

## EKSPERIMENTINIS TYRIMAS

### Evaluation of phenolic acids and phenylpropanoids in the crude drugs

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**Key words:** phenolic acids; phenylpropanoids; high-performance liquid chromatography; crude drug.

**Summary.** Phenolic acids and phenylpropanoids have an important biological activity and are therapeutic agents of crude drugs. Development of validated analysis techniques of these phytotherapeutic agents (fingerprinting and assay procedures) is an important practice for efficacy, safety, and quality control of herbal drug preparations. The aim of the present work was to study analytical capabilities of the evaluation of selected phenolic acids and phenylpropanoids: caffeic acid, chlorogenic acid, cinnamic acid, coumaric acid, ferulic acid, gallic acid, protocatechuic (3,4-dihydroxybenzoic) acid, rosmarinic acid, vanillic acid, and vanillin. Optimization and validation procedures of rapid and simple method of reversed-phase high-performance liquid chromatography were carried out. The mobile phase of the optimized chromatographic method consisted of methanol and 0.5% acetic acid solvent in water. For the application of method, two kinds of raw materials were chosen: propolis and the *Herba Origani*. Coumaric acid is the dominating phenolic acid of propolis (2785 µg/g). Results of analysis of *Herba Origani* demonstrated high quantities (6376 µg/g) of rosmarinic and protocatechuic (1485 µg/g) acids in the samples.

#### Introduction

Phenolic compounds are a wide group of substances that have particular importance in various aspects of scientific research, particularly for phytopharmacologists and phytochemists. Medical plants contain a large variety of phenolics. Phenolics, denominated as phenolic acids and phenylpropanoids, are derived from two nonphenolic molecules, benzoic and cinnamic acids, respectively. Many studies showed that phenolic acids and phenylpropanoids play a key role in antioxidative defense mechanisms in biological systems, exhibit health-promoting effects, and may have inhibitory effects on mutagenesis and carcinogenesis. Caffeic acid and gallic acid demonstrated strong antioxidant properties and act as free radical acceptors (1). The important biological activities of simple benzenoids – chlorogenic, caffeic, ferulic, gallic acids – are probably due to their cytoprotective activity and possible inhibitory effects on carcinogenesis, mutagenesis, and tumor genesis (2, 3). Phenolic acids and

phenylpropanoid compounds have showed an important antibacterial activity (4). In addition, natural phenolic compounds make a considerable contribution to the nutritional quality of fruits and fruit products, which play an important role in the daily diet (3).

The determination of phenolics is important both for their characterization in the drug and to facilitate more efficient uses of the important plant resources. Development of validated analysis techniques of these phytotherapeutic agents (fingerprinting and assay procedures) are an important practice for standardization and quality control of the herbal drugs used in clinical trials and efficacy, safety, and quality control of herbal drug preparations (5).

There are several methods for the determination of phenolic acids in herbal drugs. Various plants have been analyzed by thin-layer chromatography, high-performance liquid chromatography (HPLC), and capillary electrophoresis (3, 4, 6–9). The most reliable and applicable methods for the routine determination

of phenolics in herbal drugs are HPLC methods.

The aim of the present work was to study analytical capabilities of the HPLC separation of selected phenolic acids and phenylpropanoids: caffeic acid, chlorogenic acid, cinnamic acid, coumaric acid, ferulic acid, gallic acid, protocatechuic (3,4-dihydroxybenzoic) acid, rosmarinic acid, vanillic acid, and vanillin. The structures of analysed compounds are presented in Fig. 1.

