Simultaneous determination of coumarin derivatives in natural samples by ultra high performance liquid chromatography

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Summary
Ultra high performance liquid chromatography method coupled with ultraviolet and fluorescence detection was developed for determination of coumarin derivatives and metabolites (esculin, daphnetin, fraxetin, umbelliferone, 4-methylumbelliferone, 4-hydroxycoumarin, scoparone, coumarin, herniarin). The separations were performed using octadecyl or phenyl-hexyl silica-based analytical columns (50 mm × 4.6 mm, 1.8 μm particle size) at gradient elution with mobile phase consisting of 0.3% aqueous acetic acid : acetonitrile (9 : 1) and acetonitrile (0–35 % acetonitrile over 3.5 min). The parameters of system suitability (repeatability of retention time and peak area) and validation (linearity range, limits of detection and quantification, precision and recovery) were determined for evaluation of the method. The limits of quantification were found in micrograms per millilitre range for ultraviolet detection and in nanograms per millilitre for fluorescence detection. Practical applicability of the method was demonstrated by analysing plant and propolis samples. Accuracy of the method was assessed at three concentration levels and values of the average percentage recoveries were higher than 88 % with relative standard deviation lower than 5 %.

Keywords
simple coumarins; natural samples; ultra high performance liquid chromatography; ultraviolet detection; fluorescence detection

Analysis of complex samples and separation of a higher number of analytes are often time-consuming. There is an increasing need for fast separation methods with good chromatographic performance. Ultra high performance liquid chromatography (UHPLC) is a valuable tool for separation of analytes, which utilizes full advantages of chromatographic principles. The main advantage, improved peak separation, arises from the use of a short column packed with sorbents of smaller particles (1.7–2.0 μm) and a higher flow rate. In comparison to high performance liquid chromatography (HPLC) technique, UHPLC has enhanced sensitivity and separation power, which results in a shorter run-time of analysis [1].

Coumarins, a class of compounds that contain a 1,2-benzopyrone skeleton, occur as secondary metabolites in the seeds, roots and leaves of many plant species. Many molecules based on the coumarin ring system have been synthesized. Natural coumarins or synthetic analogs display interesting biological properties. Coumarins are used in treatment of multiple sclerosis, T-cell lymphoma, multidrug-resistant tumor, and in the treatment of nicotine addiction [2]. Besides the positive effects, some coumarins have also negative biological effects. For example, coumarin was found to be toxic to liver [3].

The most widely naturally occurring simple coumarins are coumarin, umbelliferone (7-hydroxycoumarin), esculetin (6,7-dihydroxycoumarin) and herniarin (7-methoxycoumarin). Liquid chromatographic methods with ultraviolet (UV) or mass spectrometric (MS) detection were used for determination of simple coumarins. Some of coumarins contain fluorophore in its molecule and, for this reason, the use of fluorescence (FL) detection is suitable [4]. A comparison of recent liquid chromatography methods for the determination of simple coumarins is summarized in Tab. 1. Many of the published methods were used for determination of individual coumarins [7, 8, 13] or in groups with other compounds (phenolics, furanocoumarins, pyranocoumarins and others)

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Tab. 1. Summary of recent liquid chromatography methods for separation of coumarins.

Separated compounds	Stationary phase	*T* [°C]	Mobile phase solvent	Detection	*t* [min]	*LOD* [ng·ml^{-1}]	Ref.
Ultra high performance liquid chromatography							
Esculin	C18	25	CH_3OH/H_2O with 0.1% HCOOH	UV (λ_{max} 280 nm)	4	–	[5]
Umbelliferone	C18	25	CH_3OH/H_2O with 0.1% HCOOH	UV (λ_{max} 280 nm)	4	–	[5]
				MS/MS	19	0.1	[6]
4-Hydroxycoumarin	C18	25	CH_3OH/H_2O with 0.1% HCOOH	UV (λ_{max} 280 nm)	4	–	[5]
Coumarin	C18	30	$CH_3CN/MeOH/H_2O$	UV (λ_{max} 240 nm)	2.5	50.0	[7]
			CH_3CN/H_2O with 0.1% HCOOH	MS/MS	3	20.0	[8]
High performance liquid chromatography							
Esculin	C18	25	CH_3CN/H_2O with 0.5% CH_3COOH	UV (λ_{max} 340 nm)	15	–	[9]
Daphnetin	C18	30	CH_3CN/H_2O with 0.1% HCOOH	UV (λ_{max} 325 nm)	90	40.0	[10]
Umbelliferone	C18	25	CH_3OH/0.05 mol·l^{-1} phosphate buffer (pH 5)	UV (λ_{max} 322 nm)	30	20.4	[11]
				FL (λ_{ex} 290–320 nm, λ_{em} 372–450 nm)	30	0.5	[11]
			CH_3CN/H_2O	DAD	35	50.0	[12]
		30	CH_3CN/H_2O with 0.1% HCOOH	UV (λ_{max} 325 nm)	90	83.0	[10]
4-Hydroxycoumarin	C18	25	CH_3OH/0.05 mol·l^{-1} phosphate buffer (pH 5)	UV (λ_{max} 286 nm)	30	35.6	[11]
				FL (λ_{ex} 290–320 nm, λ_{em} 372–450 nm)	30	12.5	[11]
Scoparone	C18	25	CH_3CN/H_2O	DAD	35	70.0	[12]
Coumarin	C18	40	CH_3CN/H_2O with 0.1% HCOOH	DAD	10	8.7	[13]
				ESI-MS	10	–	[13]
Herniarin	C18	30	CH_3CN/H_2O with 0.1% HCOOH	UV (λ_{max} 325 nm)	90	50.0	[10]

