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ACCURATE QUANTITATION OF 17 POLYPHENOLS FROM PROPOLIS EXTRACTS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH DIODE ARRAY DETECTION

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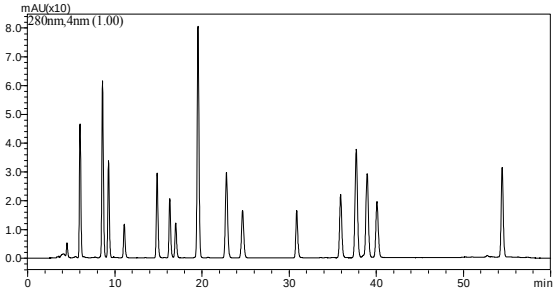
A method based on reversed-phase liquid chromatography (RP-LC) with diode array detection (DAD) has been developed and validated in order to quantitate several polyphenols from different extracts. Samples were separated on a Fortis C18 reversed-phase column (250 x 4.6 mm, 5 µm) with a gradient of acetonitrile and aqueous phosphoric acid, pH=2.5. The complete separation of seventeen polyphenols was achieved within 60 min. All calibration curves expressed good linearity ($r^2 > 0.997$) within the test range. The recovery of this method was in the range 88.12–107.87% and for the intra-day and inter-day assays, the values of deviation coefficient were less than 5%. The assay was successfully applied to the quantitation of polyphenols from 9 samples of propolis ethanolic extracts purchased from Roumanian market. The results indicated that this developed RP-LC assay could be readily utilized as a quality control method of various natural extracts.



INTRODUCTION

The propolis has been used frequently in folk medicine because of its special chemical components, strong pharmacological properties and non-toxicity. Propolis is a resinous substance collected by honeybees from the bud and bark of certain trees and plants, and stored inside their hives. Main types of chemical substances found in propolis are waxes, resins, balsams, aromatic and ethereal oils, pollen and other organic matter.[1] The chemical diversity of propolis is give by the specificity of the local flora at the site of collection and thus on the geographic and climatic characteristics of this site. The studies have shown that in temperate zones the main source of bee glue is the resinous exudate of the buds of poplar trees, especially the black poplar *Populus nigra*.[2-4] The biological properties of propolis seems to be identical even if the chemical composition differs depending on geographical origin. In many reports, the biological or pharmacological activity was associated with phenolic compounds such as flavonoids, phenolic acids and their esters especially caffeates and ferulates. For the propolis and its constituents some biological activities have been reported, such as: antibacterial,[5,6] antifungal,[7,8] antiprotozoal,[9,10] antiviral,[11,12] antitumor,[13,14] immunomodulation,[15,16] anti-inflammatory,[17,18] antioxidant,[19-22] and hepatoprotective.[23,24]

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The analysis of flavonoids and phenolic acids in propolis included over the years many methods: thin layer chromatography (TLC),[25] high performance liquid chromatography (HPLC),[26-29] gas chromatography (GC),[30-32] capillary electrophoresis (CE).[33-34] Among these methods, HPLC coupled with MS, UV or photodiode array detection is still the most used analytical technique for this purpose, while GC with the electron-capture detector or MS detection is suitable for non flavonoid compounds including aliphatic and aromatic compounds.

Propolis cannot be used as a raw material; it must be purified before analysis by extraction with suitable solvents (ethanol, methanol, acetone, hexane, chloroform, water). Extraction with ethanol (70–80%) is the most frequently used to obtain dewaxed propolis extracts rich in polyphenolic compounds. Propolis is commercially available in the Roumanian market as tinctures or tablets, which are based usually on ethanol extracts. We, therefore, investigated the polyphenolic content in nine ethanolic extracts of propolis purchased from the Roumanian commercial market between 2012 and 2013. The sensitive and complete HPLC method for determination of some phenolic acids and flavonoids studied was developed and validated.

EXPERIMENTAL

Reagents and standards

Rutin, caffeic acid, caffeic acid phenethyl ester (CAPE), p-coumaric acid, ferulic acid, cinnamic acid, chrysin, daidzein, luteolin, naringenin, rhamnetin, kaempferol, galangin, myricetin, pinostrobin, were purchased from Fluka (Germany), pinocembrin were purchased from Sigma, quercetin was purchased from Riedel-deHaën (all standards purity ≥ 98.0%). LC grade acetonitrile and methanol, ortho-phosphoric acid 85% were purchased from Merck (Germany). Before use, the solvents were filtered through a 0.45 μm Millipore membrane (type Millex-LCR, Millipore Corporation) and degassed. Ultrapure water (Milli Q system, Millipore, Bedford, MA, USA) was used throughout the experiments.

