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Development and Application of an LC-MS/MS Method for Identification of Polyphenols in Propolis Extract †

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Abstract: We identified and quantified by LC-MS/MS 11 (quercetin, galangin, pinocembrin, kaempferol, vanillin, chrysin, gallic acid, *p*-coumaric acid, trans-ferulic acid, caffeic acid, and caffeic acid phenethyl ester) out of the 21 polyphenolic compounds we looked for in ethanolic (25% and 50%) and aqueous propolis extracts by comparison with standards and literature data.

Keywords: propolis; LC-MS; polyphenolic compounds

1. Introduction

The purpose of this study is to identify the most common polyphenols found in Romanian propolis and quantify their levels in various hydroalcoholic extracts. In this regard, we have worked to develop an efficient and reliable method of analysis.

LC-MS is the method of choice in various environmental, pharmaceutical, and biochemical laboratories due to its selectivity, sensitivity, and versatility [1].

2. Materials and Methods

The LC-MS/MS analysis was carried out using a Q Trap 5500 Triple Quadrupole Mass spectrometer from Sciex with Electrospray Ionization (ESI)/Turbo Ion Spray mode. In the chromatographic analysis, a Synergi C18 (Fusion-RP 80 Å, 50 × 2 mm, particle size of 4 µm) column (Phenomenex Inc., Torrance, CA, USA) was used with an injection volume of 5 µL. The solvents used were (A) formic acid (0.5%) and (B) methanol. Gradient elution ranged from 2% to 98% B at 30 °C, and elution flow was set at 900 µL/min. The elution time was 20 min. The ionization source temperature of the MS was 500 °C; mass spectra were recorded in the negative ion mode, between 50 *m*/*z* and 500 *m*/*z* using nitrogen as the collision gas. The pressure of the gas flux to the nebulizer was set at 1000 psi.

ACS standards (quercetin, pinocembrin, galangin, kaempferol, vanillin, chrysin, gallic acid, *p*–coumaric acid, *t*-ferulic acid, caffeic acid, and caffeic acid phenethyl ester (CAPE)) were used to prepare individual 500 μg/mL stocks in ethanol. A mixed working standard, 10 μg/mL solution in ethanol, was obtained by appropriate dilution of individual stocks. Ethanol calibration solutions were prepared in a 0.08–5 μg/mL range. Automatic pipettes and class A volumetric glass flasks were used.

All solvents (ethanol, methanol, formic acid) were analytical grade and used without further purification.

Propolis extracts were prepared according to the procedure presented previously [2].

For the analysis of the samples, the type of targeted MS/MS scan was used, in which a selected ion is monitored on Q1 and a chosen fragment of the molecular ion on Q3. The sequence of analysis consisted of the injection from polypropylene filtration plates of the 2 blanks that contain mobile phase A, 7 mix solutions of polyphenols in the order of increasing the concentration 0.08, 0.1, 0.5, 1, 2, 3, 5 μg/mL; 2 blanks, 9 aqueous samples, 2 blanks, 1 calibration solution, 2 blanks, 9 25% ethanolic samples, 2 blanks samples, 1 calibration solution, 2 blanks, 9 50% ethanolic samples, 2 blanks samples, 1 calibration solution, 2 blanks, twice consecutive reinjection of a 25% ethanolic sample, 2 blanks.

3. Results and Discussion

Experimental parameters for each analyte were identified by direct injection in the MS module of individual standards, in the 0.001–0.1 μg/mL concentration range, resulting in the corresponding productions. Individual characteristics are collected in Table 1.

Table 1. MS experimental characteristics of the investigated compounds.

Compound	Parent Ion, Da	Precursor Ion, Da	DP[a], V	EP[b], V	CE[c], eV	CXP[d], V
Caffeic acid	178.9	134.9	−70	−10	−22	−13
p-Coumaric acid	162.9	118.9	−60	−10	−22	−9
Gallic acid	168.8	124.9	−65	−10	−20	−11
t-Ferulic acid	192.9	133.8	−70	−10	−22	−11
Kaempferol	284.9	92.9	−130	−10	−54	−7
Quercetin	300.9	135.8	−120	−10	−28	−11
Chrysin	253	208.9	−145	−10	−20	−17
Pinocembrin	255	212.8	−120	−10	−28	−28
Vanillin	150.9	135.8	−60	−10	−18	−9
CAPE	283	135	−120	−10	−72	−17
Gallangin	268.9	168.8	−105	−10	−36	−11

[a] Declustering potential, [b] entrance potential, [c] collision energy, [d] collision cell exit potential.

