas district)	0.30	5.15	335.30	179.20	7.48	8.42
7	Voskoniai (Kaunas district)	0.12	3.13	265.20	254.30	7.62	9.35
8	Bytvanas (Kaunas district)	0.06	1.05	297.10	52.40	8.13	8.99
9	Valerava (Kaunas district)	0.21	5.30	82.10	156.10	7.51	9.15
10	Ibenai (Kaunas district)	0.20	5.24	239.00	160.20	7.40	9.53

previous results, sonication extractions were carried out at different time intervals (5, 10, 20, 30, and 60 min) using optimum extractant concentration.

Extraction Procedures

For maceration studies approximately 1.000 g (accurate weight, weighed with 0.0001 g precision) of powdered plant material was put into a flask of 50 mL capacity and poured over with 40 mL of aqueous ethanol, then shaken in a shaker for 1 hour. After being kept away from direct sunlight for 12 hours, it was additionally shaken for 1 hour and filtered through a paper filter into a 50 mL measuring flask. After that, the remaining 10 mL of aqueous ethanol was used for 3 washings of the residue on the filter and the ethanol was poured up to the graduation. Finally, the obtained ethanol extracts were additionally diluted for 2 times.

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An ultrasonic bath, BioSonic UC100 (Coltène/Whaledent, Mahwah, NJ, USA) was used for sonication extractions. Approximately 0.25 g (accurate weight, weighed with 0.0001 g precision) of powdered plant material were sonicated with 25 mL of the aqueous ethanol for different time intervals. After centrifugation at 12,000 rpm for 10 min, the supernatant was adjusted to 25 mL in a measuring flask.

To perform the HPLC analysis of all obtained extracts, they were filtered through the membrane filter with pore size of 0.22 μm (Carl Roth GmbH, Karlsruhe, Germany). Each extraction was repeated in triplicate.

Statistical Data Analysis

The results were statistically analyzed using the program SigmaStat version 3.00 for Windows (SPSS Chicago, IL, USA) to obtain means, standard deviations, and standard errors. Graphical presentation of the results was performed using the software SigmaPlot 2004 for Windows version 9.0.

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RESULTS AND DISCUSSION

Extraction Method Optimization

For flavonoids' extraction from various matrices, the solvent is usually chosen as a function of the type of flavonoid required. Extractant polarity here plays an important role. Less polar flavonoids are commonly extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate, while alcohols or alcohol-water mixtures ensure the extraction of flavonoid glycosides and more polar aglycones.[10,21–23] Aqueous ethanol was selected as an extracting solvent, because it is commonly used for pharmaceutical preparations.[24] In order to determine the influence of the extractant polarity on the recovery of phenolics, optimized HPLC method was used. The results belonging to the preliminary assays, which aim was to select the optimal concentration of ethanol in the aqueous ethanol as extractant, are shown in Figure 1. Although some individual compounds were better extracted with concentrations other than 70%, this concentration of aqueous ethanol was selected for further analyses, as it afforded the highest total amount of identified phenolics.

In addition to the choice of extraction solvent, there are also different approaches to the actual extraction procedure.[22,25,26] A comparison of two extraction techniques was conducted for several major constituents in yarrow. On the basis of the previous results, the extractions of the same plant sample material were carried out by maceration and sonication using 70% aqueous ethanol as an extractant. The comparison of the results from these extraction approaches permits the relative assessment of extraction efficiency. Preliminary studies indicated that even a 5 min period of sonication

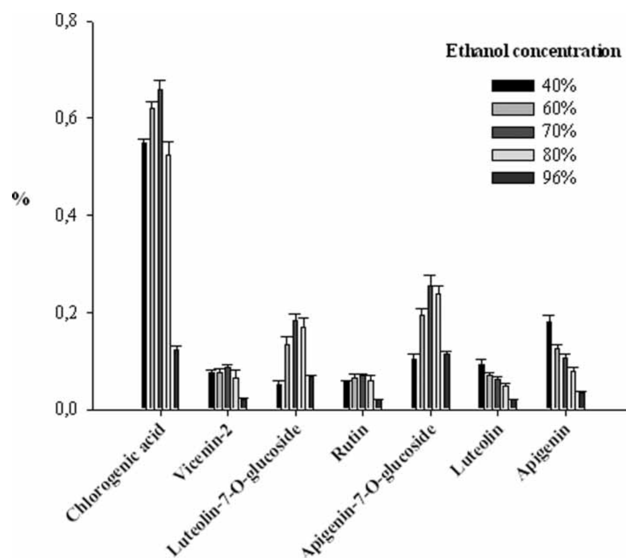

Figure 1. Mean values (%) and error bars of the identified phenolics in aqueous ethanolic extracts.

