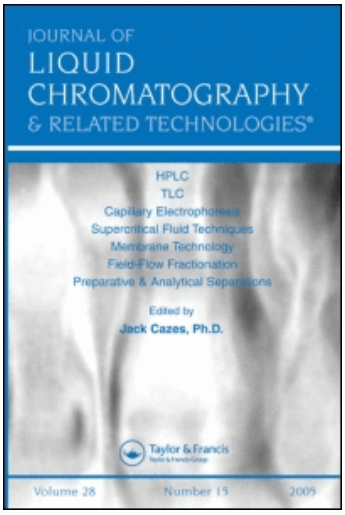

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Determination of Organic Acids in Propolis by HPLC Using Two Columns with an On-Line SPE System

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Abstract: The HPLC method consisting of two columns and an on-line SPE system was developed for analysis of propolis extracts from Slovakia. The IEC column with spectrophotometric detection was tested for the separation of acids of the "shikimate pathway" and the C_{18} column with on-line spectrophotometric (chlorogenic, rosmarinic, *p*-hydroxybenzoic acids) and fluorimetric (*p*-hydroxybenzoic acid) detection was tested for separation and determination acids in the water extract of propolis. For the preconcentration of compounds the on-line SPE on the C_{18} preseparation guard column was used. The limits of determination were $0.2\,\mu g \cdot mL^{-1}$ for shikimic acid, $20\,\mu g \cdot mL^{-1}$ for quinic acid, $0.3\,\mu g \cdot mL^{-1}$ for chlorogenic acid, $0.5\,\mu g \cdot mL^{-1}$ for rosmarinic acid, $0.3\,\mu g \cdot mL^{-1}$ for *p*-hydroxybenzoic acid (UV), and $2\,\mu g \cdot mL^{-1}$ for p-hydroxybenzoic acid (FL). On the basis of chromatographic characteristics and optical properties (UV spectra) chlorogenic acid, quinic acid, and shikimic acid were characterized in tested samples of propolis. The *p*-hydroxybenzoic acid could not be determined in the propolis extract because the interferences of unknown compounds with the same retention factor occur.

Keywords: Column liquid chromatography, Propolis, Organic acids

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INTRODUCTION

Propolis or bee glue, is a dark colored resinous substance collected by honeybees from leaf buds and cracks in the bark of various tree species. The plant origin of propolis determines its chemical diversity. Bee glue's chemical composition depends on the species of local flora present at the site of collection and, thus, in the geographic and climatic characteristics at the site. Raw propolis is composed of 50% resin (composed of flavonoids and related phenolic acids), 30% wax, 10% essential oils, 5% pollen, and 5% other organic compounds (terpenoids, steroids, aromatic alcohols, aliphatic acids and esters, sugars, amino acids, etc.). More than 300 compounds, mainly polyphenols, have been identified as constituents of propolis. Propolis has been used extensively in folk medicine since it possesses various biological activities, such as antiseptic, antifungal, antibacterial, antiviral, anti-inflammatory, anaesthethic, and antioxidant properties.[1–4] Propolis samples are complex mixtures containing a variety of compounds in different amounts. Their analysis by conventional chromatographic techniques, such as LC, is challenging. HPLC represents the most popular and reliable analytical technique for the characterization of organic acids in propolis. In most methods, HPLC is coupled with mass spectrometry, spectrophotometry, or photodiode array detection.[5–8]

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 farmacologically active molecules in propolis.[9] Quinic and shikimic acid are intermediates of the biochemically important "shikimate pathway". They are the precursors of aromatic compounds in plants and microorganisms.[10]

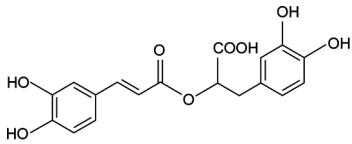
The aim of the work was analysis of five active ingredients (chlorogenic acid, rosmarinic acid, *p*-hydroxybenzoic acid, quinic acid, and shikimic acid) (Figure 1) in the water extract of propolis by HPLC with coupled C_{18} and IEC columns and on-line SPE pretreatment.