Preparation of standard solutions

Stock standard solutions (1 mg mL^{-1}) were prepared by weighting of test compounds and their dissolving in methanol. The solutions were stored at approximately 4°C in the dark. Working standard solutions were prepared by dilution of the stock standard solution with the mobile phase to obtain eight different concentrations within the range of interest. These solutions were freshly prepared and used for linearity investigation, evaluation of precision, repeatability, accuracy and robustness of the LC method.

Nine ethanol propolis extracts were purchased from Romanian market: commercial soft extract of propolis-Bioremed (S1); Tincture of propolis – Favisan (S2), Tincture of propolis – Flavasol (S3), Hydroalcoholic extract of propolis – Dacia Plant (S4), Tincture of propolis – Plant Extract (S5), Tincture of propolis – ICDPA (S6), Tincture of propolis – Fabiol (S7), Tincture of propolis – Santo Rafael (S8), Tincture of propolis – Larix (S9). Before LC analysis, the samples were diluted with the mobile phase and filtered through a 0.45 μm filter.

Instrumentation and conditions

LC analyses were performed on a Shimadzu LC-DAD chromatographic system (Shimadzu, Japan) consisting of: pumps (LC-10ADvp), diode array detector (SPD-M20A), degasser (DGU-20A5), column oven (CTO-10ASvp) and system controller (SCL-10Avp). Separation was achieved on a 250 mm x 4.6 mm, 5 μm particle, Fortis C18 (Fortis Technologies Ltd.) column at 35°C. The mobile phase for both commercial propolis samples and standards was a gradient prepared from water and phosphoric acid at pH 2.5 (solvent A) and acetonitrile (solvent B). Samples were eluted as follows: Step *1*: 30% to 50% B in 0.00-28.00 min; Step *2*: 50% to 62% B in 28.00-45.00 min; Step *3*: 62% to 70% B in 45.00-50.00; Step *4*: 70% to 30% B in 50.00-55.00 min; Step *5*: 30% B in 55.00-60.00 min. Flow rates were initially 0.8 mL/min, changed to 0.6 mL/min from 20.00 min to 27.00 min and then returned to 0.8 mL/min. The column was prepared for a new injection by washing the column for 10 min with 10 : 90 mixing ratio between aqueous and organic constituents, followed by an equilibration run time of 20 minutes. The injection volume was 20 μL. For chromatogram monitoring by DAD, UV spectra were recorded between 280-400 nm, at a resolution of 4.0 nm.

Compounds of interest were identified by their retention times, UV spectra and by addition of their standards. Because these compounds have different UV absorption parameters, different detection wavelengths were chosen to monitor these components for a good separation. Data were acquired and handled by LC Workstation LabSolution/LCsolution software (Shimadzu, Japan).

RESULTS AND DISCUSSION

Optimization of chromatographic performance

In order to achieve a good separation of all 17 compounds and a short analysis time, mobile phase components, gradient, flow rate and column temperature were optimized. The optimal chromatographic conditions were obtained after running different binary gradient elutions (methanol: acetate buffer; methanol: water and acetic acid; acetonitrile: water and acetic acid; acetonitrile: water and phosphoric acid) with a reversed phase C18 column. The combination acetonitrile-water-phosphoric acid, pH=2.5 was selected as the most suitable. The acetonitrile was preferred over methanol because of fast elution and the pH value of the mobile phase was selected based on pKa values of the polyphenols separated. It was noted that separation was better when the column temperature was kept at 35°C. The optimum flow rate was 0.8 mL min^{-1}, except 0.6 mL min^{-1} that was set in 20–27 min for a better separation of the peaks belonging to cinnamic acid, naringenin and kaempferol. Representative chromatograms of pure standards (280 nm) and a propolis sample are presented in Fig. 1.

LC Method validation

Validation of the LC method includes the determination of following performance parameters such as linearity, limits of detection, limits of quantitation, sensitivities, specificity, precision and accuracy, recovery and robustness.

