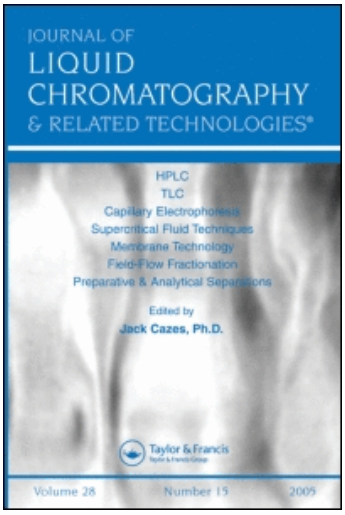

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Determination of Some Phenolic Acids in Propolis by an HPLC Method

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Abstract: The liquid chromatographic method was developed for determination of some organic acid in ethanol and water extracts of propolis from Slovakia. Analysis of chlorogenic acid, *p*-hydroxybenzoic acid, and rosmarinic acid was realized with the chromatographic column Symmetry Shield RP18 type and using a gradient of mobile phase methanol-water (pH = 2.5 adjusted with formic acid) and spectrophotometric and fluorescence detection (for *p*-hydroxybenzoic acid). Chlorogenic acid in the range from 2.7 to 56.6 μg/g and *p*-hydroxybenzoic acid from 3.9 to 150.3 μg/g was determined in tested propolis samples. Rosmarinic acid concentration in all tested propolis samples was below the limit of detection in this method. The significant differences in the acids concentrations were observed for propolis samples from east and west of Slovakia. It is probably the effect of different conditions of the collection of the resin and secrets by bees.

Keywords: HPLC, Propolis, Chlorogenic acid, *p*-Hydroxybenzoic acid, Rosmarinic acid

INTRODUCTION

Propolis is a resinous substance collected by bees from various tree buds. Bees use propolis for coating hive parts and to seal cracks and crevices in the hive.

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Propolis composition is directly related to that of bud exudates collected by bees from various trees; poplar, birch, beech, horse chestnut, alder, and various conifers. The mechanism of propolis origin is not reliably explained. The composition of propolis and its properties depend on the local floral and climatic conditions of the collection of resin and secrets by bees. Chemical composition of different samples of propolis can be changed depending on the source of propolis. On the other hand, the different samples of propolis always contain the same chemical components, independently of the plant origin.[1–3] Whereas for the determination of the geographic origin of propolis the pollen analysis is used for the determination of the botanical origin of propolis, the analytical methods (TLC, GC, HPLC, CE, and MS) are used.[4–7] HPLC represents the most popular and reliable analytical technique for the characterization of phenolic acids and flavonoids, while GC with the electron-capture detector or MS detection is suitable for nonflavonoid compounds including aliphatic and aromatic compounds.[8–13]

Raw propolis is composed of 50% resin (composed of flavonoids, and related phenolic acids), 10% essential oils, 30% wax, 5% pollen, and 5% other organic compounds.[14] More than 300 compounds, mainly polyphenols, have been identified as a constituent of propolis from different sources, and due to the modern analytical methods new compounds are identified. Flavonoids, aromatic acids, diterpene acids, and phenolic compounds appear to be the principal components responsible for the biological activities of propolis. Propolis, used extensively in folk medicine, has been reported to possess various biological activities, such as antibacterial, antifungal, antiviral, anti-inflammatory, local-anesthetic, antioxidant, and immunostimulating.[3,7,14–17]

Propolis cannot be used as a raw material, it must by purified before analysis by extraction with suitable solvents (ethanol, methanol, acetone, hexane, chloroform, water). Extraction with ethanol (70–100%) is suitable for obtaining dewaxed propolis extracts rich in polyphenolic compounds. Extraction with pure water is used for obtaining extracts containing compounds very soluble in water.[4,18]

In the present work, analysis of four samples of propolis from two regions of Slovakia (water and ethanolic extracts) was investigated. The sensitive and simple HPLC method for determination of some phenolic acids studied till now was developed.