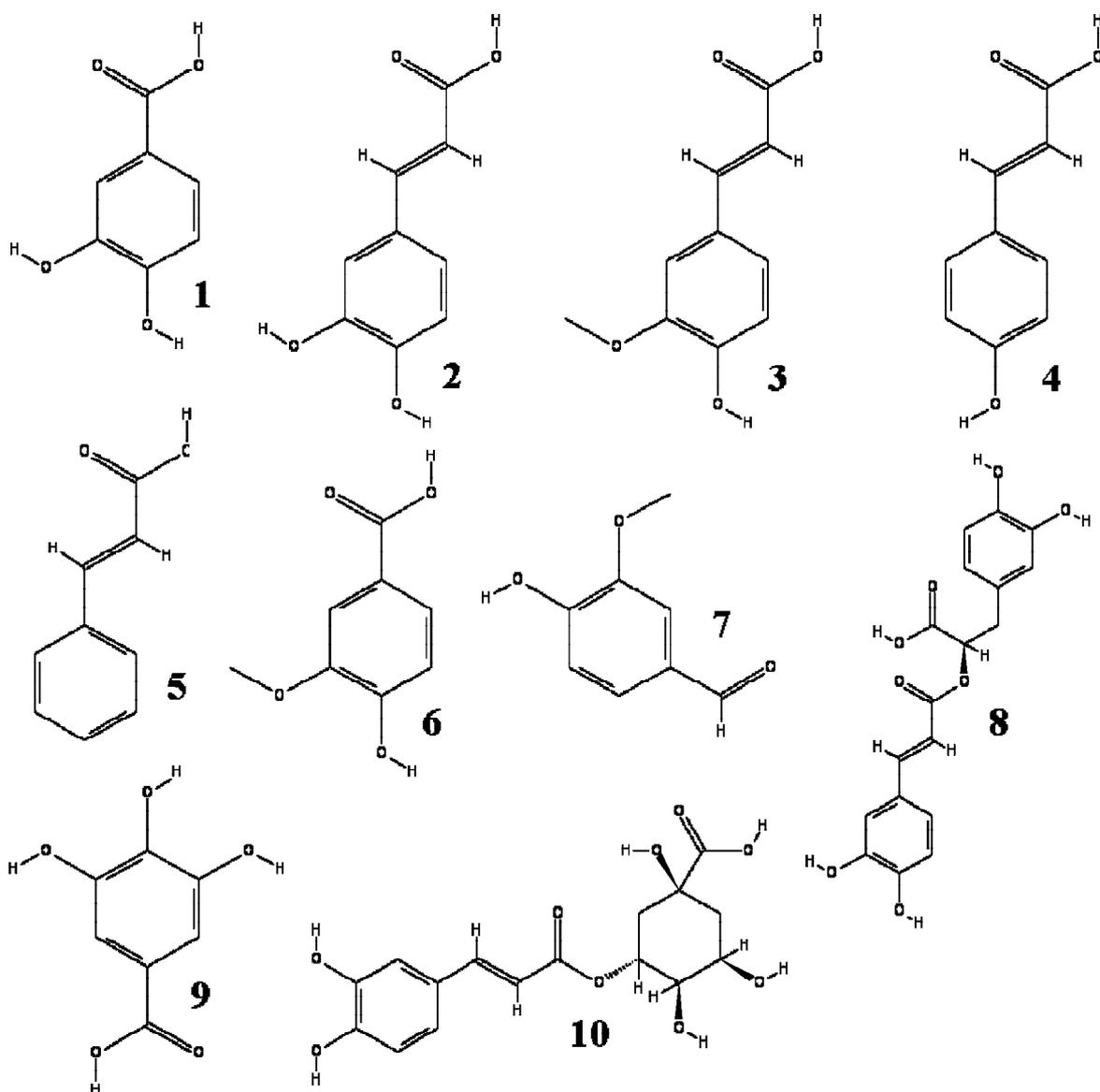
### Material and methods

**Chemicals.** Methanol for HPLC analysis was of HPLC grade and purchased from Carl Roth GmbH (Karlsruhe, Germany). Distilled water used for

preparation of solvents was filtered through the Millipore HPLC grade water preparation cartridge (Millipore, Bedford, USA) and membrane filter with a pore size of 0.45  $\mu\text{m}$ . Standards of phenolic acids and phenylpropanoids were purchased from ChromaDex (Santa Ana, USA).

**Raw material.** Samples of propolis and *Herba Origani* were collected in the territory of Lithuania. Crude drug samples passed the analysis procedures of European Pharmacopoeia. All samples were extracted with 80% (v/v) ethanol (propolis) and 70% (v/v) ethanol (*Herba Origani*) before the HPLC analysis.

**Equipment.** HPLC analysis with UV/PDA detec-



**Fig. 1.** Structure of selected phenolic acids and phenylpropanoids

1 – protocatechuic acid; 2 – caffeic acid; 3 – ferulic acid; 4 – coumaric acid; 5 – cinnamic acid; 6 – vanillic acid; 7 – vanillin; 8 – rosmarinic acid; 9 – gallic acid; 10 – chlorogenic acid.

tion was carried out using a model Waters 2690 chromatography system (Waters, Milford, USA), equipped with a Waters 2487 UV/Vis detector and Waters 996 PDA detector. For separation, a Hichrom column Hypersil H5ODS-150A 150×4.6 mm (Hichrom Ltd., Berkshire, UK) and an H5ODS-10C guard-cartridge were used. Data were collected and analyzed using personal computer and the Waters Millennium 2000® chromatographic manager system (Waters Corporation, Milford, USA).