Selectivity

The selectivity method was attempted by comparison of both UV-spectra and retention time. The peak purity of the seventeen polyphenols was evaluated by comparison of the UV spectra obtained at three points of each peak, using the LC Workstation LabSolution / LCsolution software (Shimadzu, Japan). Peaks were considered pure when their UV spectra similarity (230 to 400 nm) was greater than 95%.

Linearity, limits of detection and quantitation

Linearity over the working range of concentration was verified by regression analysis of the relative peak area as response versus standard solution concentration. The working standard solution of polyphenols was freshly prepared by dilution of the stock standard solutions with the mobile phase and analytical curves were constructed for eight concentration levels. Linear ranges were between 3-40 μg mL^{-1} for rutin and 2.5-40 μg mL^{-1} for the other compounds. The results of the regression equations and correlation coefficients are presented in Table 1. The linear equation between the standard concentrations and peak areas are presented as y = aX+b, where X is the concentration (μg mL^{-1}), y- peak area of the standard, a and b are the regression constants. Good linearities over the investigated concentration ranges were observed with the values of r^2 higher than 0.997 for all the analytes. 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 quantitation (LOQ) was measured as the lowest amount of analyte that can be reproducibly quantified above the baseline noise (S/N=10). The values obtained for LOD and LOQ for all the seventeen compounds indicate that the method exhibited a good sensitivity.

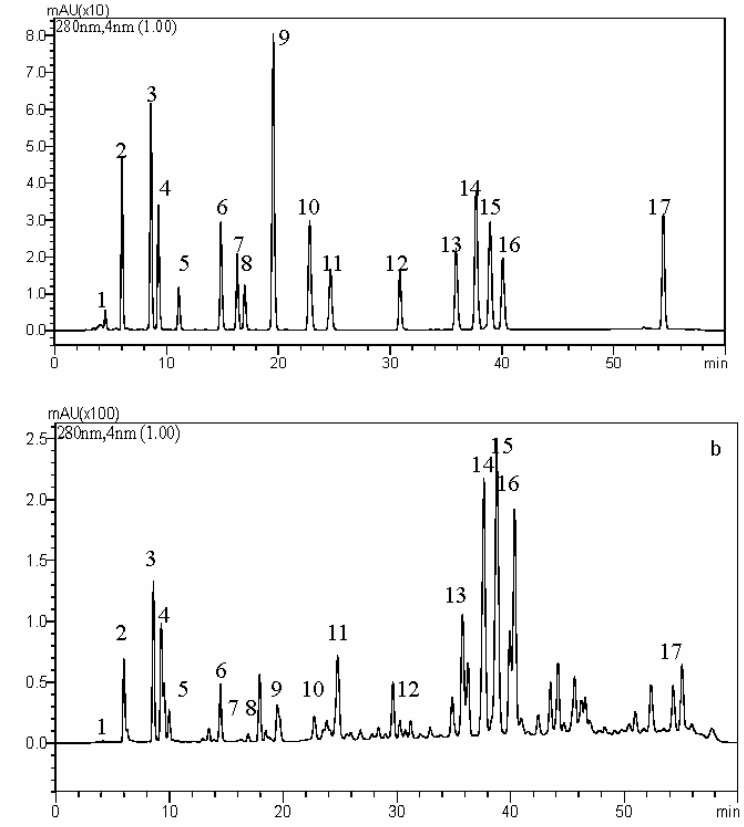

Fig. 1 – Chromatogram of standard solution at 280 nm: (a) *1* – Rutin; *2* – Caffeic acid; *3* – Coumaric acid; *4* – Ferulic acid; *5* – Myricetin; *6* – Daidzein; *7* – Luteolin; *8* – Quercetin; *9* – Cinnamic acid; *10* – Naringenin; *11* – Kaempferol; *12* – Rhamnetin; *13* – CAPE; *14* – Chrysin; *15* – Pinocembrin; *16* – Galangin; *17* – Pinostrobin; (b) Chromatogram of commercial propolis extract S1.