T – column temperature, *t* – total time of analysis, *LOD* – limit of detection, C18 – octadecyl carbon chain-bonded silica, UV – ultraviolet detection, MS/MS – tandem mass spectrometric detection, FL – fluorescence detection, DAD – diode array detection, ESI-MS – electrospray ionization mass spectrometric detection, λ_{max} – maximum of UV absorption, λ_{ex} – excitation wavelength, λ_{em} – emission wavelength.

[6, 9, 10, 12–14]. However, separation of compounds in the subgroup of simple coumarins is problematic due to their comparable polarity and similar chemical structures of the substances.

Aims of this study were: i) to find suitable conditions for simultaneous separation of nine coumarin derivatives (naturally occurring and metabolites) from the subgroup of simple coumarins, using octadecyl and phenyl-hexyl stationary phases, ii) to validate the UHPLC method with on-line ultraviolet and fluorescence detection, and iii) to apply the method for analysis of plant and propolis extracts.

MATERIALS AND METHODS

Chemicals and samples

Acetonitrile, ethanol (HPLC gradient grade) and acetic acid (99%) were purchased from Merck (Darmstadt, Germany). Ultrapure water (resistivity 18.2 MΩ·cm) was obtained from a Milli-Q (Millipore, Billerica, Massachusetts, USA) water purification system. Standards of esculin (98%), coumarin (99%), daphnetin (98%), fraxetin (98%), herniarin (98%), 4-hydroxycoumarin (98%), 4-methylumbelliferone (98%), scoparone (98%) and umbelliferone (99%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Samples of the dried medical plant, i.e. aerial parts of *Melilotus officinalis* L. (Meliloti herba; Sample I) and propolis tincture without alcohol (Sample II), were purchased from local pharmacy. Sample of crude propolis (originating in western Slovakia, 2015; Sample III) was collected from a beehive before the winter season and was stored as a solid powder at 22 °C in the dark until processing.

Standard solutions

Stock standard solutions of coumarins at a concentration of 0.1 mg·ml^{-1} were prepared in aqueous acetonitrile (1:1, v/v) and were found to be stable when stored at –18 °C. The work-

ing standard solutions (concentrations from 0.2 ng·ml^{-1} to 0.01 mg·ml^{-1}) were prepared weekly by appropriate dilution of the stock solutions with aqueous acetonitrile (1 : 1, v/v). The solutions were filtered through a syringe nylon filter of 0.22 μm pore size prior to chromatographic separations.

Sample preparation

Sample of dried powdered plant (Meliloti herba; Sample I) was prepared by mixing 5.000 g with 30 ml of distilled water. Afterwards, the sample was stirred on a mechanical shaker at 22 °C for 60 min. Finally, the mixture was centrifuged at 1 431 ×g for 10 min at 22 °C, and then the supernatant was removed and filtered through a syringe nylon filter of 0.22 μm pore size. The extract was used for chromatographic analysis.

Propolis tincture without alcohol (Sample II) was filtered through a syringe nylon filter of 0.22 μm pore size prior to chromatographic analysis.

Sample of crude propolis (Sample III) was prepared by mixing 1.000 g with 40 ml of ethanol. Afterwards, the sample was stirred at 22 °C for 72 h with a mechanical shaker. Finally, the mixture was centrifuged at 1 431 ×g for 10 min at 22 °C, and then the supernatant was removed and filtered through a syringe nylon filter of 0.22 μm pore size. The extract was used for chromatographic analysis.

The liquid extraction procedure was repeated two times.

For the validation assay, samples were spiked with working standard solutions and conditioned for at least 1 h before being used in an extraction procedure. Extracts were stored at 4 °C.