**HPLC analysis.** The mobile phase of the optimized chromatographic method consisted of solvent A (methanol) and solvent B (0.5% (v/v) acetic acid in water). The elution profile was as follows: 0 min 10% A in B, 28.6 min 60% A in B, 30 min 10% A in B. All gradients were linear. The flow rate was 1 mL/min, and injection volume was 10 µL. Absorption was measured at 290 nm. The eluted components were identified based on the retention time by comparison with retention time of reference standard. Identity of constituents was also confirmed with PDA detector by comparison with UV spectra of standards in the wavelength range of 190–400 nm.

**Quantification.** Quantification was carried out by the external standard method. Peak areas were used for quantification. For the preparation of calibration curve, the stock solution of standards were prepared freshly in methanol to obtain a set of six concentration ranges for the study of linearity. These standard solutions were measured three times, and integrated peak areas were plotted against the corresponding concentrations of the injected standards. All standard solutions were filtered through a membrane filter with pore size of 0.22 µm (membrane filters for syringes, nylon membrane, diameter of 13 mm; Carl Roth GmbH, Karlsruhe, Germany).

## Results and discussion

**Optimization of the analytical method.** Several HPLC methods with UV detection were developed for routine analysis of phenolic acids (4, 10–12). The phenolics were separated using a gradient elution and a mobile phase consisting of water and methanol or acetonitrile. To obtain a needful acidity, commonly acetic acid and rarely trifluoroacetic acid were chosen.

The following chromatographic method was optimized with respect to eluent composition. Optimization of the separation was done with respect to the chosen optimization criteria: resolution  $R_s$  should be higher than 1.5 and analysis time should not exceed 30 min. According to the literature data for chromatographic elution, empirical gradient solvent systems consisting of solvent A (methanol) and solvent B

(0.5% (v/v) acetic acid in water) were chosen. For the initial elution, solvent systems consisting of 30%, 20%, and 10% of solvent A in the 70%, 80%, and 90% solvent B, respectively, were chosen. The linear gradients were performed until mobile phase reaches 60% A in B. The gradient duration was 28.6 min.

Under two selected conditions of HPLC separation (30% and 20% of solvent A on gradient start), resolution of some peaks did not exceed 1.5 (Fig. 2). Using the first gradient (30% of solvent A on gradient start), peak resolution of protocatechuic acid and chlorogenic acid was lower than 1.5; peak resolution of vanillic acid and caffeic acid was <0.5. Using the second gradient (20% of solvent A on gradient start), peak resolution of vanillic acid and caffeic acid was lower than 1.5. Optimal resolution was achieved using the third gradient (10% of solvent A on gradient start), and it was selected as the suitable method for separation of the selected phenolics. Under the optimal separation conditions (described in Methods), analyses were done in 27 min, with the following retention times: 4.04 min, gallic acid; 6.93 min, protocatechuic acid; 11.30 min, chlorogenic acid; 12.26 min, vanillic acid; 12.93 min, caffeic acid; 13.80 min, vanillin; 16.97 min, coumaric acid; 17.95 min, ferulic acid; 22.29 min, rosmarinic acid, and 26.30 min, cinnamic acid. Optimized HPLC separation is shown in Fig. 3.

**Validation.** Validation experiments were performed. By applying optimized method, reliable parameters (resolution, linearity, limit of detection, limit of quantification, and precision) were obtained.