Precision

Intra-day and inter-day variations were utilized to determine precision of the method. The calibration samples of seventeen reference compounds were analysed during a single day (n=7) and on three consecutive days (n=6). The coefficient of variation (CV) was taken as a measure of precision and was calculated by the equation: CV (%) = (SD/mean) x 100%. All of the values were lower than 5%, which revealed a good precision of the analytical method (Tables 2 and 3).

Table 1

Calibration curve of the seventeen reference compounds (*n*=5)

No	Compound	t_R (min) ±SD	Regression equation	r^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
1	Rutin	4.614±0.057	y= 60684.2 x-157048	0.999	0.157	0.476
2	Caffeic acid	6.031±0.023	y= 171822 x-137235	0.999	0.051	0.156
3	Coumaric acid	8.620±0.026	y= 224262 x-301817	0.999	0.033	0.102
4	Ferulic acid	9.288±0.021	y= 163058 x-125490	0.999	0.050	0.152
5	Myricetin	11.081±0.035	y= 109021 x-91071.4	0.999	0.077	0.233
6	Daidzein	14.860±0.030	y= 185365 x-27243.6	0.998	0.050	0.152
7	Luteolin	16.329±0.037	y= 146959 x-131477	0.999	0.061	0.186
8	Quercetin	16.951±0.066	y= 109574 x-86573.5	0.999	0.081	0.245
9	Cinnamic acid	19.487±0.029	y= 238084 x-158236	0.999	0.037	0.111
10	Naringenin	22.745±0.071	y= 114456 x-41805.7	0.998	0.084	0.255
11	Kaempferol	24.608±0.072	y= 153828 x-100031	0.999	0.052	0.157
12	Rhamnetin	30.775±0.042	y= 132497 x-71272.9	0.998	0.067	0.203
13	CAPE	35.792±0.031	y= 160266 x-133353	0.997	0.057	0.173
14	Chrysin	37.653±0.052	y= 274839 x-240690	0.998	0.032	0.096
15	Pinocembrin	38.793±0.059	y= 148646 x-95698	0.997	0.063	0.190
16	Galangin	39.970±0.061	y= 107393 x-99083.2	0.998	0.074	0.226
17	Pinostrobin	54.295±0.037	y= 122746 x-79187.9	0.998	0.078	0.236

Table 2

Intra-day precision of the seventeen reference compounds

No	Compound	Theoretical concentration (μg mL^{-1})	Intra-day (*n = 7*)	
			Detected (μg mL^{-1})	CV (%)
1	Rutin	18	18.30±0.46	2.52
2	Caffeic acid	19.6	19.68±0.51	2.62
3	Coumaric acid	19.6	19.63±0.49	2.50
4	Ferulic acid	19.6	19.40±0.27	1.41
5	Myricetin	19.6	19.54±0.69	3.53
6	Daidzein	19.6	19.73±0.26	1.34
7	Luteolin	19.6	19.50±0.77	3.96
8	Quercetin	19.6	19.65±0.37	1.92
9	Cinnamic acid	19.6	19.66±0.20	1.01
10	Naringenin	19.6	19.21±0.26	1.40
11	Kaempferol	19.6	19.51±0.67	3.43
12	Rhamnetin	19.6	19.52±0.50	2.59
13	CAPE	19.6	18.92±0.27	1.42

Table 2 (continued)

14	Chrysin	19.6	18.36±0.31	1.73
15	Pinocembrin	19.6	19.81±0.49	2.48
16	Galangin	19.6	20.43±0.90	4.40
17	Pinostrobin	19.6	19.38±0.34	1.80

Accuracy

Accuracy was determined by recovery test. Recovery values of all of the quantitated constituents were determined using a propolis sample for which the respective chemical contents had been predetermined, spiked at three different concentration levels (2 μg mL^{-1} , 7.5 μg mL^{-1} and 12 μg mL^{-1}): recovery (%) = (amount determined – original amount)/amount added) x 100. Triplicate sample analysis was conducted for the determination of recovery at each spiked level. A good degree of accuracy was achieved for most of the compounds, only rutin, myricetin and daidzein showed smaller recovery values, 89.47%, 89.44 and 88.12% respectively. A percentage CV value was determined as the difference between measured and expected values. The average recovery with CV % values is presented in Table 4.