EXPERIMENTAL

Chemicals and Reagents

The standards of chlorogenic acid, *p*-hydroxybenzoic acid, and rosmarinic acid were obtained from ICN Biomedicals (USA). Acetonitrile, ethanol, and

methanol for HPLC (gradient grade) were provided by Merck (Germany). Acetic acid 99% p.a. and formic acid 99% p.a. were obtained from Mikrochem (Slovakia).

The samples of propolis were collected from the beehive before the winter season and were stored desiccated and in the dark until processing. The work was carried out on four samples of propolis collected in Slovakia. The samples of propolis (I–IV) were harvested in the years 2003–2005.

Sample Preparation

Propolis Samples Collected from East and West of Slovakia:

Propolis from east of Slovakia (2005) (I)

Propolis (150 g) was extracted five days with 1000 mL of water, at 25°C to obtain the extract. After filtration, the extract was evaporated to dryness under vacuum. For HPLC analysis, 1 g of dry extract was diluted in 8 mL of water. This solution was purified by SPE (Pretreatment of Propolis Extract by SPE). A sample volume of 20 μL was injected and analyzed by HPLC. The liquid extraction procedure was repeated two times. The concentration of acids in the second extract below the limit of detection was observed.

Propolis from west of Slovakia (2005) (II)

Propolis (250 g) was extracted with 500 mL of pure ethanol for three days at 25°C. The extract was filtered. The solution was next cleaned by SPE (Pretreatment of Propolis Extract by SPE), and the volume of 20 μL was injected into the liquid chromatograph. The liquid extraction procedure was repeated two times. The concentration of acids in the second extract below the limit of detection was observed.

Propolis from east of Slovakia (2004 (III), 2003 (IV))

Propolis (150 g) was extracted with 500 mL of pure ethanol for three days at 25°C. The extract was centrifuged at 1000 × for 10 min. The obtained solution was cleaned by SPE (Pretreatment of Propolis Extract by SPE), and a volume of 20 μL was injected into the liquid chromatograph. The liquid extraction procedure was repeated two times. In the second extract, the concentration of acids below the limit of detection was observed.

Pretreatment of Propolis Extract by SPE

The water and ethanol extracts of propolis were cleaned by the SPE procedure before HPLC analysis. The SPE precolumns tested were Sep-Pak C18 (Waters, USA), Chromabond C18ec (Maherey-Nagel, Germany), and Oasis HLB (Waters, USA). The precolumns were conditioned with 3 mL of methanol, 3 mL of acidic water (pH = 2.5 with formic acid), and subsequently, 2 mL of water extract of propolis (or 2 mL of ethanolic extract diluted with water 1:3) was applied. The SPE precolumns were washed with 1 mL of acidic water (pH = 2.5 with formic acid) and phenolic acids were eluted with 1 mL of methanol. The volume of 20 μL was used for HPLC analysis on a reversed-phase column.

Instrumentation

Experiments were conducted on a Hewlett Packard (series 1100) HPLC system consisting of a quaternary pump equipped with an injection valve (Rheodyne), diode array detector, and thermostat. A Shimadzu model RF551 fluorescent detector was also used for detection. Chromatographic columns tested were Symmetry Shield RP18 (150 × 3.9 mm I.D., 5 μm) (Waters, USA), Reprosil 100 C18 (125 × 3 mm I.D., 5 μm) (Watrex, Slovakia), Chromolith Performance RP-18e (100 × 4.6 mm I.D.) (Merck, Germany), and guard columns Symmetry Shield RP18 (20 × 3.9 mm I.D., 5 μm) (Waters, USA) or Separon SGX C18 (10 × 4 mm I.D., 7 μm) (Watrex, Slovakia).

The mobile phases for separation of phenolic acids were a mixture of methanol or acetonitrile and water containing acetic acid (2%) or formic acid (pH = 2.5) with different gradient profiles.

