



LC–QQQ and LC–QTOF MS methods for comprehensive detection of potential allergens in various propolis extracts

Lara Saftić¹ · Željka Peršurić¹ · Sandra Kraljević Pavelić¹

Received: 16 January 2019 / Revised: 23 April 2019 / Accepted: 30 May 2019
© Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract

Propolis is a highly complex matrix, in which phenolics are the mostly analyzed constituents. However, detailed propolis analysis is often lacking due to the high price of the commercially available phenolic standards and the lack of standards of phenols that are causing allergic reaction to propolis. Therefore, it is crucial to develop methods for detection and quantification of these constituents so that, afterwards, propolis extractions can be adjusted to the desired final application. Developed mass spectrometry-based methods were used for the quantification of 25 known phenols as well as for the identification and quantification of 27 additional phenolic derivatives, for which the standards are not readily available. We demonstrated that these newly discovered phenolic derivatives may have a high share in propolis, among which hydroxycinnamic are the most important ones as they are responsible for allergenic properties of propolis-based products. To track the changes in hydroxycinnamic acid derivatives, different propolis extract were prepared and analyzed with developed protocol. All obtained data were processed using chemometric tools to gain insight into allergens content in different propolis extracts. The presented protocol is in line with the food and pharma industry's growing needs for a comprehensive knowledge on the propolis composition, as it is used in a variety of functional and medicinal products. Knowing that some phenolic components may exert unwanted side effects, a precise selection of a fraction of compounds from propolis is essential for the development of safe-end products.

Keywords Propolis · Allergens · Phenolic compounds · LC–QQQ · LC–QTOF · Green extraction

Introduction

Propolis is known as a highly complex matrix composed of different molecular components, including wax, resins, balsams, pollen, some organic acids, phenolic compounds, vitamins, and minerals. Due to its hard and sticky structure, it is also called “bee glue”. It is made of resinous material from trees that is collected and enzymatically transformed by honeybees. In pharmacology, it is typically used as an ethanol tincture due to its proven antimicrobial, anti-inflammatory,

and anticancer activity [1]. All of its beneficial pharmacological activities are attributed to a high and diverse phenolic content. In the European type of propolis—poplar type propolis, which is the one that was used as a model in our research—several studies reported high content of flavonoids, phenolic acids and its esters, especially chrysin, pinocembrin, and ferulic and *p*-coumaric acid [2–4]. However, despite all beneficial effects of the mentioned phenolics, it was noticed that propolis can cause severe allergic reactions, especially its constituents—hydroxycinnamic acids. From all hydroxycinnamic acids, caffeic acid and its derivatives showed the highest activity in sensitization to propolis [5, 6].

Propolis phenolic fraction is, however, highly influenced by climate changes, vegetation, bees, and geographical origin. Therefore, the phenolic content (including allergens) is significantly different in propolis from different geographical areas. Accordingly, a full phenolic profile for each propolis used as raw material for the production of food supplements and/or medical products would be required to detect both

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00217-019-03308-x>) contains supplementary material, which is available to authorized users.

✉ Sandra Kraljević Pavelić
sandrakp@biotech.uniri.hr

¹ Department of Biotechnology, Centre for High-throughput Technologies, University of Rijeka, Radmile Matejčić 2, 51000 Rijeka, Croatia

phenols with beneficial effects on human health as well as those with potential side effects [7, 8]. There are few review papers describing all beneficial effects of propolis constituents on the human health and its potential in drug discovery [1, 9–13]. However, a standardization of propolis extracts is needed in modern production of medical products since propolis biological properties and chemical composition are highly variable.

Most often, propolis is standardized according to its phenolic content. Phenols, especially flavonoids and phenolic acids, are plant secondary metabolites with strong pharmacological activity [14]. Yet, due to their often unstable structure and thermal lability, the development of methods for their qualitative and quantitative analysis as well as methods for their extraction from the plant material has been a continuously demanding process [14].