Selectivity has been investigated in terms of relative standard deviations of the retention times [3]. As data in Table 2 demonstrate, they did not exceed 0.21%.

Table 2. Method specificity.

Analyte Name	Retention Time, min	Relative Standard Deviation, %
Gallic Acid	0.262	0.020
Caffeic Acid	1.88	0.080
Vanillin	2.04	0.045
p-Coumaric Acid	2.32	0.024
t-Ferulic Acid	2.66	0.090
Quercetin	4.13	0.210
Kaempferol	4.64	0.070
Pinocembrin	4.86	0.120
CAPE	5.19	0.080
Chrysin	5.25	0.010
Galangin	5.26	0.050

Calibration curves were obtained for the 0.08–5 μg/mL concentration range for all analytes of interest. Experiments run at seven concentration levels, using at least two replicate injections for each concentration level, gave linear regressions in terms of peak area, characterized by correlation coefficients larger than 0.9988, except chrysin, with a determination coefficient of 0.9822, as shown in Table 3. The calibration curve for *t*-ferulic acid is presented in Figure 1.

Table 3. Calibration parameters.

Analyte	Intercept	Slope	R^2
Quercetin	1.35×10^4	1.84×10^6	0.9998
Chrysin	7.09×10^3	4.09×10^4	0.9822
Vanillin	2.83×10^4	1.07×10^6	0.9994
Pinocembrin	4.63×10^4	1.29×10^6	0.9990
Kaempferol	2.16×10^3	1.60×10^5	0.9995
CAPE	1.51×10^4	1.29×10^5	0.9988
t-Ferulic Acid	2.26×10^4	1.26×10^6	1
p-Coumaric Acid	2.83×10^5	1.17×10^7	0.9992
Gallic Acid	7.90×10^4	5.99×10^6	0.9991
Caffeic Acid	1.51×10^5	9.99×10^6	0.9995
Galangin	9.84×10^3	6.25×10^5	0.9988

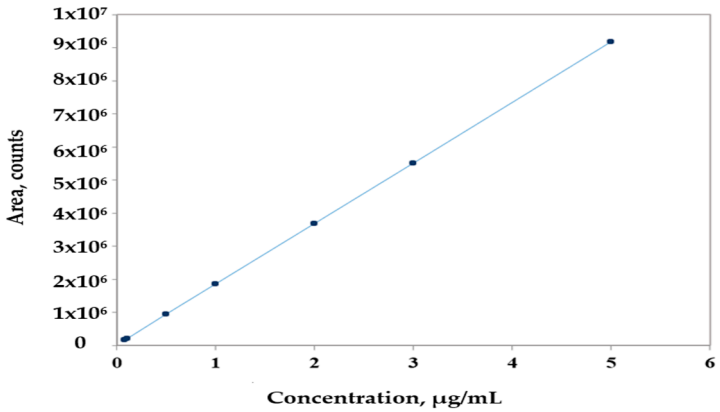

Figure 1. Calibration curve for *t*-ferulic acid.

Limit of quantitation, LOQ, and limit of detection, LOD, as shown in Table 4, were evaluated as per International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Guideline [3,4].

Table 4. Limit of quantitation (LOD) and limit of detection (LOQ) calculated for analytes of interest.

Analyte	LOD, μg/mL	LOQ, μg/mL
Quercetin	0.07	0.17
Chrysin	0.23	0.69
Vanillin	0.09	0.26
Pinocembrin	0.12	0.37
Kaempferol	0.08	0.24
CAPE	0.17	0.51
t-Ferulic Acid	0.01	0.03
p-Coumaric Acid	0.16	0.49
Gallic Acid	0.17	0.52
Caffeic Acid	0.12	0.30
Galangin	0.18	0.54

The method was applied for the analysis of Romanian propolis extracts. Figures 3 and 4 show typical LC-MS/MS chromatograms. The quantified levels of polyphenolics are collected in Table 5.