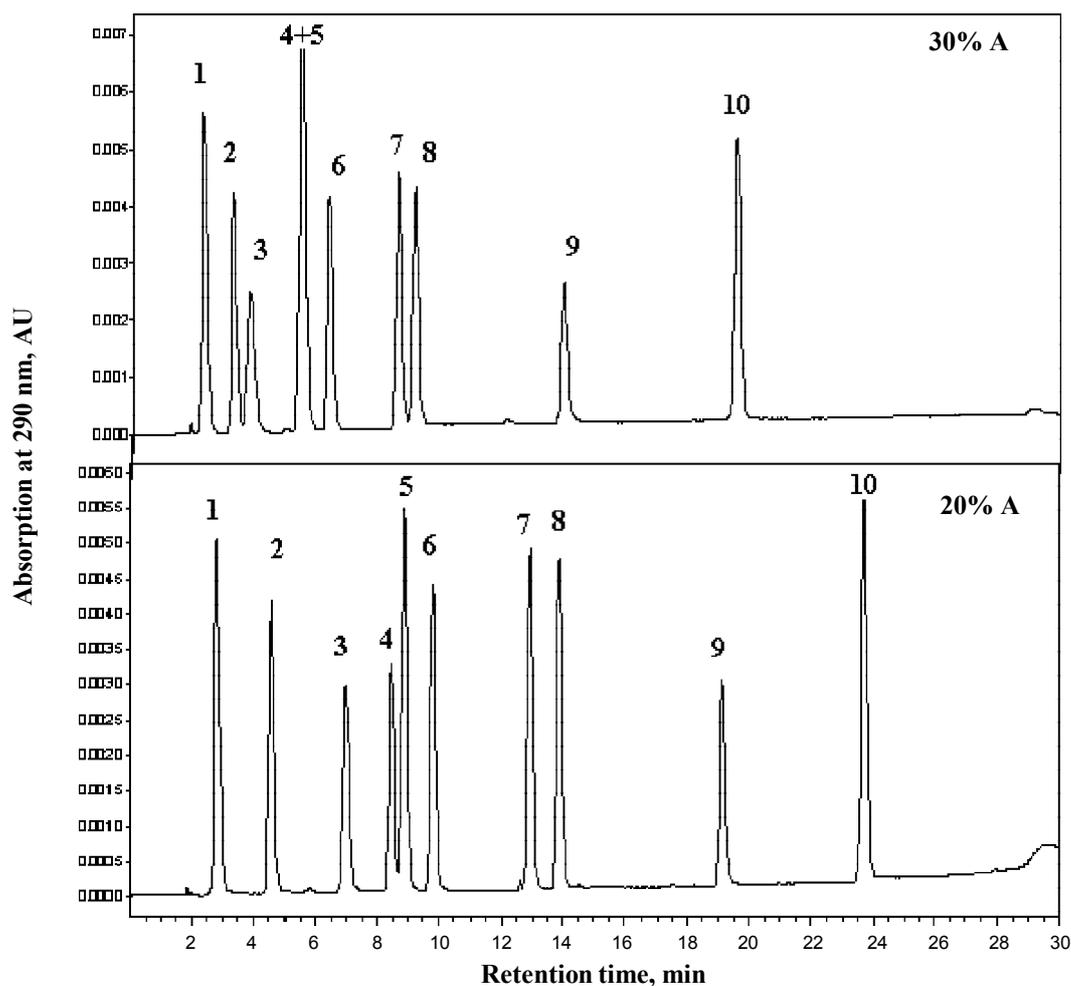
The problem of resolution was described above (in Optimization). All peaks are absolutely separated ( $R_s > 1.5$ ). Table 1 shows two values of resolution, calculated using the lowest and highest concentrations of standard solutions.

Linearity of detector response (peak area *versus* concentration) was evaluated by preparing five calibration samples. Each sample was injected in triplicate, and regression curve was obtained by plotting peak area *versus* concentration. A second-order polynomial calibration was observed. After log-transformation, the data provided a linear function for all reference standards following the equation:

$$Y = a + bX$$

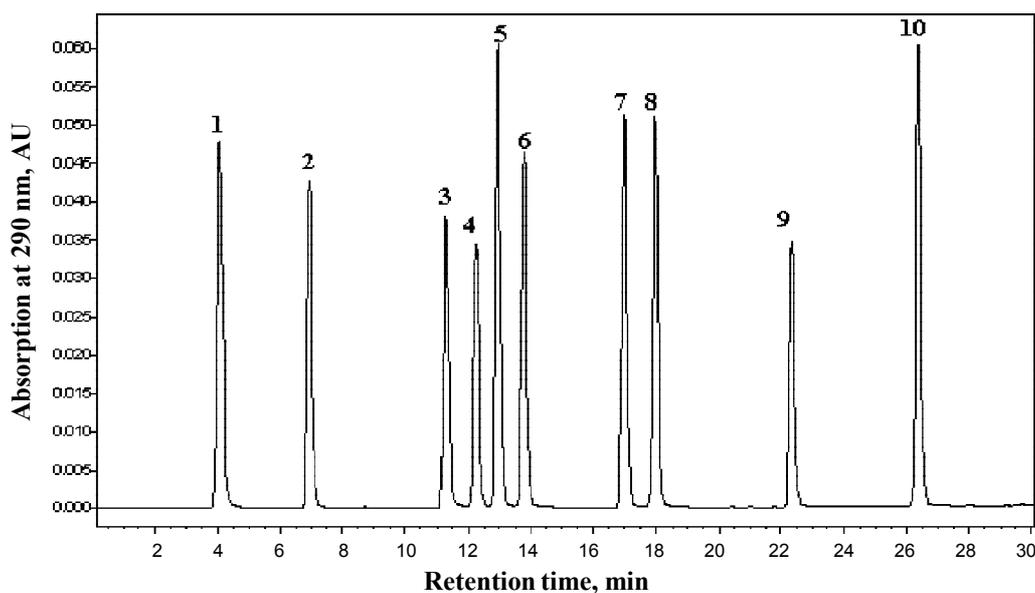
where  $Y$  is the log value of the peak area,  $X$  – the log value of sample amount,  $a$  – the intercept, and  $b$  – the slope.