In this paper, a previously developed method [15], based on liquid chromatography (LC) coupled to triple quadrupole mass spectrometry (QQQ), and a confirmation method [15], based on LC coupled to quadrupole high-resolution time-of-flight mass spectrometry (LC–QTOF), were used for a metabolomics profiling of polyphenols in propolis samples. Additional screening for phenolic derivatives was obtained using the Precursor ion scan mode of the LC–QQQ instrument to detect all compounds that have the same MS/MS fragments as researched phenols with available standards. Afterwards, a developed MS protocol was used to track changes in the potential allergen content in different propolis extracts. Special attention was paid to green extraction procedures based on the use of water and olive oil to develop cheap and environmentally friendly extracts for food and pharmaceutical application.

Materials and methods

Reagents and materials

Apigenin, pinobanksin, naringenin, *p*-coumaric acid, kaempferol, myricetin, luteolin, oleuropein, chrysin, 2,5-dihydroxybenzoic acid (2,5-DHBA), 3,4-dihydroxybenzoic acid (3,4-DHBA), caffeic acid, trans-cinnamic acid, vanillic acid, and 4-hydroxybenzoic acid (*p*HBA) were obtained from Sigma-Aldrich (MO, USA). Gallic acid was obtained from Alfa Aesar (Thermo Fischer Scientific, Massachusetts, SAD). Pinocembrin, sakuranetin, rhamnetin, ferulic acid, tectochrysin, and galangin were obtained from Extrasynthese (France). Apigenin-7-*O*-glucoside and verbascoside were obtained from HWI Analytik GMBH (Germany).

Chemical reagents, methanol (HPLC grade) and acetonitrile (LC–MS grade), were supplied by Honeywell research chemicals, and formic acid (LC–MS grade) was supplied

by Sigma-Aldrich. Ultra-pure water was obtained using the mili-Q water purification system ($< 0.058 \mu\text{S/cm}$, mili-Q model Pacific TII 12, Thermo Scientific, Massachusetts, SAD).

All sample extracts were filtrated before analysis with Chromafil cellulose acetate microfilters ($0.45 \mu\text{m}$, 25 mm) (Macherey–Nagel, Germany).

Samples

For the purpose of MS methods development, three propolis samples were collected and stored at -20°C until analysis. All of the propolis samples were from Croatia, collected directly from registered producers. Prior to the extraction, propolis samples were ground using the FreezerMill 6775 Cryogenic grinder (SPEXEurope, UK).

Propolis extraction procedure for MS methods development

30 mg of the homogenized propolis sample was extracted with 2 ml of methanol. Afterwards, samples were sonicated in an ultrasonic bath (Sonorex digitec, Bandelin, Germany) for 10 min at constant temperature of 25°C , diluted 10 times and filtrated.

Water extraction procedures

W1–W5 label extractions were prepared by dissolving 30 g of propolis in 2 ml of milli-Q water and subjected to a 10-min sonication. W1 extract was prepared using milliQ water at neutral pH, whereas, for the preparation on W2 extracts, milli-Q water was acidified with formic acid to pH 2.5. Extracts W3–W5 were prepared using milliQ water with addition of glycerol in the ratios shown in Table 1. After the sonication, all extracts were filtered and analyzed.

Olive oil extraction procedures

For the preparation of olive oil-based propolis extracts (OO1 and OO2), 100 mg of propolis was dissolved in 2 ml of extra virgin olive oil. Afterwards, samples were sonicated for 10 (OO1) or 30 min (OO2). 200 μL of the olive oil layer was mixed with 1400 μL of methanol and sonicated for 10 min and afterwards frozen at -20°C (to facilitate separation of methanol from the oily layer). The methanol layer was then filtered and analyzed.