All the separations were carried out at a flow rate of 0.5 mL/min and the column temperature was 25°C. The injection volume was 20 μL. For quantitative analysis, UV wavelengths of 255 nm for *p*-hydroxybenzoic acid and 330 nm for chlorogenic and rosemarinic acid were used. The fluorescence detector was operated at $\lambda_{Ex} = 265$ nm and $\lambda_{Em} = 350$ nm. The peak area of related compounds was used for quantitative calculations. The standards were dissolved in water, and filtered with a 0.45 μm filter when necessary.

RESULTS AND DISCUSSION

Polyphenols (including flavonoids, phenolic acids, and their esters), due to their proven ability to inhibit specific enzymes, to simulate some hormones and neurotransmitters and to scavenge free radicals, are considered to be the main pharmacologically active molecules in propolis.[19]

The aim of the work was to develop an HPLC method for determination of three, till now studied, active ingredients (chlorogenic acid, rosmarinic

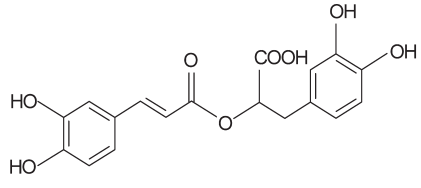
acid, *p*-hydroxyphenolic acid) in water and ethanolic extracts of propolis. Molecular structures of the studied acids are shown in Figure 1.

HPLC Separation

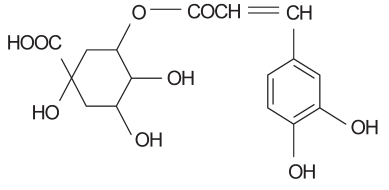
First, suitable chromatographic conditions - stationary phase, mobile phase, and type of detection, were selected. Next, the sample cleaning methods were tested.

For the purpose of the study, several columns with different stationary phases were tested. Reprosil 100 C18 column is a conventional reversed-phase end-capped silica based column monomeric covered with C_{18} groups (carbon content 17%). The packing of Symmetry Shield RP18 column is made by a sorbent with a high covering of silanol groups and reduction of trace metals content (carbon content 17%). The mobile phase with a high amount of water can be used for separation. The Chromolith Performance RP-18e column is made from a highly porous monolithic rod of silica with a bimodal pore structure providing a unique combination of macropores and mesopores.

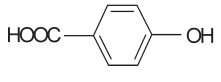
The mobile phase composed of methanol or acetonitrile and water (pH = 2.5 adjusted with formic acid) or 2% acetic acid with different gradient elution profiles was tested. The chlorogenic acid, rosmarinic acid, and *p*-hydroxyphenolic acid were separated on tested stationary and mobile



Rosmarinic acid



Chlorogenic acid



p-Hydroxybenzoic acid

Figure 1. Chemical structures of the studied acids.

phases. For all types of stationary phases (the same mobile phase and gradient), the effect on the time of analysis and symmetry of peaks were evaluated.

The best separation with the Reprosil 100 C18 column with guard column Separon SGX C18 was obtained using the mobile phase composed of acetonitrile - water (pH = 2.5 adjusted with formic acid) and gradient 0–5 min 25–40% acetonitrile, 5–10 min 40–70% acetonitrile. The run time of analysis was 11 min. The values of asymmetry were from 0.9 to 1.06.

The best separation with the Symmetry Shield RP18 (connect with guard columns Symmetry Shield RP18) was observed using gradient elution (0–5 min 25–50% methanol, 5–10 min 50–60% methanol, 10–15 min 60–90% methanol) of mobile phase methanol - water (pH = 2.5, adjusted with formic acid). The run time of analysis was 14 min and the values of asymmetry ranged from 0.96 to 1.22.