Liquid chromatography

Analyses were performed on Agilent 1290 Series LC system (Agilent Technologies, Santa Clara, California, USA) equipped with a degasser, a binary solvent delivery system, an autosampler, a column thermostat, a diode array detector and a fluorescence detector. The analytical columns Zorbax Eclipse Plus phenyl-hexyl HT (50 mm × 4.6 mm internal diameter, 1.8 μm particle size) and Zorbax Eclipse Plus C18 HT (50 mm × 4.6 mm internal diameter, 1.8 μm particle size, Agilent Technologies) were used for chromatographic separations. The mobile phase consisted of acetonitrile and water containing 0.3 % acetic acid (1 : 9, v/v) (A) and acetonitrile (B). Gradient elution at a constant flow rate of 2.0 ml·min^{-1} was used according to the following program, starting at 0 % B and rising linearly to 35 % B over 3.5 min, then to 100 % B over 0.5 min. The composition was held at 100 % B for further 1 min and returned to the initial conditions. The column was re-equilibrated for 1 min. The diode array detector was set at 280 nm and 323 nm, and UV spectrum was recorded at 190–400 nm. The fluorescence detector was set at 320 nm excitation wavelength and 450 nm emission wavelength. Emission spectrum was recorded at 250–500 nm. Injection volume was 5 μl and the column temperature was 30 °C.

Qualitative analysis of coumarins in sample extracts was done by comparison of retention times, ultraviolet and fluorescence spectra with those of coumarins standards. Spectra were also used to confirm the purity of coumarin peaks separated from other compounds present in extracts. Quantitative analysis of coumarins was done using the external standard method.

System suitability test and validation of the method

System suitability parameters (repeatability of retention time, repeatability of peak area) were evaluated under the optimized chromatographic conditions using the standard mixture of coumarins at the test concentration of 5.0 μg·ml^{-1}.

Linearity was evaluated using mixed standard solutions for each compound, separately for UV and separately for FL detector. Concentration ranges were from limit of quantification (*LOQ*) of coumarins obtained for UV detector to 100 μg·ml^{-1}, and from *LOQ* of coumarins obtained for FL detector to 100 ng·ml^{-1} (seven mixed standard solutions). Seven preparations of each solution were analysed and the values of peak areas of each analyte were recorded. The calibration curve of analyte was obtained by plotting a graph of mean peak area versus corresponding concentration of analyte.

The limit of detection (*LOD*) and *LOQ* values were calculated utilizing specific calibration curves in the concentration range starting from 0.3 μg·ml^{-1} (depending on analyte *LOQ*) to 10 μg·ml^{-1} for UV detection, and starting from 0.3 ng·ml^{-1} (depending on analyte *LOQ*) to 30 ng·ml^{-1} for FL detection, according to equations 1 and 2:

$$LOD = 3.3 \times \frac{\sigma}{b} \quad (1)$$

$$LOQ = 10 \times \frac{\sigma}{b} \quad (2)$$

where σ is the standard deviation of the intercept and b is the slope of the calibration curve.

Precision of the proposed method was tested for seven preparations of spiked Sample II at the test concentration of 1.0 μg·ml^{-1} (for umbelliferone, scoparone, coumarin, esculin, 4-methylumbelliferone, herniarin) or 5.0 μg·ml^{-1} (for

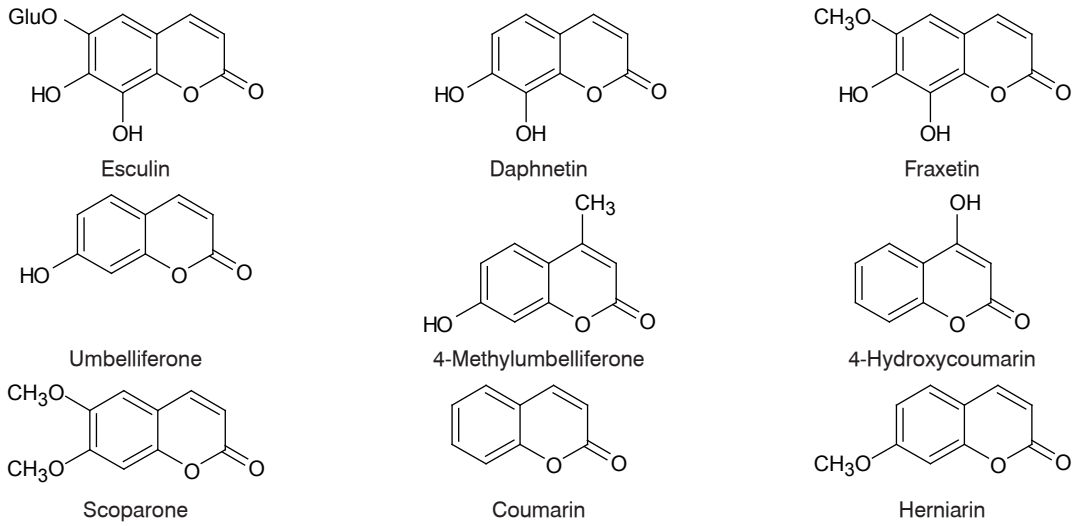

Fig. 1. Structures of analysed coumarins.

daphnetin, fraxetin, 4-hydroxycoumarin), making triplicate injections under the working conditions. For inter-day variation, the measurements were conducted on three consecutive days. Results were expressed as percentage relative standard deviation (*RSD*).