EXPERIMENTAL

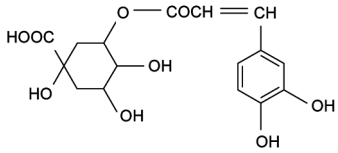
Materials

The standards of chlorogenic acid, *p*-hydroxybenzoic acid, rosmarinic acid, shikimic acid, and quinic acid (Figure 1) were obtained from ICN Biomedicals (Irvine, CA, USA).

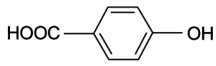
Methanol for HPLC (gradient grade) was purchased from Merck (Darmstadt, Germany). Sulphuric acid p.a. 96% was obtained from Mikrochem (Bratislava, Slovakia).



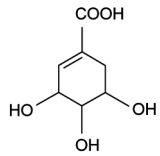
Rosmarinic acid



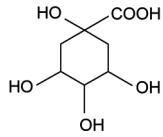
Chlorogenic acid



p-Hydroxybenzoic acid



Shikimic acid



Quinic acid

Figure 1. Chemical structures of the studied acids.

The sample of propolis was collected from the beehive before the winter season and was stored, desiccated and in the dark, until processing. The sample of propolis was harvested in the year 2006 from East of Slovakia.

Sample Preparation

Powdered raw propolis (150 g) was extracted by shaking on laboratory mixer for 7 days with 1000 mL of water, at temperature 25°C to obtain the water extract. After filtration of nonsoluble part of propolis, the extract was evaporated to dryness under vacuum at temperature 25°C (we obtained the constant weight of 2.50 g of the dry part of extracted compounds). For HPLC analysis, 1 g of dry extract was diluted in 8 mL of water. A sample volume of 20 μL was injected and analyzed by HPLC.

Apparatus and Chromatographic Conditions

Experiments were conducted on Agilent Technologies (Waldbronn, Germany) series 1100 HPLC system consisting of a quaternary pump equipped with an injection valve model 7125 (Rheodyne, Cotati, CA, USA), diode array detector, and thermostat. Shimadzu models RF551 fluorescent detector (Tokyo, Japan), SPD-10A spectrophotometric detector (Shimadzu, Tokyo, Japan) and pump LC-10AD (Shimadzu, Tokio, Japan) were also used. Chromatographic columns were Symmetry Shield RP18 (150×3.9 mm I.D., 5 μm) (Waters, Milford, MA, USA), column A, and Polymer IEX H-form (250×8 mm I.D., 8 μm) (Watrex, Bratislava, Slovakia), column B. A Preseparation guard column used was Symmetry Shield RP18 (20×3.9 mm I.D., 5 μm) (Waters, Milford, MA, USA). The mobile phase for separation of phenolic acids on column A was a mixture of methanol and water containing sulphuric acid ($9\,\text{mmol} \cdot \text{L}^{-1}$) with gradient profile (0–5 min 25–50% methanol, 5–10 min 50–60% methanol, 10–15 min 60–100% methanol). The mobile phase for separation of quinic acid and shikimic acid on column B was sulphuric acid ($9\,\text{mmol} \cdot \text{L}^{-1}$). The scheme of the column system is shown in Figure 2. The sample was injected (injection valve I) on the preseparation guard column conditioned with sulphuric acid ($9\,\text{mmol} \cdot \text{L}^{-1}$) as mobile phase. Quinic acid and shikimic acid was subsequently separated on column B with mobile phase for column B (The injection valve II was in the position for the guard column to be connected with column B.) After 5 min of sample injection on the guard column, the injection valve II was turned to the position in which the guard column was connected