The Chromolith Performance RP-18e (with guard column Separon SGX C18) was tested thereafter with the same mobile phases, and enabled the faster analysis of all studied acids. The best separation occurred with acetonitrile and water (pH = 2.5, adjusted with formic acid) (gradient: 0–4 min 25–30% methanol, 4–6 min 30–40% methanol, 6-8 min 40-100% methanol, 8-14 min 100% methanol) as mobile phase. The run time of analysis was 13 min. The values of asymmetry were from 0.89 to 1.10.

A comparison of analyses performed with the above mentioned C_{18} columns by use of suitable mobile phases are shown in (Figure 2).

Final Conditions

A Symmetry Shield RP18 column connected with a guard column packed with the same sorbent, mobile phase composed of methanol-water (pH = 2.5, adjusted with formic acid) with gradient elution (0–5 min 25–50% methanol, 5–10 min 50–60% methanol, 10–15 min 60–100% methanol) at a flow rate 0.5 mL/min was finally used for the chromatography of selected phenolic acids in the propolis samples. The chlorogenic acid, *p*-hydroxybenzoic acid, and rosmarinic acid (trans isomers) were separated from the transformation product of rosmarinic acid (*cis* isomer) and the obtained retention factors were 2.95 for chlorogenic acid, 3.25 for p-hydroxybenzoic acid, 4.59 for *cis*-rosmarinic acid, and 4.91 for *trans*-rosmarinic acid. The resolution for chlorogenic acid/*p*-hydroxybenzoic acid was 2.8, for *p*-hydroxybenzoic acid/rosmarinic acid/(*cis*) 8.9 and cis/trans rosmarinic acid 2.7, respectively (shown in Table 1).

Detection

Detection wavelength was chosen according to absorbance spectra of all separated compounds. The chromatograms of analyte separation were