Recovery studies were performed with spiked samples (spiked with identified coumarins) at three concentration levels of coumarins. Spiked and unspiked samples were treated by the same procedure. Seven independent extractions of each were carried out for real samples.

Robustness of the analytical method was evaluated by testing the influence of column temperature (± 2 °C) and the wavelength (± 2 nm).

RESULTS AND DISCUSSION

Coumarin and its derivatives (Fig. 1), belonging to the group of simple coumarins, were selected for this study. Coumarins occur naturally in many herbal plants, in other natural spices and in foods. They can be also present in bee products [14, 15].

UHPLC separation and detection of coumarins

The separation of nine selected coumarin derivatives was performed in a reversed-phase chromatographic mode. The UHPLC system was equipped with columns packed with 1.8 mm particle size stationary phases of octadecyl or phenyl-hexyl type. Both tested stationary phases are suitable for separation of acidic, neutral, and weakly basic compounds, while the phenyl-hexyl phase has better selectivity for analytes containing phenyl groups. Identical gradient profiles and flow rates for both tested analytical columns were applied. Efficient separation was obtained in the gradient mode with mobile phase consisting of acetonitrile and 0.3% aqueous acetic acid.

Acetic acid as an additive played an important role in symmetry of peaks. The increase in the acetic acid concentration from 0.1% to 0.3% resulted in an increase of symmetry factor of peaks (A_s = 0.91–0.97 for phenyl-hexyl column, A_s = 0.69–0.86 for octadecyl column; Tab. 2).

The column temperature was kept slightly above the laboratory temperature (30 °C) to decrease viscosity of the mobile phase, which helped to significantly reduce the column back-pressure. The values of pressure in the chromatographic separation system (at the composition of mobile phase corresponding to the beginning of gradient and at the flow rate used) were close to the upper limit of applicability of both tested columns (maximum pressure 60 MPa) if the working temperature was 23 °C. The increase of column temperature resulted in a reduction of the back-pressure to 48 MPa (at the beginning of gradient).

The main advantages of the developed UHPLC method were faster analysis, faster equilibration of the column to the initial conditions and lower consumption of organic solvents. The total analysis time for separation of selected compounds was less than 6 min (in comparison with 10–90 min for HPLC analyses; Tab. 1).

The suitability of the chromatographic system for the separation of target coumarins was evaluated by values of resolution, hight equiva-

lent of theoretical plate, repeatability of retention times and repeatability of peak areas (Tab. 2). The retention times were in the interval 0.72–2.79 min for phenyl-hexyl column and 0.85–3.22 min for octadecyl column. Both columns exhibited good stability of retention times (*RSD* being lower than 0.2 %) and good repeatability of peak areas (*RSD* being lower than 1.7 %). UHPLC chromatograms of a selected group of coumarins obtained on tested columns are shown in Fig. 2, demonstrating baseline separation of all studied compounds. The values of resolution were comparable for both columns.

Another advantage of the UHPLC method (in comparison with HPLC) was the improvement of column efficiency. The separation based on utilization of the stationary phase with particle size of 1.8 μm resulted in reduction of the height equivalent of the theoretical plate value (by more than 4 times in comparison to the stationary phase with particle size of 5 μm; data not shown). Slightly better results, from the aspect of separation efficiency, were obtained for phenyl-hexyl column in comparison with octadecyl column, and this column was used for analysis of real samples.

The important characteristic of coumarins is the absorbance and fluorescence in the UV light range. UV detection wavelength was chosen according to the absorbance spectra of separated compounds, which display UV absorption maxima in the range 280–335 nm. The wavelength 280 nm was optimal for the detection of three selected coumarins, namely, 4-hydroxycoumarin, coumarin and scoparone, while the wavelength 323 nm was optimal for the detection of esculin, daphnetin, fraxetin, umbelliferone, 4-methylumbelliferone and herniarin. The utilization of FL detection in coumarin analysis had a positive effect on selectivity of detection and sensitivity of determination. Five of coumarin derivatives studied, namely, esculin, umbelliferone, 4-methylumbelliferone, scoparone and herniarin, show fluorescence. The optimal fluorescence excitation wavelength (λ_{ex}) and emission wavelength (λ_{em}) maxima are 320 nm and 450 nm, respectively. Chromatograms of separation of coumarins obtained by using FL detection are shown in Fig. 2B, Fig. 2D.

System suitability test and validation results

The system suitability parameters (repeatability of retention time, repeatability of peak area) and validation parameters (linearity, *LOD*, *LOQ*)

Tab. 2. Comparison of separation parameters for UHPLC separation of coumarins on different columns.