The parameters of the calibration curves and their coefficients of determination demonstrate good linearity in the ranges of standard concentrations analyzed (Table 1).



**Fig. 2.** HPLC chromatograms of different gradient elution (30% and 20% of solvent A on gradient start)

Analytes: 1 – gallic acid; 2 – protocatechuic acid; 3 – chlorogenic acid; 4 – vanillic acid; 5 – caffeic acid; 6 – vanillin; 7 – coumaric acid; 8 – ferulic acid; 9 – rosmarinic acid; 10 – cinnamic acid.



**Fig. 3.** Optimized HPLC separation of phenolic acids and phenylpropanoid standards

Analytes: 1 – gallic acid; 2 – protocatechuic acid; 3 – chlorogenic acid; 4 – vanillic acid; 5 – caffeic acid; 6 – vanillin; 7 – coumaric acid; 8 – ferulic acid; 9 – rosmarinic acid; 10 – cinnamic acid.

Table 1. Parameters of resolution and calibration curves for separated phenolics

No.	Analyte	Fixed linear range ( $\mu\text{g/mL}$ )	Resolution		Equation	$r^2$	LOD ( $\mu\text{g/mL}$ )	LOQ ( $\mu\text{g/mL}$ )
			$R_{S, \min}$	$R_{S, \max}$				
1	Gallic acid	0.17–6.80	–	–	$Y=1.90 \times 10^4 X - 4.43 \times 10^2$	0.99985	0.021	0.070
2	Protocatechuic acid	0.13–5.24	8.77	8.66	$Y=1.82 \times 10^4 X - 6.48 \times 10^2$	0.99990	0.017	0.057
3	Chlorogenic acid	0.10–4.00	16.12	14.99	$Y=2.14 \times 10^4 X - 5.81 \times 10^2$	0.99988	0.019	0.064
4	Vanillic acid	0.08–3.23	3.75	3.42	$Y=2.27 \times 10^4 X + 1.05 \times 10^2$	0.99990	0.009	0.031
5	Caffeic acid	0.08–3.20	2.35	2.36	$Y=4.12 \times 10^4 X - 3.74 \times 10^2$	0.99989	0.008	0.026
6	Vanillin	0.07–2.80	3.30	3.18	$Y=3.59 \times 10^4 X - 3.34 \times 10^2$	0.99995	0.006	0.021
7	Coumaric acid	0.04–1.60	11.73	11.26	$Y=7.06 \times 10^4 X + 3.08 \times 10^2$	0.99988	0.004	0.014
8	Ferulic acid	0.07–2.80	3.94	3.48	$Y=3.91 \times 10^4 X - 6.21 \times 10^2$	0.99989	0.018	0.058
9	Rosmarinic acid	0.09–3.60	18.27	15.96	$Y=2.03 \times 10^4 X - 5.03 \times 10^2$	0.99988	0.016	0.054
10	Cinnamic acid	0.05–2.00	15.21	14.44	$Y=6.85 \times 10^4 X + 3.23 \times 10^2$	0.99994	0.004	0.014

$R_{S, \min}$  and  $R_{S, \max}$  – resolution values calculated using minimal and maximal concentrations of standard solutions; LOD – limit of detection; LOQ – limit of quantitation.