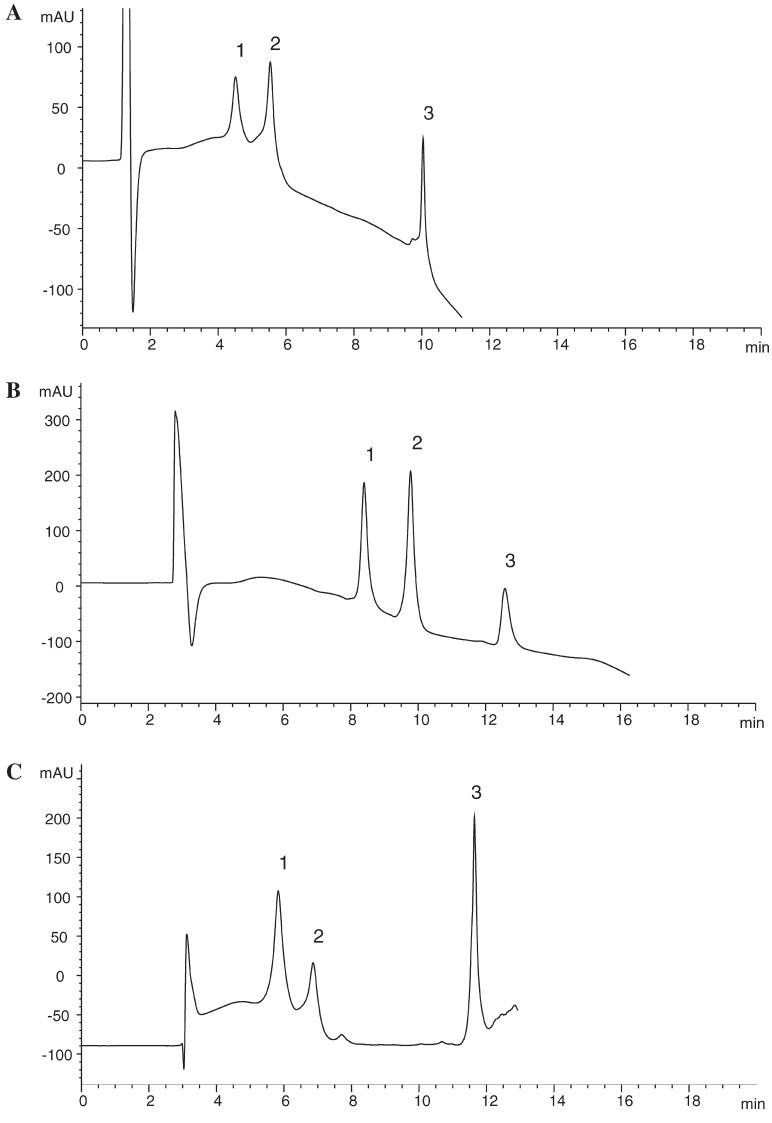


Figure 2. HPLC chromatograms for the separation of standard solution of phenolic acids (chlorogenic-1, *p*-hydroxybenzoic-2, *trans*-rosmarinic-3) on Reprosil 100 C18 (A), Symmetry Shield RP18 (B), and Chromolith Performance RP-18e (C) stationary phases. Chromatographic conditions: see text, flow rate: 0.6 mL/min.

evaluated at the wavelength of maxima of UV spectra (330 nm for chlorogenic and rosmarinic acid, 255 for *p*-hydroxybenzoic acid). The suitable wavelength for all three studied phenolic acids was 230 nm. Due to the interferences in propolis samples, the fluorescence detection was tested for *p*-hydroxybenzoic

Table 1. Method validation results of chlorogenic acid, *p*-hydroxybenzoic acid and rosmarinic acid

Parameter	Chlorogenic acid	*p*-Hydroxy-benzoic acid	Rosmarinic acid
Repeatability-t_R (%)[a]	1.5	1.5	1.3
Repeatability-A (%)[a]	0.80	0.73	0.92
Theoretical plates[b]	8665	12317	20960
Resolution[b]	2.8	8.9	2.7 (*cis-trans*)
Asymmetry[b]	1.14	1.03	0.95
Precision RSD (%)[c]	1.69	1.55	1.78
Linearity (r)	0.9996[d]	0.9992[e] 0.9988[f]	0.9985[d]
Accuracy RSD (%)[c]	2.30	2.58	2.62
LOD	0.17 μg/mL[d]	0.18 μg/mL[e] 0.35 μg/mL[f]	0.18 μg/mL[d]
LOQ	0.51 μg/mL[d]	0.53 μg/mL[e] 1.06 μg/mL[f]	0.55 μg/mL[d]

[a]Made in six replicates.
[b]Made in three replicates.
[c]Six samples injected three times each.
[d]UV 330 nm.
[e]UV 255 nm.
[f]FL λ_{Exc} 265 nm λ_{Em} 350 nm.

acid. The appropriate excitation and emission wavelengths were 265 nm and 350 nm.

The response of the UV detector at 330 nm (for chlorogenic acid and rosmarinic acid) and 255 nm for *p*-hydroxybenzoic acid and fluorescence detector at 265 nm (exc) and 350 nm (em) (for *p*-hydroxybenzoic acid) showed a good linearity for standard solutions in the concentration range 0.51 μg/mL–50.0 μg/mL for chlorogenic acid, 0.55 μg/mL–50.0 μg/mL for rosmarinic acid, 0.53 μg/mL–50.0 μg/mL (spectrophotometric detection), 1.06 μg/mL–50.0 μg/mL (fluorescence detection) for *p*-hydroxybenzoic acid. The regression equation had correlation coefficients over 0.998.

Figure 3 shows the chromatograms of separation and UV spectra of phenolic acids (chlorogenic, *p*-hydroxybenzoic, rosmarinic) using reversed-phase column with photodiode array and fluorescence detection.