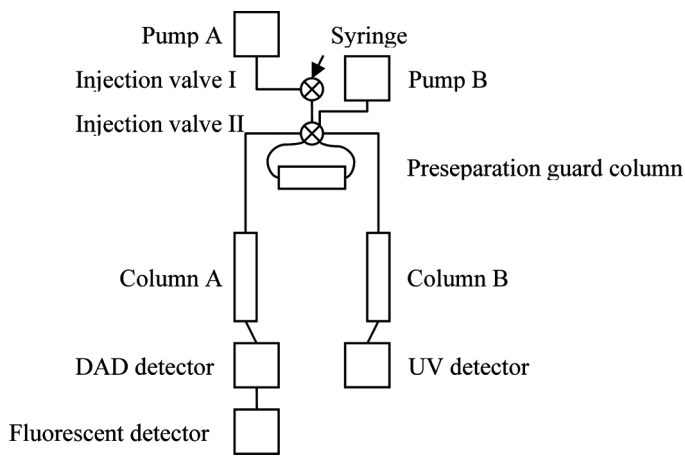


Figure 2. Sketch of the column system.

with column A and phenolic acids were eluted from the guard column with gradient of mobile phase for column A. All the separations were carried out at a flow rate of $0.6\,mL \cdot min^{-1}$ and the column temperature was 25°C. The injection volume was 20 μL. The monitored wavelengths were 255 nm for *p*-hydroxybenzoic acid, 330 nm for chlorogenic and rosemarinic acid, 210 nm for quinic and shikimic acid. The fluorescence detector was operated at $\lambda_{Ex} = 265\,nm$ and $\lambda_{Em} = 350\,nm$. The stock solutions were prepared by dissolving standards substances in water to obtain a concentration of $10\,mg \cdot mL^{-1}$ and filtered with a 0.45 μm filter when necessary.

RESULTS AND DISCUSSION

HPLC Separation

In our previous work,[11,12] we presented the separation and determination of some phenolic acids and acids of the "shikimate pathway" by individual HPLC methods with off-line SPE pretreatment.

In the present work, we attempt to analyze selected acids in one run on the coupled LC columns system containing RP18 and IEC columns in parallel connection. The main objective of coupled column LC system was to increase the number of the sample compounds that can be separated in a single run.[13] The problem was compatibility of mobile phases. The mobile phases used in individual methods described above was not suitable because the tailing of peaks was observed (values of asymmetry were higher then 1.5 for all peaks). The mobile phase recommended by the manufacturer of the IEC stationary phase is sulphuric acid $5–20\,mmol \cdot L^{-1}$ with addition of a maximum 10% of methanol or acetonitrile. Since the mobile phases with higher content of organic modifier is not suitable for the IEC stationary phase, we can not use the connection of RP18 and IEC column in step by step for analysis of the studied compounds. For this reason the mobile phase for the RP18 column was adjusted to the mobile phase for the IEC column and was modified with sulphuric acid instead formic acid. The suitable mobile phase for separation of phenolic acids on the RP18 column was a mixture of methanol and water containing sulphuric acid ($9\,mmol \cdot L^{-1}$) with gradient profile as is presented in the Experimental section. The mobile phase for separation of quinic and shikimic acid on the IEC column was sulphuric acid ($9\,mmol \cdot L^{-1}$) without organic modifiers, because the presence of methanol in mobile phase (5%) caused decreasing of retention of phenolic acids on the SPE RP18 preseparation guard column.

The RP18 preseparation guard column placed on-line before the analytical columns was used for the pretreatment of analytes from the

water extract of propolis. After the sample injection on the guard column, the quinic acid and shikimic acid were not retained and they were eluted directly on the IEC column (mobile phase $9\,mmol \cdot L^{-1}$ sulphuric acid). The phenolic acids were retained on the top of the RP18 preseparation guard column and after washing with $9\,mmol \cdot L^{-1}$ sulphuric acid (5 min) the phenolic acids were eluted with the gradient of mobile phase on the RP18 analytical column in back-flush mode. The break through volumes of the phenolic acids under study were determined in water at a spiking compound level $10\,\mu g \cdot mL^{-1}$ by using a UV detector operated at 255 nm for *p*-hydroxybenzoic acid and 330 nm for chlorogenic acid and rosmarinic acid, at flow rate $0.6\,mL \cdot min^{-1}$. Figure 3 demonstrated the typical break through curves of phenolic acid with break through after 23 mL for chlorogenic acid and 6 mL for *p*-hydroxybenzoic acid. The break through volume of rosmarinic acid was higher than 43 mL.