Table 2. Within-day and between-day precision and accuracy of assay for determination of phenolics in standard solution

Analyte	Injected concentration ( $\mu\text{g/mL}$ )	RSD, % for peak area		Mean concentration measured ( $\mu\text{g/mL}$ )		RSD, % for amount		Recovery, %	
		WD	BD	WD	BD	WD	BD	WD	BD
Gallic acid	3.400	0.1	2.2	3.483	3.419	0.1	1.5	102.3–102.6	97.3–102.4
Protocatechuic acid	2.624	0.5	3.5	2.785	2.706	0.5	2.6	105.6–106.4	97.8–106.4
Chlorogenic acid	2.000	2.0	3.1	2.023	1.998	1.9	2.1	100.0–103.8	95.1–100.9
Vanillic acid	1.615	0.7	3.5	1.702	1.657	0.7	2.4	104.7–106.2	97.3–105.3
Caffeic acid	1.600	0.5	3.2	1.681	1.638	0.5	2.2	104.5–105.4	97.7–105.4
Vanillin	1.400	0.3	3.5	1.485	1.439	0.3	2.6	105.7–106.3	97.8–106.2
Coumaric acid	0.800	0.4	3.3	0.841	0.821	0.4	2.2	104.7–105.5	97.4–105.5
Ferulic acid	1.400	0.4	2.7	1.455	1.427	0.4	1.7	103.6–104.4	97.5–104.4
Rosmarinic acid	1.800	0.4	1.8	1.805	1.781	0.4	1.8	99.9–100.7	97.2–100.2
Cinnamic acid	1.000	0.2	3.2	1.055	1.031	0.2	2.4	105.2–105.6	98.2–105.2

RSD – relative standard deviation; WD – within-day precision; BD – between-day precision.

The limit of detection (LOD) and limit of quantification (LOQ), defined as the lowest concentration of the analyte that can be clearly detected or quantified, were calculated by multiplying the signal to noise ratio by 3 (LOD) and by 10 (LOQ). The limits were set at the concentration of lowest calibration (diluted with methanol 2–4 times). The limits of detection and the limits of quantification were between 0.004–0.021 and 0.014–0.07, respectively (Table 1).

The precision and accuracy of the method were assessed by within-day and between-day run validations. Method precision was determined by measuring repeatability and precision of peak area. The repeatability (within-day) precision of the method was determined by performing replicate injection ( $n=4$ ). The relative standard deviation (RSD, %) was also calculated by comparing the observed concentrations. In Table 2, RSD values are given for peak area and amount. The between-day precision was evaluated by injecting three sets of controls on three separate days. In all cases, RSD was lower than 3.5% for peak area and 2.6% for amount. The results show that repeatability of peak area was suitable to assay analyses. The within-day and between-day accuracy was also measured in the range of 95.1–106.4%.

**Application.** For the application of method, samples of propolis and *Herba Origani* were chosen. Prop-

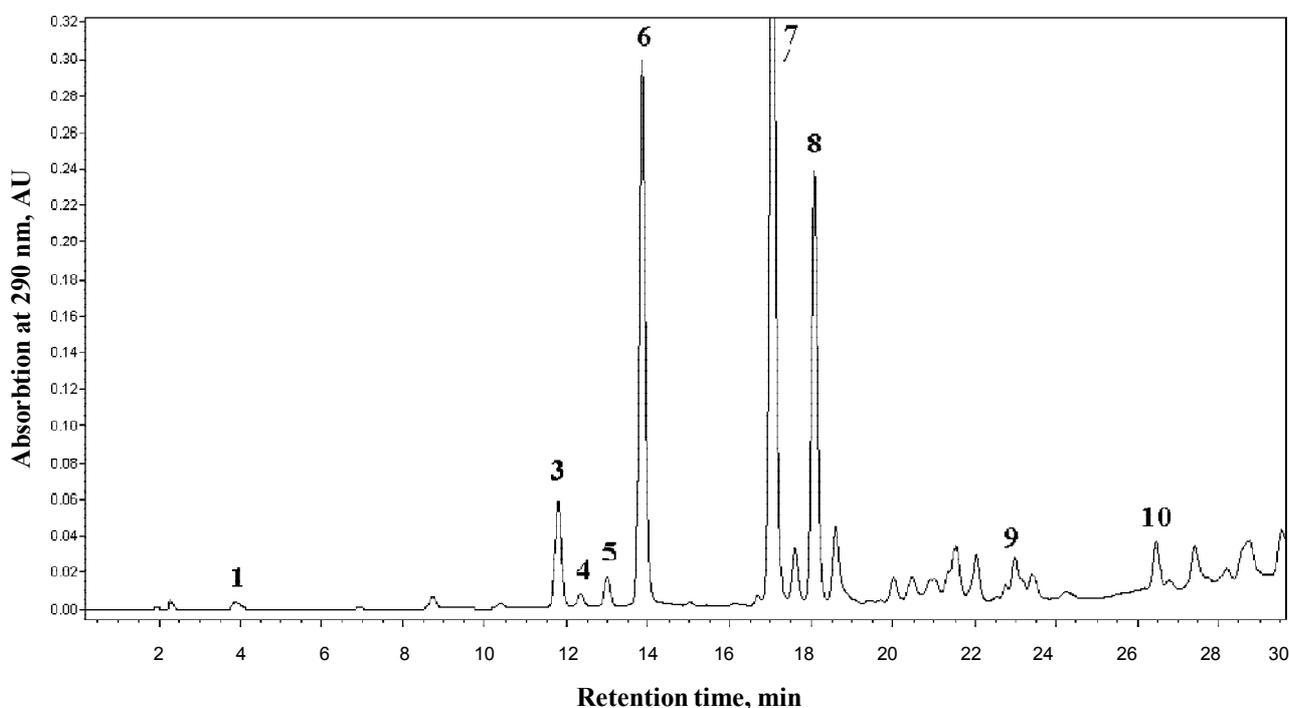
olis is a natural product, produced by bees, and has antibacterial, antiviral, antifungal, anti-inflammatory, anesthetic, and immunomodulating properties (13). Recent findings report antimicrobial, fungicidal, and antioxidant properties of essential oil of *Origanum vulgare* and crude drug *Herba Origani* (14). Volatile compounds of Lithuanian propolis and *Origanum vulgare* samples were analyzed earlier by method of gas chromatography/mass spectrometry (13, 14).