Sample Preparation

The water and ethanolic extracts of propolis samples were purified and preconcentrated by solid phase extraction. Three types of sorbents were used

for the sample cleanup, C_{18} cartridge (Sep-Pak C18), end-capped C_{18} cartridge (Chromabond C18ec), and cartridge with hydrophilic lipophilic properties (Oasis HLB). For the tested elution media, the suitable elution conditions were determined. Two elution agents were tested: methanol–water

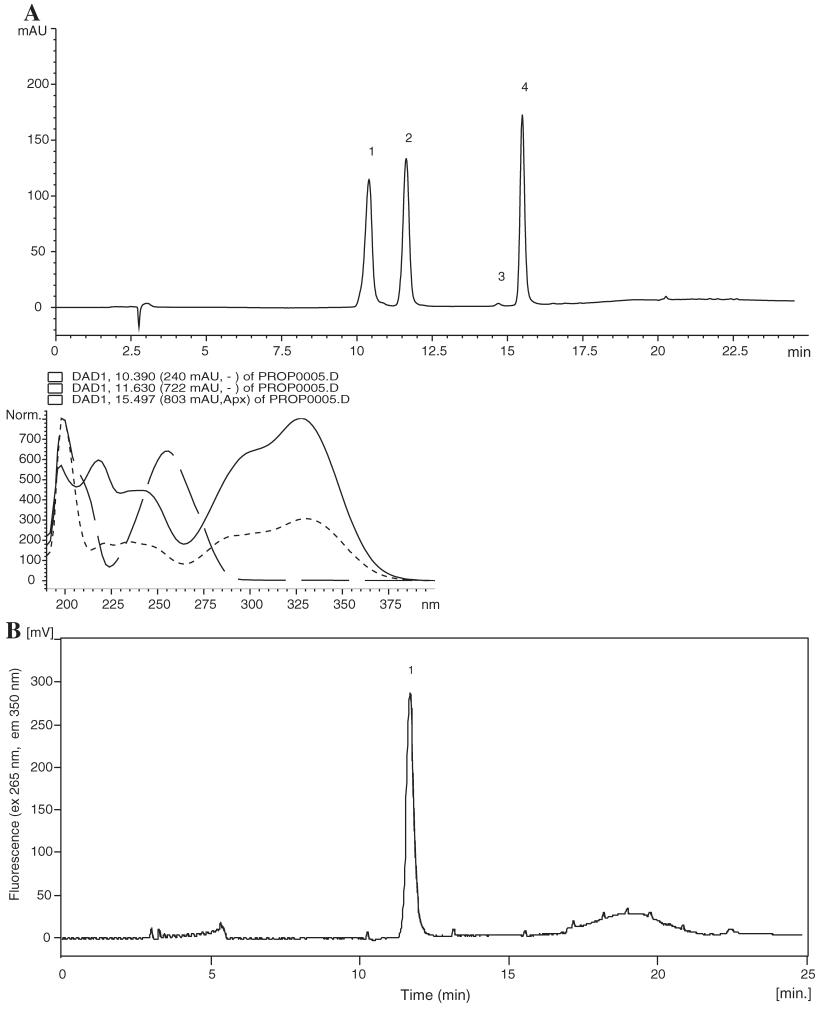


Figure 3. HPLC chromatograms and UV spectra for the separation of standard solution of phenolic acids (chlorogenic-1, *p*-hydroxybenzoic-2, *cis*-rosmarinic-3-rosmarinic-4) using a reversed-phase column with spectrophotometric detection (A) and fluorescence detection (B). Chromatographic conditions: Symmetry Shield RP18, mobile phase: methanol-water (pH = 2.5) with gradient elution (0–5 min 25–50% methanol, 5–10 min 50–60% methanol, 10–15 min 60–100% methanol), flow rate 0.5 mL/min, UV detection at 230 nm (A), FL detection at 265 nm (ex) 350 nm (em) (B).