As was presented in previous papers,[12] for the detection of *p*-hydroxybenzoic acid the spectrophotometric detector ($\lambda_{max} = 255\,nm$) was suitable. Due to the interferences in propolis samples, the fluorescence detection in on-line connection with DAD was tested for *p*-hydroxybenzoic acid. The appropriate excitation and emission wavelengths were 265 nm and 350 nm.

The typical chromatograms for a standard solution of the six analytes are shown in Figure 4. The total time of preseparation and analysis of acids on both parallel columns was 20 min ($k_{chlroregenic\ acid} = 1.68$, $k_{p\text{-hydroxybenzoi cacid}} = 2.08$, $k_{cis\text{-rosemarinic acid}} = 2.64$, $k_{trans\text{-rosemarinic acid}} = 2.90$, $k_{quinic\ acid} = 0.51$, $k_{shikimic\ acid} = 0.76$).

Analytical Data

For the validation of the employed analysis system and method, the parameters of suitability of 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. The results are presented in Table 1. The precision was measured for three days using spiked water extract of propolis (two concentration levels). The accuracy of the method was determined by replicate analysis of spiked water extract of propolis. The two concentration levels of quinic acid (0.25 and $2.5\,mg \cdot mL^{-1}$), shikimic acid (2.5 and $25\,\mu g \cdot mL^{-1}$), and chlorogenic acid, *p*-hydroxybenzoic acid, rosmarinic acid (3 and $10\,\mu g \cdot mL^{-1}$) in samples were tested by using six replicates. The precision in all cases was less than 6% and the accuracy was less than 5% for all studied analytes. Linearity of the detection response was determined at six different concentrations. The dependencies

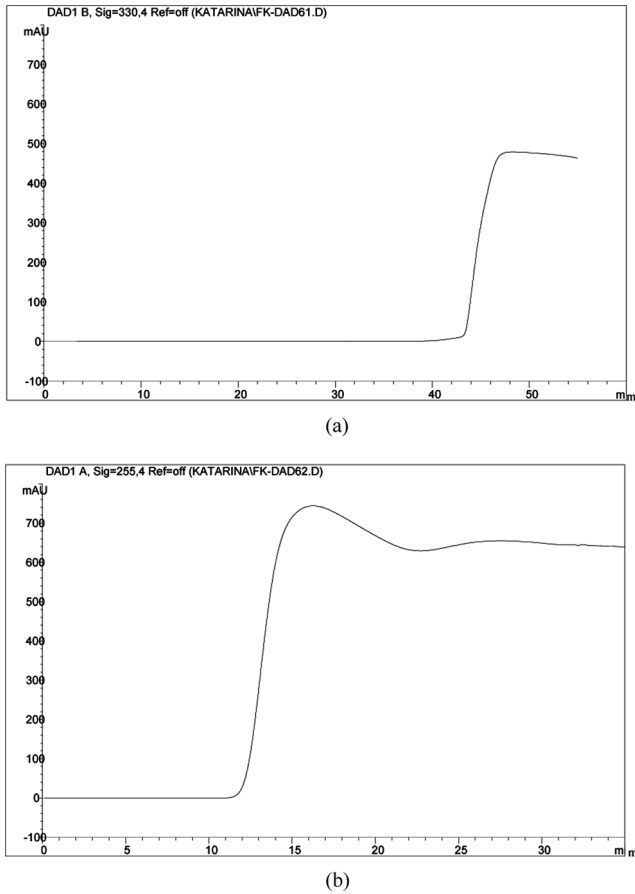


Figure 3. The break through curves of chlorogenic acid (a) and *p*-hydroxybenzoic acid (b). Chromatographic conditions: preseparation guard column Symmetry Shield RP18 ($20 \times 3{,}9$ mm I.D., 5 μm), water at a spiking level $10\,\mu g \cdot mL^{-1}$ of standards, flow rate $0.6\,mL \cdot min^{-1}$, UV detection at 255 and 330 nm.

of peak areas versus concentration of acids were linear with the correlation coefficients of 0.9983–0.9996 in the concentration range $0.5–100\,\mu g \cdot mL^{-1}$ for chlorogenic acid, *p*-hydroxybenzoic acid, rosmarinic acid, and shikimic acid, and $0.05–10\,mg \cdot mL^{-1}$ for quinic acid (Table 1). Limit of detection (LOD) was measured as the lowest amount of the analyte that may be detected to produce a response that is different from that of a blank ($S/N = 3$). Limit of quantification (LOQ) was measured as the lowest amount of analyte that can be reproducibly quantified above the baseline noise ($S/N = 10$).