Methanol extraction procedures

Methanol-based extracts were prepared by dissolving 30 g of propolis in 2 ml of 100% methanol (M1) or a 50:50 v:v milli-Q:methanol (M2–M5) solution. For M3–M5 extracts,

Table 1 Codes and parameters of individual extraction procedure

Code	Water (%)	Methanol (%)	USE (min)	Glycerol (%)	Olive oil (%)	pH
W1	100		10			Neutral
W2	100		10			2.50
W3	95		10	5		Neutral
W4	90		10	10		Neutral
W5	80		10	20		Neutral
OO1			10		100	Neutral
OO2			30		100	Neutral
M1		100	10			Neutral
M2	50	50	10			Neutral
M3	50	50	10			2.5
M4	50	50	30			2.5
M5	50	50	45			2.5
A1	100		10			11.5
A2	50	50	10			11.5
A3	50	50	30			11.5

milli-Q was acidified with formic acid to pH 2.5. All extracts were sonicated for 10 min (M1–M3), 30 min (M4), or 45 min (M5) (Table 1) and were afterwards filtered, diluted 10 times with methanol, and analyzed.

Alkaline extraction procedures

The alkaline extract A1 was prepared by dissolving 30 g of propolis in 2 ml of alkalinized milli-Q pH 11.5. Extracts A2 and A3 were prepared using an alkalinized solution (pH 11.5) of 50:50 v:v milli-Q:methanol. All extracts were sonicated for 10 or 30 min, as indicated in Table 1. After the sonication, the extracts were neutralized by HCl until pH 7, filtered, and analyzed.

LC–QQQ analysis

An Agilent 1260 series HPLC chromatograph equipped with a degasser, binary pump, auto-sampler, and column oven coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with a jet stream electrospray (AJS ESI) source (Agilent Technologies, Palo Alto, CA, USA) was used for quantitative analysis of the propolis samples. All data processing was obtained using the Mass Hunter workstation software (version B.07.00, Agilent Technologies, Palo Alto, CA, USA).

Chromatographic analyses were carried out on Zorbax SB-C18, Rapid resolution HT, 600 bar column (2.1 mm × 50 mm I.D., 1.8 µm, Agilent Technologies). The mobile phase was composed of (A) 0.1% formic acid in milliQ water and (B) 0.1% formic acid in acetonitrile. The gradient elution was as follows: 0–0.9 min linear gradient from 1% to 10%B, 0.9–3 min from 10% to 20%B, 3–4.5 min from 20% to 25%B, 4.5–6 min from 25% to 30%B, 6–7.5

30%B, 7.5–9 min from 30% to 90%B, 9–9.30 90%B, 9.30–9.60 from 90% to 10%B and 9.60–15 min from 10 to 1%B. The post time was set to 2 min. The flow rate was 0.33 ml/min. The column oven was maintained at 30 °C. The sample injection volume was 2.5 µL. All samples were injected in duplicates. For AJS-ESI-QQQ, the parameters were set as follows: capillary voltage was 3.5 kV in both the positive and negative mode, nozzle voltage 0.5 kV, ion source temperature was set to 300 °C, gas flow was 5 l/min, nebulizer pressure was 45 psi, drying gas temperature was 250 °C and sheath gas flow was 11 l/min. Nitrogen was used as collision gas. The collision energies were in the range between 0 V and 40 V. The precursor and product ions, fragmentor, and collision energies are listed in Table 2.

For the quantification of phenolic compounds without available standards on LC–QQQ instrument, analysis was carried out over the precursor phenolic compound and set with the same parameters as in the previously described method, but in the dynamic MRM (DMRM) mode. The DMRM mode showed upgrading in the field of data quality by obtaining the analysis in the specific time with a delta retention time for each analyzed compound. The fragmentor and collision energies for these compounds were the same as those previously determined for the available standards by means of which the quantification was carried out. The quantification was carried out using the calibration curve of the phenolic compound with a similar chemical structure, but the final result was multiplied by the molecular weight correction factor. The molecular weight correction factor was calculated by dividing the molecular weight of the phenolic derivative to be quantified with that of the standard used for its quantification. Multiplication with the factor was used to convert all phenolic derivatives to a form which has an existing standard (Table 3).