Table 2. Recovery of phenolic acids for different sorbents

Analyte	Recovery (%)		
	Chromabond C18ec	Waters SEP-Pak C18	Oasis HLB
Chlorogenic acid	14.5 ± 0.2	15.0 ± 0.1	75.7 ± 0.4
p-Hydroxybenzoic acid	23.8 ± 1.2	23.0 ± 0.3	99.0 ± 0.2
Rosmarinic acid	40.1 ± 1.3	63.5 ± 0.6	98.0 ± 1.3

Made in three replicates.

pH = 2.5 (adjusted with formic acid) (80:20) and methanol. The yields of phenolic acids from water with the standard addition of studied compounds (concentration 30 μg/mL) obtained with the use of these elution media were evaluated. In the case of methanol–water pH = 2.5 (80:20) as elution medium, poor recovery was achieved (in the interval 10–50% depending on the sorbent). To obtain recovery more than 70%, the increase of volume of elution medium was necessary (min. three times - 3–5 mL of methanol–water pH = 2.5 (80:20)). The results of recovery obtained for elution medium pure methanol for tested sorbents are summarized in Table 2. The higher values of recovery were obtained using sorbent made of macroporous copolymer of lipophilic divinylbenzene and hydrophilic *n*-vinylpyrolidone (Oasis HLB) (75–99%). This type of cartridge was used for pretreatment of water extract of propolis samples. In the case of ethanol extract of propolis, the very low extraction recoveries were obtained for all tested sorbents and elution media (less than 5%). For that reason, the extracts were diluted with water (1:3). The recoveries obtained by using Oasis HLB sorbent were in the interval of 70–95%.

Table 3. Assay results for chlorogenic acid and *p*-hydroxybenzoic acid in propolis samples from Slovakia

Propolis	Chlorogenic acid (μg/g)	*p*-Hydroxybenzoic acid (μg/g)
I	2.7 ± 0.3	3.9 ± 0.2
II	56.6 ± 3.1	108.2 ± 5.2
III	4.2 ± 0.3	45.3 ± 2.6
IV	16.5 ± 0.8	150.3 ± 5.5

Made in three replicates.