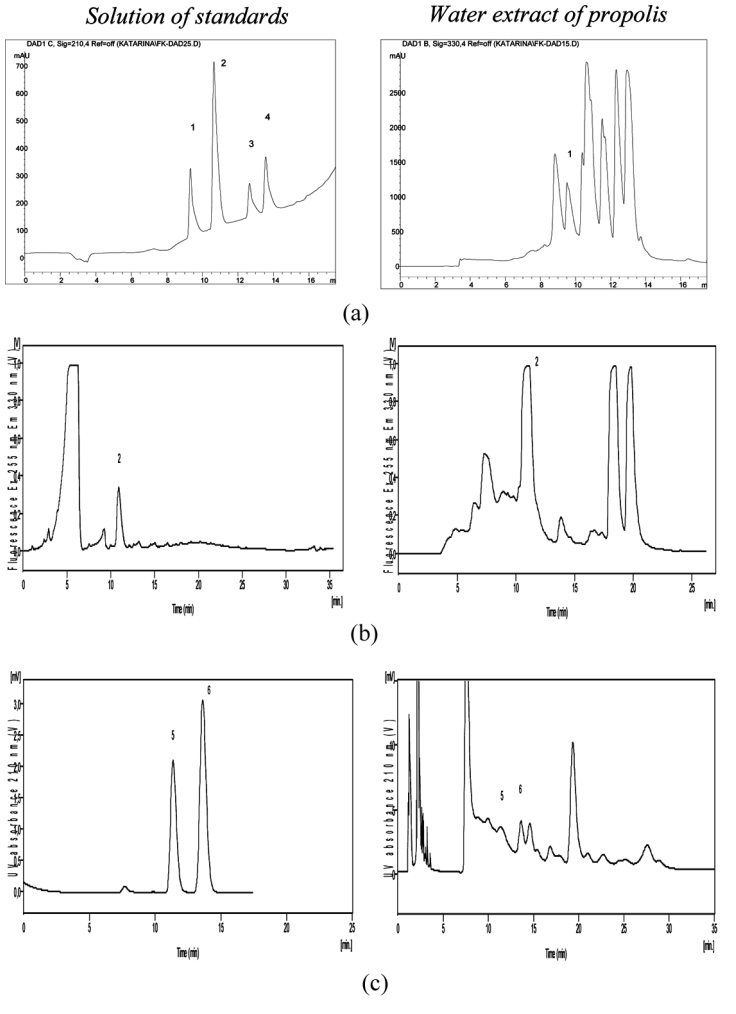


Figure 4. HPLC chromatograms of separation of standard solution of acids and water extract of propolis analyzed on RP18 column with photodiode array (a) and fluorescent (b) detector and IEC column with spectrophotometric detector (c). Column (a,b), Symmetry Shield RP18; mobile phase, methanol and water containing sulphuric acid ($9\,\text{mmol}\cdot\text{L}^{-1}$) with gradient profile (0–5 min 25–50% methanol, 5–10 min 50–60% methanol, 10–15 min 60–100% methanol); flow rate, $0.6\,\text{mL}\cdot\text{min}^{-1}$; detection, DAD (255 nm, 330 nm) FL ($\lambda_{Ex} = 265$ nm, $\lambda_{Em} = 350$ nm); temperature, 25°C. Column (c), Polymer IEX H-form; mobile phase, sulphuric acid ($9\,\text{mmol}\cdot\text{L}^{-1}$); flow rate, $0.6\,\text{mL}\cdot\text{min}^{-1}$; detection, UV (210 nm); temperature, 25°C. Preseparation guard column Symmetry Shield RP18.