Table 2 LC–QQQ method parameters of phenolic acids and flavonoids’ analysis of propolis extracts

Phenolic compound	RT (min)	Polarity	Fragmentor (V)	m/z precursor ion	m/z product ion ^a	Collision energy (V)	$Y = ax + b$		Linearity range (µg/ml)	R^2	LOD (µg/ml)	LOQ (µg/ml)
							Slope (a)	Intercept (b)				
2,5-DHBA	5.27	–	80	152.8	108.0^a 81.8 53.0	20 16 20	15,806.66	33.47	0.01–7.5	0.995	0.0070	0.0212
3,4-DHBA	1.76	–	90	152.9	108.0 81.0 53.0	20 16 18	3761.51	15.01	0.05–7.5	0.998	0.0132	0.0399
Apigenin	10.33	–	160	268.9	149.0 116.9	24 22	41,665.02	7.67	0.001–0.5	0.995	0.0006	0.0018
Apigenin- <i>O</i> -7-glucoside	7.55	–	200	430.9	310.9 268.1	24 36	90,425.92	–7.26	0.001–0.5	0.997	0.0003	0.0008
Caffeic acid	5.35	–	100	178.8	135.0 116.9 88.9	12 24 34	27,277.16	206.79	0.05–5.0	0.991	0.0250	0.0758
Chrysin	11.32	+	170	254.8	153.3 103.4 69.3	30 36 50	18,930.41	59.95	0.05–5.0	0.992	0.0105	0.0317
Ferulic acid	6.70	–	100	192.9	177.9 149.0 134.0	8 6 12	2289.69	4.91	0.01–10.0	0.997	0.0071	0.0214
Galangin	11.37	–	170	268.9	252.0 168.9 142.9	24 24 30	2707.41	530.78	0.25–1.0	0.995	0.6470	1.9605
Gallic acid	0.86	–	100	168.8	125.0 78.9	10 20	11,611.28	58.78	0.05–7.5	0.998	0.0167	0.0506
Kaempferol	10.56	+	200	286.8	241.6 153.5 121.3	30 36 32	6487.14	56.22	0.05–2.5	0.997	0.0286	0.0867
Luteolin	9.11	–	180	284.9	150.9 133.0	24 36	26,778.66	63.89	0.01–5.0	0.994	0.0079	0.0239
Myricetin	7.831	–	150	316.8	151.0	22	8864.58	481.24	0.5–2.5	0.992	0.1792	0.5429
Naringenin	10.15	–	140	270.9	151.0 118.9	12 24	33,448.04	10.74	0.005–0.5	0.998	0.0011	0.0032
<i>p</i> -Coumaric acid	6.27	–	90	162.9	119.0 92.8 64.9	12 36 48	30,897.42	769.42	0.1–2.5	0.991	0.0822	0.2490
<i>p</i> HBA	4.29	–	80	136.9	93.0 64.8	12 34	18,186.76	29.97	0.01–1.0	0.992	0.0054	0.0165

Table 2 (continued)

Phenolic compound	RT (min)	Polarity	Fragmentor (V)	<i>m/z</i> precursor ion	<i>m/z</i> product ion ^a	Collision energy (V)	$Y = ax + b$		Linearity range (µg/ml)	R^2	LOD (µg/ml)	LOQ (µg/ml)
							Slope (<i>a</i>)	Intercept (<i>b</i>)				
Pinobanksin	10.10	–	140	271.0	252.9	18	10,928.37	27.17	0.01–1.0	0.998	0.0082	0.0248
					225.0	18						
					196.6	24						
					160.7	24						
Pinocembrin	11.30	–	140	254.8	212.9	16	1910.20	8.66	0.025–0.5	0.999	0.0150	0.0453
					150.8	20						
Quercetin	9.15	–	150	300.9	178.8	14	17,670.76	–23.67	0.01–1.0	0.992	0.0044	0.0134
					151.0	18						
					120.9	24						
					165.0	18						
Rhamnetin	8.31	–	160	314.9	121.0	24	47,328.35	–666.83	0.05–10.0	0.995	0.0465	0.1409
					118.9	28						
Sakuranetin	11.12	–	110	284.9	164.9	12	1272.05	–24.48	0.1–0.5	0.989	0.0635	0.1924
					92.8	10						
					226.6	34						
					131.1	18						
Trans-cinnamic acid	5.22	–	90	166.9	102.9	2	260.86	19.43	0.5–5.0	0.991	0.2458	0.7448
					151.8	10						
					122.9	6						
					107.9	14						
Verbascosid	6.96	–	200	623.0	461.4	28	16,911.73	–8.68	0.005–10.0	0.994	0.0017	0.0051
					161.0	38						