Table 3

Inter-day precision of the seventeen reference compounds

No	Compound	Theoretical concentration (μg mL^{-1})	Inter-day (n=6)					
			Day 1		Day 2		Day 3	
			Detected (μg mL^{-1})	CV (%)	Detected (μg mL^{-1})	CV (%)	Detected (μg mL^{-1})	CV (%)
1	Rutin	5.00	5.19±0.10	2.04	5.01±0.07	1.47	4.87±0.16	3.28
		7.50	8.09±0.19	2.35	7.931±0.15	1.85	8.65±0.07	0.84
		13.00	12.82±0.23	1.85	12.53±0.11	0.91	13.60±0.33	2.48
2	Caffeic acid	5.00	5.4±0.04	0.88	5.48±0.17	3.19	5.17±0.20	3.97
		9.00	9.18±0.14	1.59	8.95±0.14	1.62	9.49±0.06	0.62
		14.50	15.02±0.17	1.17	14.21±0.10	0.76	15.32±0.21	1.40
3	Coumaric acid	5.00	5.41±0.21	4.04	5.45±0.20	3.81	5.16±0.24	4.69
		9.00	9.51±0.46	4.90	9.22±0.37	3.98	9.71±0.45	4.62
		14.5	14.17±0.52	3.72	14.75±0.53	3.63	15.70±0.63	4.04
4	Ferulic acid	5.00	5.70±0.11	1.98	5.47±0.08	1.55	5.13±0.23	4.53
		9.00	9.91±0.21	2.12	9.68±0.23	2.32	10.46±0.20	1.89
		14.50	15.43±0.26	1.68	14.96±0.18	1.24	15.51±0.28	1.83
5	Myricetin	5.00	5.10±0.02	0.45	5.19±0.02	0.55	5.04±0.07	1.38
		9.00	9.65±0.12	1.20	9.69±0.17	1.77	9.72±0.11	1.13
		14.5	14.47±0.17	1.20	13.16±0.06	0.47	13.53±0.15	1.11
6	Daidzein	5.00	5.27±0.05	0.94	5.12±0.05	1.05	5.34±0.09	1.74
		9.00	9.34±0.12	1.31	9.04±0.37	4.08	10.07±0.07	0.71
		14.50	15.52±0.19	1.25	13.73±0.08	0.60	14.83±0.16	1.10
7	Luteolin	5.00	5.62±0.04	0.80	5.19±0.05	0.98	5.12±0.06	1.24
		9.00	9.32±0.11	1.17	9.31±0.21	2.21	10.01±0.09	0.86
		14.50	14.86±0.17	1.20	14.00±0.06	0.45	14.89±0.15	1.02
8	Quercetin	5.00	5.59±0.04	0.76	5.12±0.08	1.69	5.01±0.07	1.43
		9.00	9.73±0.12	1.27	9.86±0.07	0.67	10.21±0.14	1.37
		14.50	14.49±0.20	1.41	14.23±0.05	0.41	15.64±0.14	0.91
9	Cinnamic acid	5.00	5.04±0.03	0.67	5.22±0.12	2.31	5.10±0.10	1.97
		9.00	9.63±0.13	1.34	9.32±0.22	2.39	10.07±0.06	0.62
		14.50	15.43±0.17	1.15	14.52±0.09	0.64	15.78±0.18	1.14
10	Naringenin	5.00	5.18±0.048	0.92	5.42±0.07	1.29	5.10±0.10	2.11
		9.00	9.27±0.12	1.26	8.85±0.38	4.34	9.881±0.05	0.51
		14.50	14.67±0.20	1.36	13.85±0.08	0.63	14.59±0.16	1.09

Table 3 (continued)