Nine analytes were identified in the propolis with the comparison of retention times and PDA spectra of standards. Only protocatechuic acid was not detected in the samples. Typical chromatogram is presented in Fig. 4. Amounts of phenolics are given in the Table 3. On the ground of the presented quantities, coumaric acid was established as the dominating phenolic acid of propolis.

Results of analysis of *Herba Origani* demonstrated high quantity of rosmarinic acid in the samples (Table 4). Fig. 5 presents the typical chromatogram of *Herba Origani* sample. Six phenolic compounds of this crude drug could be assayed with the validated method.

### Conclusions

Optimization and validation procedures of rapid and simple method of reversed-phase high-



**Fig. 4.** HPLC chromatogram of propolis sample

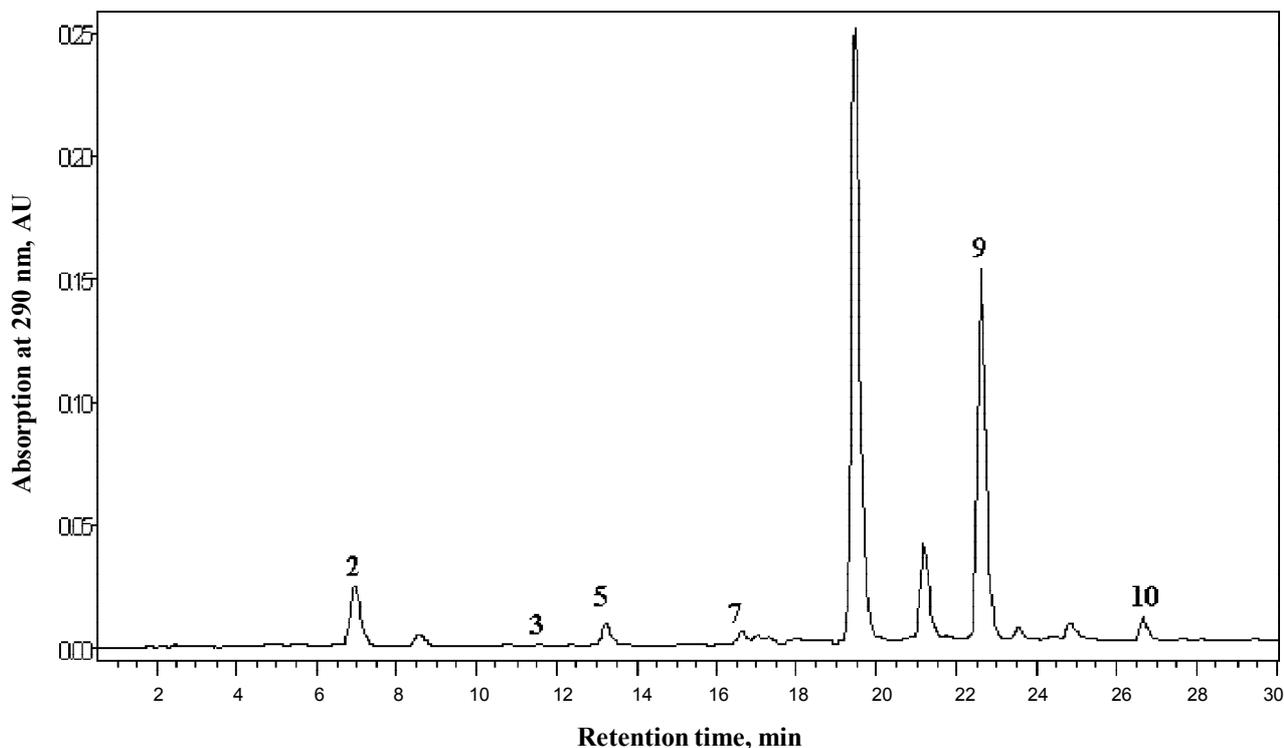
Analytes: 1 – gallic acid; 3 – chlorogenic acid; 4 – vanillic acid; 5 – caffeic acid; 6 – vanillin; 7 – coumaric acid; 8 – ferulic acid; 9 – rosmarinic acid; 10 – cinnamic acid.