^aQuantifier ions are denoted in bold

Table 3 List of phenolic compounds without standard available and their MRM parameters for LC–QQQ analysis

Phenolic derivative	RT (min)	Polarity	<i>m/z</i> precursor ion	<i>m/z</i> product ion ^a	MWCF	Calibration curve
Caffeic acid cinnamyl ester	11.4	–	295	178.0 134.0^a 112.0	1.64	Caffeic acid standard calibration curve
Caffeic acid phenylethyl ester	11.2	–	283	179.0 163.0 135.0	1.58	
Caffeic acid benzyl ester	11.2	–	269	178.0 161.0 134.0	1.50	
Caffeic acid prenyl ester	11.1	–	247	179.0 161.0 135.0 112.0	1.38	
Dymethyl caffeic acid	7.7	+	209	163.0 141.0 133.0 120.0 119.0	1.15	
Caffeic acid methyl ester	7.0	+	196	178.0 177.0 134.0 89.0	1.09	
Caffeic acid derivative 1	10.6	–	457	179.1 161.0 135.0	2.55	
Caffeic acid derivative 2	8.4	–	414.9	253.0 178.9 163.0 160.7 134.6	2.32	
Cinnamic acid derivative 1	6.2	–	387	343.0 284.0 269.0 225.0 193.1 148.1 133.9	2.01	Ferulic acid standard calibration curve
Cinnamic acid derivative 2	6.6	–	375	178.9 160.7 135.0	2.10	Caffeic acid standard calibration curve
<i>p</i> -Coumaric acid prenyl ester	11.4	+	233	163.0 146.6 118.8 92.9	1.41	<i>p</i> -Coumaric acid standard calibration curve
<i>p</i> -Coumaric acid cinnamyl ester	7.5	+	281	241.0 192.6 162.1 147.0 118.6	1.71	
<i>p</i> -Coumaric acid derivative 1	7.1	–	329	162.8 145.1 119.0 84.7	1.42	
<i>p</i> -Coumaric acid derivative 2	7.2	–	359	195.0 163.0 145.0 119.0	1.71	

Table 3 (continued)

Phenolic derivative	RT (min)	Polarity	<i>m/z</i> precursor ion	<i>m/z</i> product ion ^a	MWCF	Calibration curve
<i>p</i> -Coumaric acid derivative 3	6.1	–	279	163.0 145.0 119.0	2.02	
<i>p</i> -Coumaric acid derivative 4	10.3	–	382.9	219.0 163.0 145.0 119.0	2.20	
<i>p</i> -Coumaric acid derivative 5	6.1	–	357	192.9 162.8 133.8 118.6 81.8	1.71	
<i>p</i> -Coumaric acid derivative 6	10.9	–	441	179.0 163.0 118.8	2.35	
Pinobanksin-5-methyl-ether-3- <i>O</i> -acetate	10.8	–	327	284.8 267.0 239.0 224.0 195.8	1.20	Pinobanksin standard calibration curve
Pinobanksin-3- <i>O</i> -acetate	11.2	–	313	270.1 252.9 224.5 196.8	1.15	
Pinobanksin-3- <i>O</i> -butyrate	11.5	–	341	271.0 252.9 196.7 146.5	1.26	
Pinobanksin-3- <i>O</i> -propionate	11.48	–	327	271.0 253.0 225.0 209.0 181.0	1.20	
Pinobanksin-5-methyl-ether	10.5	–	285	271.0 251.9 240.9 223.9	1.05	
Pinobanksin-3- <i>O</i> -hexanoate	12.2	–	367	271.5 252.6 200.8	1.35	
Pinobanksin-3- <i>O</i> -pentanoate or 2-methylbutyrate	12.05	–	355	271.2 253.0	1.31	
Quercetin-3,7-dimethyl ether	10.6	–	329	314.0 301.0 285.0 271.0	1.05	Quercetin standard calibration curve
Quercetin-3-methyl ether	9.0	–	315	300.0 270.7 255.0	1.64	