Analyte	t_r [min]	R_s	A_s	*HEPT* [%]	*RSD*-t_r [%]	*RSD-A* [%]
Phenyl-hexyl column						
Esculin	0.72	12.19	0.91	9.46	0.2	1.4
Daphnetin	1.19	1.21	0.95	3.46	0.1	1.6
Fraxetin	1.24	9.35	0.94	2.81	0.1	1.6
Umbelliferone	1.62	9.79	0.95	1.65	0.1	1.3
4-Methylumbelliferone	2.03	1.58	0.96	1.03	0.1	0.8
4-Hydroxycoumarin	2.11	3.57	0.95	1.47	0.1	1.5
Scoparone	2.23	2.21	0.95	1.42	0.1	1.2
Coumarin	2.34	5.30	0.97	1.11	0.1	1.3
Herniarin	2.78		0.97	0.90	0.1	1.5
Octadecyl column						
Esculin	0.84	9.79	0.69	34.47	0.1	1.2
Daphnetin	1.45	1.62	0.82	3.27	0.2	1.7
Fraxetin	1.54	9.20	0.82	2.81	0.1	1.4
Umbelliferone	1.94	1.48	0.86	2.00	0.1	1.2
4-Methylumbelliferone	2.44	1.56	0.82	1.69	0.1	1.5
4-Hydroxycoumarin	2.51	2.23	0.76	1.98	0.1	1.4
Scoparone	2.60	1.56	0.84	3.53	0.2	1.1
Coumarin	2.68	5.02	0.77	1.29	0.1	1.3
Herniarin	3.21		0.74	0.88	0.1	1.2

Mixture of coumarin standards at concentrations of 5.0 $\mu g \cdot ml^{-1}$ was used for optimization of chromatographic conditions. Relative standard deviation was calculated for seven preparations of standard mixture and analysed three times within a day. t_r – retention time, R_s – peaks resolution, A_s – symmetry factor of peak, *HEPT* – height equivalent of theoretical plate, *RSD*-t_r – relative standard deviation of retention time repeatability, *RSD-A* – relative standard deviation of peak area repeatability.