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

Parameter	Chlorogenic acid	*p*-Hydroxy-benzoic acid	Rosmarinic acid	Quinic acid	Shikimic acid
Repeatability-t_R (%)[a]	2.7	2.9	2.5	1.7	1.5
Repeatability-A (%)[a]	2.8	2.7	2.9	1.5	1.7
Theoretical plates[b]	7981	8566	9409	3357	3973
Resolution[b]	2.86	5.08		2.76	
Asymmetry[b]	1.26	1.16	1.35	0.96	1.05
Precision RSD (%)[c]	5.89	5.85	5.78	5.10	4.92
Accuracy RSD (%)[c]	4.29	3.98	3.82	3.59	3.87
Linearity (r)	0.9992[d]	0.9992 0.9985	0.9983[d]	0.9995[g]	0.9996[g]
LOD	0.1 $\mu g \cdot mL^{-1}$[d]	0.1 $\mu g \cdot mL^{-1}$[e] 0.5 $\mu g \cdot mL^{-1}$[f]	0.2 $\mu g \cdot mL^{-1}$[d]	8 $\mu g \cdot mL^{-1}$[g]	0.1 $\mu g \cdot mL^{-1}$[g]
LOQ	0.3 $\mu g \cdot mL^{-1}$[d]	0.3 $\mu g \cdot mL^{-1}$[e] 2.0 $\mu g \cdot mL^{-1}$[f]	0.5 $\mu g \cdot mL^{-1}$[d]	20 $\mu g \cdot mL^{-1}$[g]	0.2 $\mu g \cdot mL^{-1}$[g]

[a]Made in six replicates.
[b]Made in three replicates.
[c]Three samples injected three times each.
[d]UV 330 nm.
[e]UV 255 nm.
[f]FL λ_{Exc} 265 nm λ_{Em} 350 nm.
[g]UV 210 nm.

Sample Analysis

On the base of preliminary tests, 1, 3, 5, 7, and 9 days were chosen as test extraction times of propolis with water. The yield of extraction was determined by analysis of quinic and shikimic acids in extracts. After 7 days of extraction, the concentration of studied acids in water extracts was constant.

Under the suitable conditions, chlorogenic acid, quinic acid, and shikimic acids were determined by HPLC in water extracts of propolis. The chromatograms of water extracts of propolis from Slovakia analyzed on the chromatographic system containing RP18 (a,b) and IEC column (c) with on-line SPE pretreatment are shown in Figure 4. The peaks were characterized by UV spectra and by addition of standards. The method with UV and alternative, fluorescent detection was not suitable for analysis of *p*-hydroxybenzoic acid in the tested propolis sample, because the interferences of unknown compounds with the same retention factor occurring (Figure 4b). The change of separation conditions did not improve the separation of *p*-hydroxybenzoic acid from unknown compounds.

Quantitative analysis of analytes was achieved by using the corresponding calibration curves. The concentration of chlorogenic acid was $3.7 \pm 0.3\,\mu g \cdot g^{-1}$, quinic acid $0.4 \pm 0.02\,mg \cdot g^{-1}$, and shikimic acid $5.8 \pm 0.5\,\mu g \cdot g^{-1}$ in the raw propolis. Rosmarinic acid concentration in the tested propolis sample was below the limit of determination of the used method.

CONCLUSIONS

The chromatographic system with the RP18 and IEC analytical columns in parallel connection with on-line SPE was established for the analysis of acids in the water extract of propolis. The limits of determination were about $\mu g \cdot mL^{-1}$ concentration level for all studied organic acids. The advantage of the present method, in comparison to individual separation methods, was reduction of total analysis time and simultaneous analysis of acids of different polarity at two chromatographic columns.

On the base of chromatographic characteristics and optical properties (UV spectra), chlorogenic acid, quinic acid, and shikimic acid were identified in tested samples of propolis. Rosmarinic acid concentration in the tested propolis sample was below the limit of detection of the used method.

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