^aQuantifier ions are denoted in bold

Reference phenolic compounds were dissolved in methanol and diluted in appropriate concentrations to establish the calibration curves. For quantitative analysis, 15 different concentrations for each standard were made

and linearity ranges of their calibration curves are shown in Table 2. The concentrations of all the standards were injected into triplicates. Calibration curves were constructed using the linear regression and were not forced

to pass through zero. Also, a $1/\times$ statistical weight was applied to obtain the most reliable calibration curves for all phenolics. Calibration curves ranges were achieved on the basis of the linearity of the responses for each individual metabolite (Table 2). Linearity was determined using the coefficient of determination (R^2). The limit of detection (LOD) and the limit of quantification (LOQ) were determined using a quantifier transition according to the International conference on Harmonization (ICH) guidelines [16].

LC–QTOF analysis

For qualitative analysis, an Agilent 1290 series UPLC chromatograph equipped with a degasser, binary pump, autosampler, and column oven coupled to an Agilent 6550 iFunnel quadrupole time-of-flight mass spectrometer equipped with dual AJS ESI source (Agilent Technologies) was used.

The chromatographic conditions, column and gradient were the same as in the LC–QQQ method. For the AJS–ESI–QTOF, the parameters were set as follows: capillary voltage was 3.5 kV in both the positive and negative mode, nozzle voltage 1 kV, ion source temperature was set to 200 °C, gas flow was 14 l/min, nebulizer pressure was 35 psi, drying gas temperature was 350 °C, and sheath gas flow was 11 l/min. Accurate mass measurements were obtained by means of ion mass correction techniques using reference masses at m/z 121.0509 (protonated purine) and 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921] in positive ion mode, while m/z 112.9856 [deprotonated trifluoroacetic acid (TFA)] and 1033.9881 (TFA adducted HP-921) were used in the negative ion mode. The reference solution was introduced into the AJS ESI source and the compounds were present in each spectrum.

Statistical data processing

Principal component analysis (PCA) was performed using the UNSCRAMBLER software version 10.4 (Computer Aided Modeling (CAMO), Trondheim, Norway).

Results and discussion

MS methods development

Propolis samples for the MS methods development were prepared with an optimized methanol extraction procedure. The methanol extraction enhanced with ultrasound was chosen because the obtained extracts had the highest phenolic content. Table 2 summarizes all phenolic compounds analyzed and identified with the reference standard, while Table 3

summarizes all phenols that were analyzed with Precursor and Product ion scans on the LC–QQQ instrument (using the MS/MS parameters of phenolics that were previously determined with reference standards). These compounds were identified by the MS/MS data published in the literature or by means of diagnostic fragments not reported in the literature. All compounds without an available reference standard available were also detected with the LC–QTOF instrument and, for compounds not reported in the literature, tentative identification and molecular formula were provided using the instrument MassHunter software (Online Resource 1).