11	Kaempferol	5.00	5.74±0.06	1.08	5.25±0.05	1.06	5.05±0.15	3.06
		9.00	9.86±0.11	1.15	9.45±0.31	3.29	10.43±0.11	1.02
		14.50	14.88±0.20	1.37	14.65±0.07	0.53	16.08±0.15	0.95
12	Rhamnetin	5.00	5.66±0.04	0.81	5.20±0.05	0.99	4.89±0.19	3.96
		9.00	9.34±0.12	1.33	9.84±0.16	1.60	9.95±0.12	1.16
		14.5	14.59±0.204	1.40	15.01±0.09	0.61	14.92±0.12	0.85
13	CAPE	5.00	5.20±0.08	1.65	5.19±0.12	2.32	5.22±0.16	3.17
		9.00	9.61±0.12	1.27	9.34±0.16	1.72	9.83±0.20	2.061
		14.50	14.93±0.23	1.55	14.52±0.15	1.05	14.83±0.25	1.68
14	Chrysin	5.00	5.05±0.04	0.85	4.94±0.11	2.24	4.97±0.10	2.05
		9.00	9.63±0.12	1.30	9.34±0.37	3.97	11.26±0.07	0.67
		14.50	14.77±0.19	1.28	13.14±0.08	0.67	12.76±0.11	0.92
15	Pinocembrin	5.00	5.28±0.03	0.72	5.07±0.11	2.18	5.13±0.11	2.28
		9.00	9.42±0.15	1.56	9.23±0.23	2.47	9.99±0.06	0.61
		14.50	14.99±0.25	1.71	14.26±0.08	0.61	14.79±0.16	1.12
16	Galangin	5.00	5.98±0.04	0.80	5.45±0.11	2.11	5.22±0.06	1.18
		9.00	9.78±0.13	1.37	9.64±0.31	3.20	10.59±0.08	0.72
		14.5	12.86±0.18	1.44	14.08±0.09	0.68	14.74±0.11	0.80
17	Pinostrobin	5.00	6.61±0.03	0.58	5.35±0.12	2.25	5.30±0.11	2.13
		9.00	9.58±0.15	1.52	9.13±0.09	1.05	9.55±0.08	0.84
		14.50	14.55±0.20	1.43	14.78±0.11	0.80	15.53±0.15	0.99

Table 4

Recovery of the seventeen reference compounds (*n=3*)

Compound	Original (µg)	Added (µg)	Determined (µg)	Recovery (%)	Mean (%)	CV (%)
Rutin	2.65	2	4.47	91.17	89.47±1.52	1.70
		7.5	9.32	88.99		
		12	13.68	88.24		
Caffeic acid	7.79	2	9.86	103.38	104.60±1.06	1.01
		7.5	15.69	105.34		
		12	20.92	105.09		
Coumaric acid	4.90	2	6.99	104.32	100.84±4.54	4.51
		7.5	12.59	102.53		
		12	16.87	95.70		
Ferulic acid	2.28	2	4.34	103.25	98.63±4.70	4.76
		7.5	9.69	98.80		
		12	14.01	93.85		
Myricetin	0.92	2	2.73	90.48	89.44±0.90	1.01
		7.5	7.60	89.03		
		12	12.22	90.41		
Daidzein	3.19	2	4.96	88.12	88.12±0.43	0.48
		7.5	9.84	88.56		
		12	14.16	87.70		
Luteolin	1.18	2	3.04	92.9	92.96±1.01	1.09
		7.5	8.23	94.01		
		12	12.68	91.98		
Quercetin	2.61	2	4.57	98.25	97.63±4.52	4.63
		7.5	10.24	101.81		
		12	14.21	92.83		
Cinnamic acid	2.81	2	4.65	91.67	93.35±4.59	4.83
		7.5	10.33	100.27		
		12	14.46	93.15		
Naringenin	2.76	2	4.67	95.35	98.86±3.04	3.08
		7.5	10.32	100.81		
		12	15.32	100.42		

Table 4 (continued)

Kaempferol	3.29	2	5.14	92.22	96.16±4.75	4.94
		7.5	10.90	101.44		
		12	15.15	94.83		
Rhamnetin	2.46	2	4.61	107.40	103.70±4.78	4.61
		7.5	10.37	105.41		
		12	14.75	98.30		
CAPE	22.68	2	24.70	100.85	103.48±2.30	2.22
		7.5	30.57	105.14		
		12	35.74	104.45		
Chrysin	23.29	2	25.29	99.88	99.23±4.40	4.43
		7.5	31.04	103.27		
		12	35.19	94.54		
Pinocembrin	25.71	2	27.84	107.53	107.87±3.00	2.78
		7.5	34.04	111.03		
		12	38.84	105.05		
Galangin	19.56	2	21.39	91.33	95.67±4.37	4.60
		7.5	27.05	99.78		
		12	31.26	93.61		
Pinostrobin	9.66	2	11.73	103.35	102.43±3.66	3.57
		7.5	17.58	105.55		
		12	21.96	98.40		