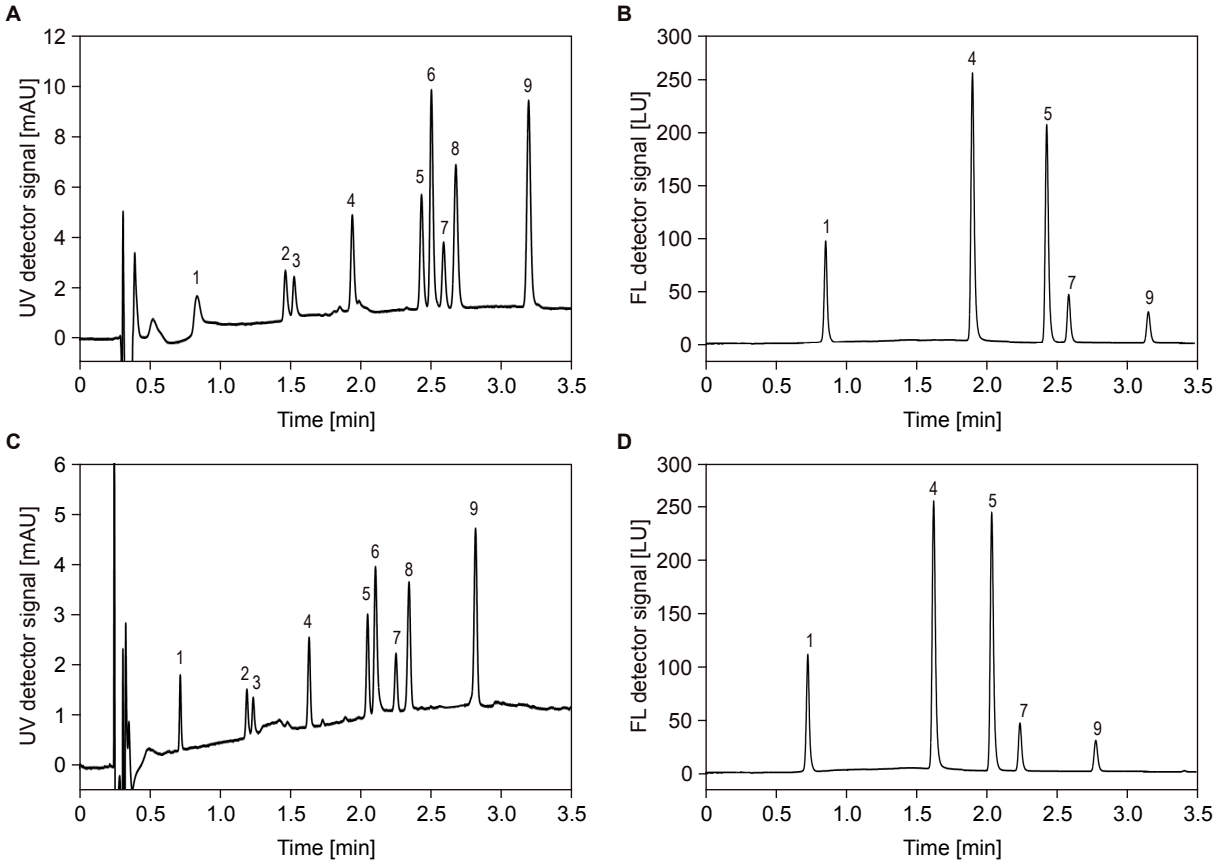

Fig. 2. Chromatograms of separation of coumarins on different columns.

A – octadecyl column (UV detector), B – octadecyl column (fluorescence detector), C – phenyl-hexyl column (UV detector), D – phenyl-hexyl column (fluorescence detector).
UV detection was carried out at a wavelength of 280 nm. Fluorescence detection was carried out at excitation wavelength 320 nm and emission wavelength 450 nm).
1 – esculin, 2 – daphnetin, 3 – fraxetin, 4 – umbelliferone, 5 – 4-methylumbelliferone, 6 – 4-hydroxycoumarin, 7 – scoparone, 8 – coumarin, 9 – herniarin.

Tab. 3. Analytical parameters of UHPLC with a phenyl-hexyl stationary phase.

Compound	Linear range		R^2		*LOD*		*LOQ*		*RSD-P* [%]
	UV [μg·ml^{-1}]	FL [ng·ml^{-1}]	UV [μg·ml^{-1}]	FL [ng·ml^{-1}]	UV [μg·ml^{-1}]	FL [ng·ml^{-1}]	UV [μg·ml^{-1}]	FL [ng·ml^{-1}]	
Esculin	1.0–100	2.9–100	0.999	0.990	0.3	1.0	1.0	2.9	3.3
Daphnetin	1.5–100	–	0.998	–	0.5	–	1.5	–	3.8
Fraxetin	1.5–100	–	0.999	–	0.4	–	1.5	–	2.6
Umbelliferone	0.3–100	0.3–100	0.999	0.998	0.1	0.1	0.3	0.3	2.6
4-Methylumbelliferone	0.3–100	1.0–100	0.999	0.985	0.1	0.3	0.3	1.0	2.3
4-Hydroxycoumarin	1.5–100	–	0.998	–	0.5	–	1.5	–	3.2
Scoparone	1.2–100	6.0–100	0.999	0.985	0.4	2.0	1.2	6.0	3.0
Coumarin	0.5–100	–	0.998	–	0.2	–	0.5	–	2.9
Herniarin	0.6–100	10–100	0.998	0.985	0.2	3.0	0.6	10.0	2.9

Method precision was evaluated for spiked Sample II at the concentration of 1.0 μg·ml^{-1} (for umbelliferone, scoparone, coumarin, esculin, 4-methylumbelliferone and herniarin) or 5.0 μg·ml^{-1} (for daphnetin, fraxetin and 4-hydroxycoumarin), making triplicate injections under the working conditions.
R^2 – coefficient of determination, *LOD* – limit of detection, *LOQ* – limit of quantitation, *RSD-P* – relative standard deviation of precision, UV – ultraviolet detection at absorption maximum λ_{max} (λ_{max} = 323 nm for esculin, daphnetin, fraxetin, umbelliferone, 4-methylumbelliferone, scoparone, and herniarin, λ_{max} = 280 nm for 4-hydroxycoumarin and coumarin), FL – fluorescence detection at excitation wavelength λ_{ex} = 320 nm and emission wavelength λ_{em} = 450 nm.

Tab. 4. The contents of coumarin derivatives in real samples.

		Meliloti herba (Sample I)			Propolis tincture without alcohol (Sample II)			Crude propolis (Sample III)		
		Content [μg·g^{-1}]	Recovery [%]	*RSD* [%]	Content [μg·g^{-1}]	Recovery [%]	*RSD* [%]	Content [μg·g^{-1}]	Recovery [%]	*RSD* [%]
Umbelliferone	Original	< *LOD*		–	0.03		1.5	0.10		3.2
	Spiked	0.05	95.0	3.7	0.05	98.4	1.6	0.05	93.3	4.7
		0.10	96.3	3.9	0.10	97.3	3.0	0.10	90.0	2.9
		1.00	91.1	4.6	1.00	97.1	2.6	1.00	91.8	4.0
Scoparone	Original	< *LOD*		–	0.3		1.8	5.7		2.1
	Spiked	5.0	95.7	2.9	1.0	96.9	3.0	1.0	91.9	3.6
		10.0	95.1	3.2	5.0	97.6	2.1	5.0	87.8	4.2
		50.0	93.4	4.0	50.0	96.2	3.8	50.0	93.5	4.5
Coumarin	Original	6.0		2.1	< *LOD*		–	< *LOD*		–
	Spiked	5.0	94.6	3.6	1.0	95.7	2.9	1.0	95.0	3.7
		10.0	93.4	4.5	5.0	95.1	3.2	5.0	96.3	3.9
		50.0	93.7	3.9	50.0	93.4	4.0	50.0	91.1	4.6