afforded the higher total amount of identified phenolics (1.52%) than the extraction yields with maceration (1.42%). Furthermore, extraction efficiency was observed to increase with time for sonication extractions (Figure 2). The profile of the extracts obtained with different periods of sonication was

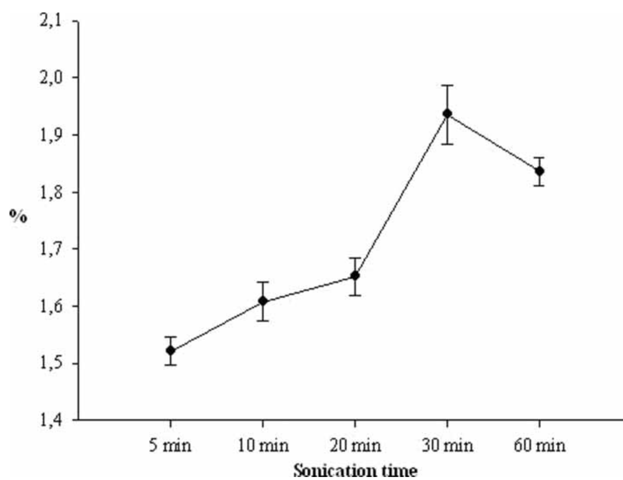

Figure 2. Mean values (%) and error bars of total content of the identified phenolics in the yarrow extracts obtained with different periods of sonication.

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the same in all cases. However, when the time was increased to 60 min, the extraction yield decreased. Thus, a 30 min period of sonication was selected for further analyses, as it afforded the highest total amount of identified phenolics.

Chromatographic Separation Optimization

Broad characterization of plant matrix bioactive constituents was achieved using a gradient elution method performed with a binary solvent system. Preliminary experiments were performed to obtain the best peak resolution and separation for a mixture of standards. Usually, acetonitrile-water or methanol-water mixtures with or without small amounts of acid are the solvents of the prior selection for RP-HPLC analysis of flavonoids.[21,22] Because of the complex botanical matrix containing analytes of various polarities, a stronger organic solvent, acetonitrile, was chosen. Moreover, in order to suppress the ionization of phenolic hydroxyl groups that results in higher chromatographic retention of analytes,[27] trifluoroacetic acid was used. Two chromatographic columns, such as XTerra® RP18 (150 × 3.9 mm, 3.5 μm) Waters (Milford, MA, USA) and Ascentis™ RP-Amide (150 × 4.6 mm, 5 μm) Supelco (Bellefonte, PA, USA), were screened with 0.1% TFA solution in water (solvent A) and 0.1% TFA solution in acetonitrile (solvent B) as mobile phase. The best selectivity and resolution was observed on the Ascentis RP-Amide column. The better selectivity of the latter column might be due to increased interaction of analytes with phenolic groups with polymeric amide-bonded phase.[28] Therefore, this column was selected. Furthermore, in order to obtain sufficient resolution of real extract, which is more difficult to separate compared to model mixtures due to the effects of a complex matrix of the real samples, the gradient elution method was additionally optimized. The chromatographic separation of 70% (v/v) ethanolic extract of yarrow herb obtained using optimal HPLC conditions is illustrated in Figure 3. Table 3 shows that sufficient resolution (R_S) of analytes was obtained in the mixture of standards. Moreover, optimization of the chromatographic method enabled an efficient separation of luteolin-7-*O*-glucoside and rutin in real extracts with a resolution higher than 2.0. Linearity, limit of detection (LOD), and limit of quantification (LOQ) were evaluated for quantitative purposes (Table 4). Limits of detection and quantification were determined by calculation of the signal-to-noise ratio. Signal-to-noise ratios of approximately 3:1 and 10:1 were used for estimating the detection limit and quantification limit, respectively, of the method. Thus, LOD and LOQ values ranged from 0.04 to 0.46 μg/mL, and from 0.15 to 1.52 μg/mL, respectively, which suggested full capacity for the quantification of each bioactive compound investigated. R^2 values of the analytes were higher than 0.99, thus confirming the linearity of the method. To verify the precision of the proposed HPLC method, five replicate injections of a 70% ethanolic yarrow