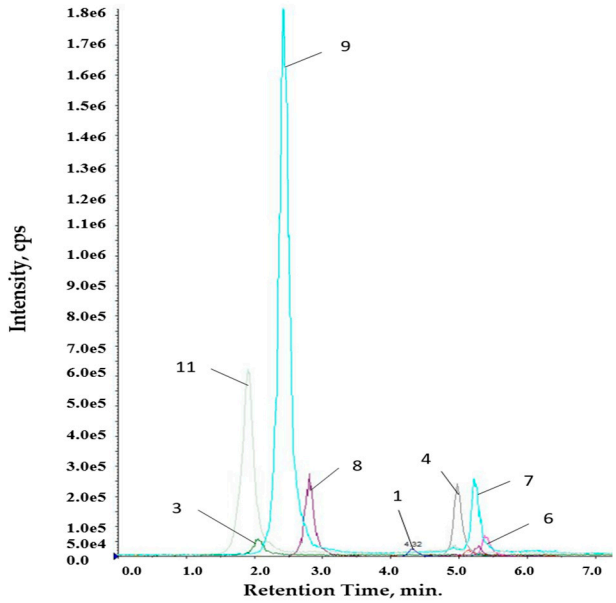

Figure 2. Chromatogram for ethanolic extract.

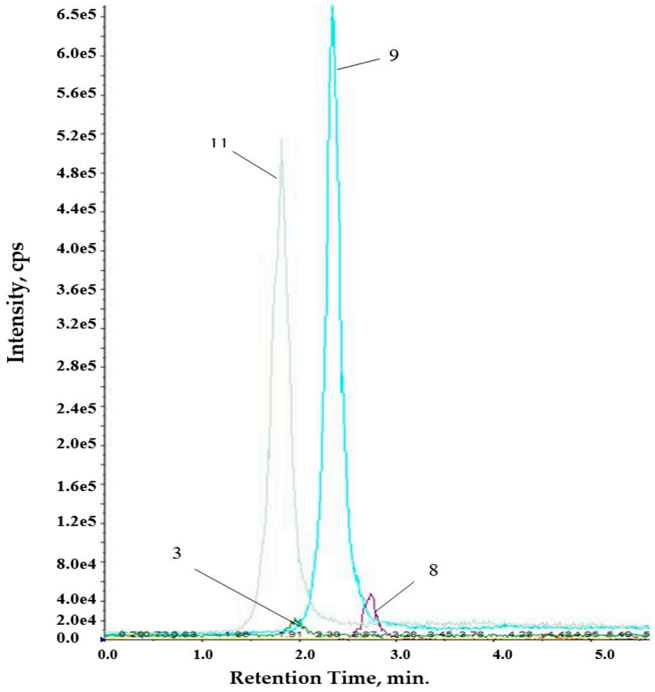

Figure 3. Chromatogram for aqueous extract.

The polyphenols identified in the propolis under study fall into two categories: compounds that are not extracted in water (quercetin, chrysin, pinocembrin, kaempferol, galangin, CAPE) and compounds that are extracted both in water and ethanolic solutions (*p*-coumaric acid, trans-ferulic acid, caffeic acid, vanillin).

Table 5. Polyphenolics in ethanolic and aqueous extracts.

Code	Compound Name	Ethanolic Extract		Aqueous Extract	
		Retention Time, min	Concentration, µg/mL	Retention Time, min	Concentration, µg/mL
1	Quercetin	4.311	0.834	-	-
2	Chrysin	-	-	-	-
3	Vanillin	2.040	3.589	1.964	0.292
4	Pinocembrin	4.978	10.50	-	-
5	Kaempferol	4.699	0.990	-	-
6	Galangin	5.389	6.781	-	-
7	CAPE	5.276	4.579	-	-
8	*t*-Ferulic Acid	2.808	13.26	2.744	0.794
9	*p*-Coumaric Acid	2.427	10.80	2.340	1.261
10	Gallic Acid	-	-	-	-
11	Caffeic Acid	1.910	4.873	1.821	1.330

4. Conclusions

The system used for the analysis of phenolic compounds in propolis extracts consisted of an Ultra High-Performance Liquid Chromatograph coupled with a 5500 Triple Quadrupole Mass Spectrometer and the mass spectra were recorded in the negative ion mode.

Calibration curves were obtained by injecting mixtures of exactly known concentrations (0.08; 0.1; 0.5; 1; 2; 3; 5 µg/mL), resulting in correlation coefficients larger than 0.9988, except for chrysin.

Relative standard deviations of the retention times were below 0.2%. The values corresponding to the detection limits were between 0.01 and 0.23 µg/mL and limits of quantitation had values in the range 0.03 µg/mL (for *t*-ferulic acid)—0.69 µg/mL (chrysin).

The use of the LC-MS analysis method proposed proved effective in quantifying 11 polyphenolics in aqueous and ethanolic extracts of propolis.

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Conflicts of Interest: The authors declare no conflict of interest.

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