Recovery data obtained by analysis of tested samples fortified with standards of identified coumarins at three spiking levels. *RSD* – relative standard deviation calculated for seven individual preparations of samples, *LOD* – limit of detection.

were evaluated under the optimized chromatographic conditions using a mixture of coumarin standards. Method precision was tested for Sample II and recovery results were evaluated for sample extracts and analytes identified in tested samples. Recovery values for Sample II spiked with standards of nine coumarins at three concentration levels were higher than 88 % (data not shown).

Repeatability of the injection of a mixture of coumarin standards was satisfactory, with *RSD* lower than 0.2 % for retention times and lower than 1.7 % for peak areas (Tab. 2).

Analytical characteristics of UHPLC method including *LOD*, *LOQ*, linearity range and precision are summarized in Tab. 3. The calibration curves presented a satisfactory correlation between analyte concentration and peak area (coefficient of determination R^2, for all curves being higher than 0.982). *LOD*s and the *LOQ*s of tested compounds from the subclass of simple coumarins was 0.1–0.5 μg·ml^{-1} and 0.3–1.5 μg·ml^{-1} for UV detection, and 0.1–3 ng·ml^{-1} and 0.3–10 ng·ml^{-1} for FL detection, respectively.

The developed method with UV detection provided *LOD*s comparable to other reported methods [7, 12]. The use of the more sensitive FL detection had been previously published only for determination of umbelliferone and 4-hydroxycoumarin. The *LOD* value for umbelliferone in the presented study is lower than the value reported for Noni fruit samples utilizing HPLC with FL detection [11]. The *LOD* values obtained were comparable to values reported previously for coumarin derivatives using UHPLC with MS detection [16]. Method precision was checked from the seven preparations of spiked Sample II and triplicate injections of each under the working conditions. The interday *RSD* values were less than 4 %.

The efficiency of extraction was determined based on the recovery of coumarins from spiked plant and propolis samples. The values of recovery ranged from 88 % to 98 % with *RSD*s lower than 5.0 % (Tab. 4).

The change of column temperature (± 2 °C) and detection wavelength (± 2 nm) was used for evaluation of robustness of the method. The peak resolution remained similar despite the different conditions.

Analysis of real samples

The samples of a medicinal herb *Meliloti herba* (Sample I) and propolis (tincture without alcohol Sample II and crude propolis Sample III) were selected to demonstrate the applicability of UHPLC method for analysis of real samples. Pharmaceutical studies revealed that the important biologically active compounds of plant and propolis are coumarins, primarily those from the subclass of simple coumarins [15, 17, 18].

Two of coumarins, umbelliferone and scoparone, were identified in tested propolis samples (Tab. 4). Since UV detection was less selective and sensitive, FL detection was used as a better alternative for detection of umbelliferone and scoparone. Content of coumarins in Sample II

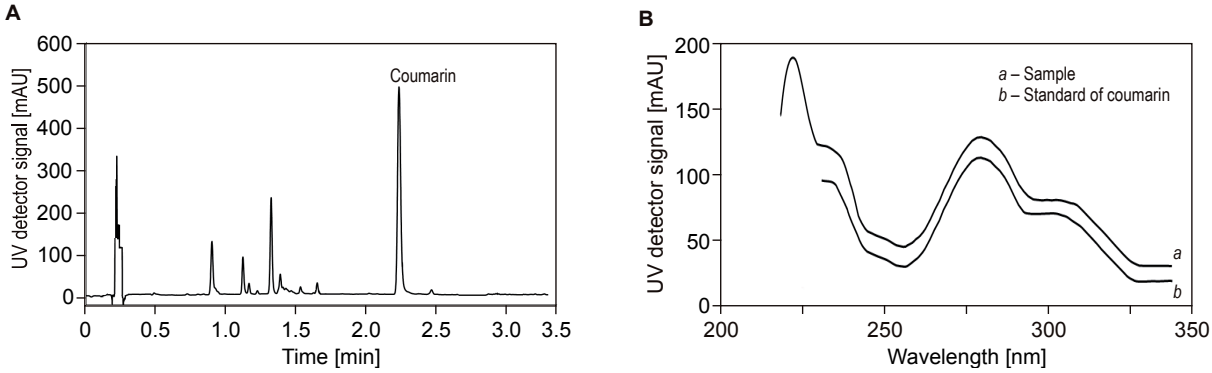

Fig. 3. *Meliloti herba* extract analysis.