Robustness

Robustness is defined as the capability of an analytical procedure to remain unaffected by small but deliberate changes in the method parameters. To ensure that the LC method is insensitive to minor changes in the experimental conditions it is important to demonstrate the robustness of the method. When the pH value of mobile phase was changed by 0.20 units of pH, from 2.50 to 2.30 and 2.70, this alteration caused no significant changes in the resolution of the standards (5μg mL^{-1}). The same minor changes were observed when the temperature was changed from 35°C to 30°C and 40°C. In the ranges examined were small deviations from the method settings, and corresponding responses in the peak area ratio considered were observed. The coefficient of variation (CV%) of retention time and peak area counts were calculated for each parameter and the CV values were found to be in agreement to the robustness of the method in the range 0.11-5.67%.

Resolution was affected when the method was tested on a column Kromasil C18, 250 mm x 4.6 mm, 5 μm particles, also minor change of percentage of acetonitrile in mobile phase and minor change of flow which had a great influence on both retention time and resolution.

Analysis of polyphenols in samples

The nine ethanolic propolis samples from Romanian market were analysed by the gradient RP-LC method with DAD. Samples compounds were identified by comparing their retention times and UV spectra with those of standard compounds (Fig. 1). From the corresponding calibration curve was calculated the content of each analyte. The content of polyphenols in samples using the proposed RP-LC method is shown in Table 5. There was a significant variability in the content of phenolic acids and flavonoids of the nine ethanolic propolis samples. For example, pinocembrin was a major compound and its content varied from 0.56 to 485.10 mg mL^{-1}. The maximum amount of total constituents was found in the sample 1, whereas the minimum amount, in the sample S5. Rutin was quantified only in S1, S8 and S9. All the samples have a high content of flavonoids; the results were in accordance with those reported by other studies on the composition of propolis from temperate zones.[22, 35-37]

CONCLUSIONS

An RP-HPLC method coupled with DAD for the simultaneous quantitation of seventeen polyphenols in propolis extracts has been developed and validated. From our information it is the first time when the commercial Roumanian propolis extracts were evaluated for polyphenolic contents. The LC method described in this paper is very suitable for qualitative and quantitative analysis of propolis extracts (and other extracts) owing to its acceptable precision and accuracy.

Table 5

The contents of phenolics in commercial propolis samples

Compound	Content (mg mL^{-1} , n=3)								
	S1	S2	S3	S4	S5	S6	S7	S8	S9
Rutin	26.66	-	-	-	-	-	-	0.32	2.06
Caffeic acid	98.64	1.12	0.91	1.26	0.32	2.06	1.31	0.85	2.70
Coumaric acid	122.39	1.64	2.29	2.73	0.85	2.70	1.48	0.70	2.69
Ferulic acid	135.03	1.81	2.02	2.52	0.70	2.69	1.56	-	0.10
Myricetin	9.43	0.10	0.10	0.10	-	0.10	0.10	0.10	0.81
Daidzein	31.83	0.37	0.36	0.02	0.10	0.81	0.38	0.10	0.19
Luteolin	13.50	0.15	0.12	0.12	0.10	0.19	0.16	0.12	0.44
Quercetin	30.04	0.38	0.19	0.19	0.12	0.44	0.45	0.16	0.68
Cinnamic acid	37.13	0.39	0.45	0.53	0.16	0.68	0.50	0.15	1.06
Naringenin	47.58	0.61	0.44	0.68	0.15	1.06	0.62	0.13	0.62
Kaempferol	37.28	0.41	0.26	0.29	0.13	0.62	0.46	0.10	0.41
Rhamnetin	27.42	0.32	0.18	0.17	0.10	0.41	0.37	0.60	4.75
CAPE	238.88	2.49	1.84	2.38	0.60	4.75	2.92	0.71	5.39
Chrysin	252.46	3.45	2.13	2.57	0.71	5.39	3.84	1.05	9.24
Pinocembrin	485.10	5.76	3.61	4.58	1.05	9.24	6.75	0.56	4.15
Galangin	238.59	2.39	1.52	1.62	0.56	4.15	2.77	0.32	2.21
Pinostrobin	121.77	1.35	1.16	1.31	0.32	2.21	1.36	0.32	2.06

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