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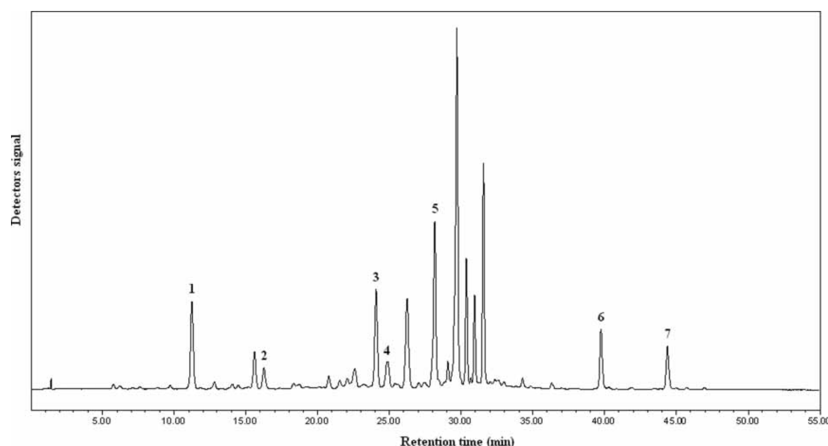

Figure 3. HPLC chromatographic separation of 70% (v/v) ethanolic extract of yarrow herb. Peaks identified: 1-chlorogenic acid, 2-vicenin-2, 3-luteolin-7-*O*-glucoside, 4-rutin, 5-apigenin-7-*O*-glucoside, 6-luteolin, 7-apigenin.

extract were examined. It can be seen that repeatability for retention times (Table 5) is high and does not exceed 0.99%, while this statistical parameter for peak area is acceptable, and does not exceed 4.97%.

Analyses of Yarrow Extracts

The raw material of yarrow is one of the oldest and most important drugs widely used both in folk and official Lithuanian medicine.[29] It is usually

Table 3. Resolution (R_S) of analytes in mixture of standards

R_S	
	Chlorogenic acid
14.56	
	Vicenin-2
22.31	
	Luteolin-7-*O*-glucoside
2.14	
	Rutin
10.07	
	Apigenin-7-*O*-glucoside
38.87	
	Luteolin
14.04	
	Apigenin

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Table 4. Regression curves, linearity, limit of detection (LOD) and limit of quantification (LOQ)

Compound	Regression equation	Correlation coefficient (R^2)	LOD, (μg/mL)	LOQ, (μg/mL)
Chlorogenic acid	$Y = 2.7 \cdot 10^3 \times - 2.29 \cdot 10^3$	0.9940	0.46	1.52
Vicenin-2	$Y = 5.29 \cdot 10^3 \times - 2.63 \cdot 10^3$	0.9970	0.28	0.93
Luteolin-7-*O*-glucoside	$Y = 1.2 \cdot 10^4 \times - 2.92 \cdot 10^3$	0.9988	0.13	0.42
Rutin	$Y = 9.4 \cdot 10^3 \times - 9.56 \cdot 10^3$	0.9997	0.21	0.63
Apigenin-7-*O*-glucoside	$Y = 1.13 \cdot 10^4 \times - 2.68 \cdot 10^3$	0.9992	0.24	0.79
Luteolin	$Y = 3 \cdot 10^4 \times - 1.5 \cdot 10^3$	0.9993	0.04	0.15
Apigenin	$Y = 1.52 \cdot 10^4 \times - 2.26 \cdot 10^3$	0.9991	0.11	0.37

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gathered from wild populations and only rarely derives from cultivated plants. In recent years, the requirements are increasing on quality, safety, and efficiency of herbal medicinal products and plant raw material.[30] Due to that, the assessment of phytochemical composition is of crucial importance for the evaluation of quality of the local raw yarrow material, as well as for its purposive selection and conservation of resources from over-exploitation.

The established HPLC method was applied to the quantification of the biologically active compounds in different samples of *A. millefolium* collected from natural populations in 10 localities of the central Lithuania. The results of the phytochemical analysis of these materials for their contents of bioactive compounds are presented in Table 6. The mean values of total contents of the identified phenolics in the samples varied from 1.24 to 1.95% (Figure 4). As it can be seen from this figure, the total amounts of the identified phenolics varied within a small range from 1.24 to 1.58%, while the highest percentage of the identified phenolics (1.95%) was observed in yarrow collected in the Zuikine habitat.

Generally, the patterns of phenolic distribution in the analysed samples of *Millefolii herba* were very similar. Apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, and chlorogenic acid were the main compounds among the identified analytes, accompanied with minor flavonoids as rutin, apigenin, and luteolin. In accordance with earlier investigations,[20,31] in addition to the characteristic composition of the flavonoid complex with the predominant formation of flavonol- and flavone-*O*-glycosides, the trend to the synthesis of flavone-*C*-glycosides was also underlined by the minor compound vicenin-2.