**Table 3. Amounts of phenolics in propolis**

No.	Analyte	Amount in the air-dried propolis ( $\mu\text{g/g}$ )
1	Gallic acid	105.4
3	Chlorogenic acid	540.5
4	Vanillic acid	88.1
5	Caffeic acid	166.0
6	Vanillin	1094.8
7	Coumaric acid	2784.6
8	Ferulic acid	939.9
9	Rosmarinic acid	140.8
10	Cinnamic acid	104.1

**Table 4. Amounts of phenolics in oregano (*Herba Origani*) sample**

No.	Analyte	Amount in the air-dried oregano ( $\mu\text{g/g}$ )
2	Protocatechuic acid	1485.7
3	Chlorogenic acid	89.9
5	Caffeic acid	195.6
7	Coumaric acid	175.4
9	Rosmarinic acid	6375.9
10	Cinnamic acid	171.3

**Fig. 5. HPLC separation of oregano (*Origanum vulgare*) phenolics**

Analytes: 2 – protocatechuic acid; 3 – chlorogenic acid; 5 – caffeic acid; 7 – coumaric acid; 9 – rosmarinic acid; 10 – cinnamic acid.

performance liquid chromatography were carried out. By applying optimized method, reliable parameters (resolution, linearity, limit of detection, limit of quantification, and precision) were obtained. For the application of method, two kinds of raw materials – propolis

and the *Herba Origani* – were chosen. Coumaric acid is the dominating phenolic acid of propolis (2785  $\mu\text{g/g}$ ). Results of analysis of *Herba Origani* demonstrated high quantities (6376  $\mu\text{g/g}$ ) of rosmarinic acid in the samples.

## Fenolinių rūgščių ir fenilpropanoidų įvertinimas vaistinėje žaliavoje

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**Raktažodžiai:** fenolinės rūgštys, fenilpropanoidai, efektyvioji skysčių chromatografija, vaistinė žaliava.

**Santrauka.** Fenolinės rūgštys ir fenilpropanoidai pasižymi reikšmingu biologiniu aktyvumu ir yra vaistinių žaliavų veiklieji komponentai. Įteisintų tyrimo metodikų tiems junginiams nustatyti (kokybiniam ir kiekybiniam žaliavų tyrimui) tobulinimas ypač svarbus augalinių vaistinių preparatų veiksmingumo, saugumo ir kokybės kontrolei.

*Tyrimo tikslas* – ištirti galimybę analitiniais metodais įvertinti šias fenolines rūgštis ir fenilpropanoidus: chlorogeno rūgštį, cinamono rūgštį, galio rūgštį, ferulo rūgštį, kavos rūgštį, kumaro rūgštį, protokatecho (3,4-dihidroksibenzoinę) rūgštį, rozmarino rūgštį, vaniliną ir vanilino rūgštį. Atlikti tyrimai paprastos ir greitos atvirkštinių fazių efektyviosios skysčių chromatografijos metodikos optimizavimui ir įtiesinimui. Sukurto chromatografinio metodo judrioji skystoji fazė yra metanolis ir acto rūgšties 0,5 proc. vandeninis tirpalas. Kiekybinis metodo pakartojamumas yra didesnis nei 2,6 proc. Metodo taikymui pasirinktos dvi vaistinės žaliavos: propolis ir raudonėlio žolė (*Herba Origani*). Nustatyta, jog propolyje vyrauja kumaro rūgštis (2785 µg/g) ir vanilinas (1094 µg/g). Raudonėlio žolės (*Herba Origani*) bandinių tyrimo duomenys rodo didelį (6376 µg/g) rozmarino rūgšties ir protokatecho rūgšties (1485 µg/g) kiekį žaliavoje.

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