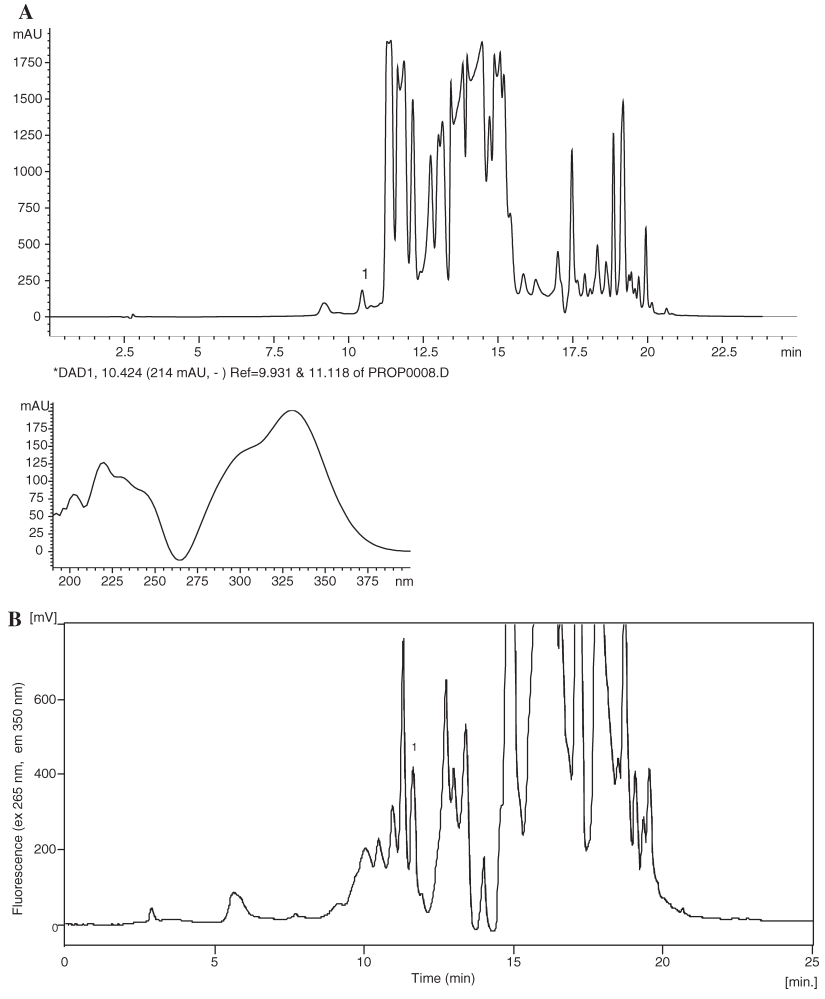


Figure 4. HPLC chromatograms and UV spectra for the separation of water extract of propolis (I) using a reversed-phase column with spectrophotometric detection (chlorogenic acid-1) (A) and fluorescence detection (*p*-hydroxybenzoic acid-1) (B). Chromatographic conditions: Figure 3.

Method Validation

The developed method was validated. The parameters of the suitability of the method (repeatability of elution times and peak areas, number of theoretical plates, resolution, and asymmetry) and validation parameters (precision, linearity, accuracy, limit of detection, and determination) were examined.

Linearity of the detection response was determined at eight different concentrations. Accuracy of the method was determined by using a water and ethanol solution of acids at concentration 30 μg/mL of each studied phenolic acid. The limit of detection (LOD) and the limit of quantification (LOQ) were determined. Limit of detection was measured as the lowest amount of the analyte that may be detected to produce a response, which is significantly different from that of a blank (S/N = 3). Limit of quantitation was measured as the lowest amount of analyte that can be reproducibly quantified above the baseline noise (S/N = 10). The method validation results obtained for final separation and detection conditions are shown in Table 1.

Determination of Acids in Propolis

Propolis or "bee glue" is a generic name for the resinous hive product collected by bees from various plant sources. Propolis usually contains a variety of chemical compounds, such as polyphenols (flavonoids, phenolic acids, and their esters), terpenoids, steroids, aromatic alcohols, aliphatic acids and esters, sugars, and amino acids,[1–7] and its composition depends on the vegetation at the site of collection.

The four samples of propolis (water or ethanol extracts) from Slovakia were analysed by the gradient reversed-phase HPLC method with on-line spectrophotometric and fluorescence detection. The chromatograms of the samples indicate the presence of chlorogenic acid in the range from 2.7 to 56.6 μg/g and *p*-hydroxybenzoic acid from 3.9 to 150.3 μg/g. Rosmarinic acid concentration in all tested propolis samples was below the detection limit of the method used. The results are shown in Table 3. It is clear that the significant differences in the acid concentrations were obtained for propolis samples from the east (samples I, III, IV) and west (sample II) of Slovakia. It is probably the effect of different local floral and climatic conditions of the collection of resin and secrets by bees. Figure 4 shows the chromatograms of the water extract of propolis obtained by HPLC methods.

CONCLUSION

The HPLC-DAD and HPLC-DAD-FL developed methods are suitable and valid for the determination of groups of phenolic acids (chlorogenic acid, *p*-hydroxybenzoic acid, rosmarinic acid) in propolis samples obtained from the east and west of Slovakia.

The chromatographic column Symmetry Shield RP18, gradient of mobile phase (methanol–water pH = 2.5), and spectrophotometric and fluorescence detection was used for analysis of chlorogenic acid, *p*-hydroxybenzoic acid, and rosmarinic acid. In propolis samples, chlorogenic and *p*-hydroxybenzoic

were determined. The differences in the concentration of studied phenolic acids were observed for propolis samples from the east and west of Slovakia.

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