The specific patterns of flavonoid accumulation in each plant species are determined both by the intricate system of genetically controlled enzymes and by environmental factors.[32] The biosynthesis of secondary metabolites can be induced by solar radiation[33] as well, as it is enhanced in response to increased exposure to various pollutants.[34] Thus, the *A. millefolium* habitats

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Table 5. Repeatability of HPLC method[a]

Compound	RSD (%)					
	Retention time			Integrated area		
	Run-to-run	Day-to-day	Extraction-to-extraction	Run-to-run	Day-to-day	Extraction-to-extraction
Chlorogenic acid	0.41	0.96	0.99	0.52	1.64	2.67
Vicenin-2	0.22	0.62	0.94	1.08	1.54	3.83
Luteolin-7-*O*-glucoside	0.12	0.53	0.56	1.16	1.56	3.62
Rutin	0.09	0.48	0.52	1.18	1.47	4.15
Apigenin-7-*O*-glucoside	0.07	0.39	0.46	1.17	2.28	4.97
Luteolin	0.06	0.29	0.55	1.10	1.18	3.39
Apigenin	0.06	0.27	0.18	1.08	1.55	2.32

[a]n = 5 for run-to-run and day-to-day repeatability, and n = 3 for extraction-to-extraction repeatability.

Table 6. Amounts of phenolic compounds in herb of *A. millefolium* (n = 10)

Compound	Quantity (%)			Std. Error
	Min	Max	Mean	
Chlorogenic acid	0.548	1.028	0.719	0.024
Vicenin-2	0.054	0.175	0.094	0.012
Luteolin-7-*O*-glucoside	0.148	0.285	0.196	0.007
Rutin	0.027	0.068	0.047	0.003
Apigenin-7-*O*-glucoside	0.229	0.351	0.288	0.006
Luteolin	0.027	0.054	0.039	0.001
Apigenin	0.034	0.056	0.044	0.001

were assayed for the concentrations of soil nutrients (Table 2). As it can be seen, analysed soils were poor in nitrogen (0.06–0.49%) and amounts of potassium and phosphate varied within ranges 20.90–255.60 mg/kg and 55.90–991.70 mg/kg, respectively. The soil pH usually ranged from neutral to slightly alkaline in reaction and quantities of humus varied from 1.05 to 7.58%. Nevertheless, taken together, the results from phytochemical and soil assays appeared to be insufficient to evaluate the nature of accumulation trends dependence on environmental impact. Consequently, further investigations are required to comprehensively analyze the dependence of the phenolic compounds accumulation in yarrow on different environmental factors.

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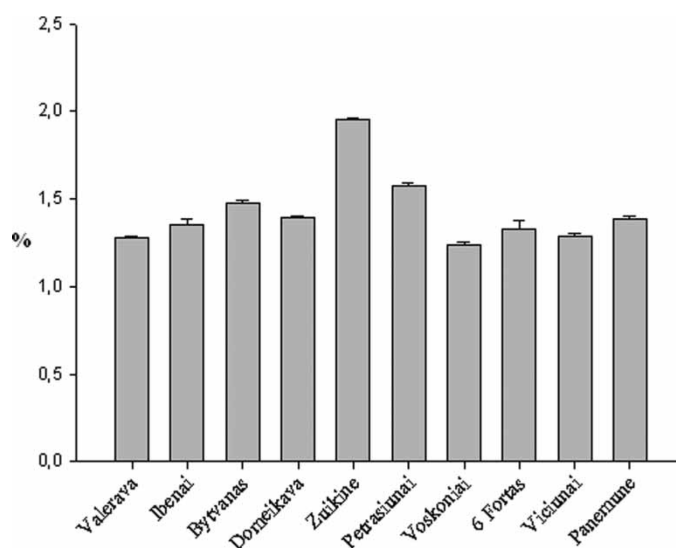

Figure 4. Mean values (%) and error bars of total content of the identified phenolics in the herb of yarrow obtained from different habitats.

CONCLUSIONS

The RP-HPLC method for separation of the flavonoid and phenolcarbonic acid complex in ethanolic extract of yarrow was developed. The method proved to be suitable for determination of the qualitative and quantitative phenolic composition in the ethanolic extracts of the herb of yarrow. Furthermore, the method shows a good selectivity, linearity, and precision, and it might be useful for the quality control analysis of yarrow raw materials and preparations. It was determined, that the highest levels of phenolic compounds were achieved using 70% aqueous ethanol through ultrasonic extraction of botanical samples for a 30 min period of sonication. Total contents of the identified phenolics in the plant material varied from 1.24 to 1.95%.

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