A – Chromatogram obtained on phenyl-hexyl column using UV detection at a wavelength of 280 nm, B – UV spectrum of coumarin.

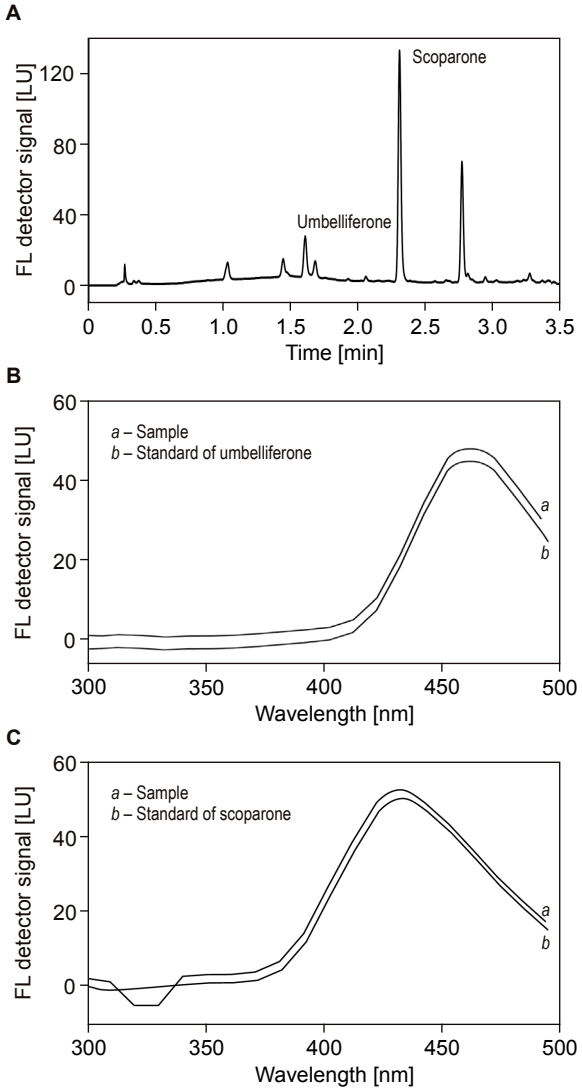

Fig. 4. Crude propolis extract analysis.

A – Chromatogram obtained on phenyl-hexyl column using fluorescence detection (excitation wavelength 320 nm, emission wavelength 450 nm), B – Fluorescence spectrum of umbelliferone, C – Fluorescence spectrum of scoparone.

(propolis tincture without alcohol) was found to be significantly lower than in the crude propolis sample, which was probably connected with the preparation of this commercial product. The procedure applied for preparation of Sample III (crude propolis) was typical for preparation and use of propolis tincture in traditional folk medicine. The presence of coumarins in propolis samples could have varied since its chemical composition depends on the provenance of the sample, flora surrounding the hive, climatic and geographic characteristics of the site, bee species and other factors [19, 20]. Coumarin was determined in tested sample of *Meliloti herba* (family *Fabaceae*) (Tab. 4). Simple coumarins are bioactive compounds naturally occuring also in many other plant families, for example *Apiaceae* and *Rutaceae* [21]. The content of other coumarin derivatives under study in tested real samples were lower than *LOD*. Chromatograms of herbal plant (Sample I) and propolis (Sample III) extracts are shown in Fig. 3A and Fig. 4A, respectively. Coumarins in tested samples were identified on the basis of comparison of their retention factors, UV spectra (Fig. 3B) and/or FL spectra (Fig. 4B and Fig. 4C) using spectra of standards.

CONCLUSION

In summary, validation and applicability of UHPLC method for simultaneous separation of coumarin, its metabolites and related compounds was presented. Octadecyl and phenyl-hexyl silica-based analytical columns packed with 1.8-μm particles were tested for separation performance. The mobile phase and gradient profile for both columns were identical and contained 0.3% aqueous acetic acid/acetonitrile (9:1) and aceto-

nitrile. Better results, from the aspect of peak resolution and peak symmetry, were obtained for phenyl-hexyl column, and this column was subsequently applied for analysis of real samples. The developed method was rapid, precise, accurate, sensitive and suitable for analysis of plant and propolis extracts, where three compounds (coumarin in plant sample, umbelifferone and scoparone in propolis samples) out of the set of nine analytes were detected and quantified. The more sensitive approach with fluorescence detection facilitated the detection of certain analytes at very low concentration levels. Values of *LOD* for selected coumarins were in the range of nanograms per millilitres for FL detection and in the range of micrograms per millilitres for UV detection. The method could be a useful tool in medicinal chemistry applications, since there is currently an interest in characterizing the bioactive compounds in natural products used in traditional folk medicine.

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