Results showed that most of detected flavonoids in propolis samples were aglycones [2], which is in accordance with previous research on the composition of the European poplar type propolis [8, 17]. As a lot of researches were focused on the fragmentation pathways of flavonoids and phenolic acids, these will be mentioned below very briefly to better understand the occurrence and confirmation of the structure of new phenolic compounds [18–22]. Tables 2 and 3 contain lists of detected and quantified phenols in propolis samples and, as it can be seen, most of the flavonoid glycosides found in the samples are derived from pinobanksin. All pinobanksin derivatives were identified via a specific fragment m/z of 271 in the negative ion mode that correspond to the deprotonated pinobanksin ion, and further m/z ion at 253 that stays as a loss of H_2O group. Afterwards, their structures were additionally confirmed with LC–QTOF and correlated with the data in the literature and confirmed [2, 23, 24]. The quercetin-3-methyl ether and quercetin-3,7-dimethyl ether were detected over the LC–QQQ mode Precursor ion scan using m/z values obtained from the quercetin standard optimization. The fragmentation pattern for flavonol glycosides is well described in the literature, so all the obtained results were compared and confirmed [2, 25]. They all have the characteristic 301 m/z fragment that indicates the deprotonated quercetin ion and ions 271 m/z and 151 that were confirmed by fragmentation of the quercetin reference standard.

Phenolic acids are divided into two subgroups: hydroxycinnamic and hydroxybenzoic acids. All analyzed benzoic acids had available standards (Tables 2 and 3), and the propolis fraction of hydroxycinnamic acids and their derivatives will be therefore presented. In our research, we managed to detect a high proportion of hydroxycinnamic acids. The fragmentation of reference standards of the hydroxycinnamic phenolic acid revealed that this group of compounds has a common fragmentation pattern—loss of the carboxyl group that corresponds to the loss of -44 Da. Among them, caffeic and *p*-coumaric acid derivatives had the highest share. The MS/MS pathway and side effects of the mentioned caffeic acid derivatives, i.e., propolis allergens are already described in the literature and their presence is analyzed in our propolis samples [3, 4, 22–24]. We confirmed the presence of the caffeic acid cinnamyl ester, caffeic acid phenylethyl ester

(CAPE), caffeic acid benzyl ester, caffeic acid prenyl ester, dimethyl caffeic acid (DMCA), caffeic acid methyl ester, and two other possible caffeic acid derivatives.

These derivatives were identified using the Precursor ion scan of the LC–QQQ instrument by looking for specific fragments of the caffeic acid fragmentation pathway: m/z 178, 179, 135, and 134. Caffeic acid phenylethyl ester and caffeic acid prenyl ester were identified due to their two specific fragments: 179 m/z that is $[M-H-2CH_3]^-$ ion and 135 m/z that is $[C_9H_7O_4-CO_2]^-$. Caffeic acid cinnamyl ester, caffeic acid benzyl ester, and caffeic acid methyl ester were identified due to m/z 178 $[C_9H_6O_4-H]^-$ and m/z 134 that is $[C_9H_6O_4-H-CO_2]^-$ ion. Other caffeic acid derivatives were also confirmed, such as dimethyl caffeic acid and caffeic acid methyl ester, both in the positive ion mode (Tables 2 and 3). Dimethyl caffeic acid showed the MS/MS fragment at m/z 163 corresponding to the loss of the H_2O-CO section and m/z 133 corresponding to loss of the $H_2O-CO-2CH_3$ section. These fragments had also been confirmed previously [24]. Also, their retention times (Table 2) were the same as provided in the literature [3]. The caffeic acid had the retention time 5.35 min, and after caffeic acid, methyl and then dimethyl caffeinate were detected. Other caffeinates were detected in the following RT order: prenyl ester < benzyl ester < phenylethyl ester < cinnamyl ester. However, other unconfirmed derivatives of caffeic acid were also spotted. First, the caffeic acid derivative with m/z 457.1153 was detected in the negative ion mode, with confirmatory caffeinate fragments m/z 179, 161, and 135 and the tentative molecular formula $C_{23}H_{22}O_{10}$. Another possible caffeic acid derivative was detected at m/z 415.1036 with the same fragments that had previously been confirmed for caffeinates m/z 179 and 135. Furthermore, two phenolic acids with hydroxycinnamic acid residues were observed, and their fragments are provided in Table 3. The hydroxycinnamic acid derivative 1, due to the obtained fragment m/z 193 that is probably the deprotonated ferulic acid $[C_{10}H_{10}O_4]^-$, was quantified via the ferulic acid standard calibration curve. The hydroxycinnamic acid derivative 2 had fragments m/z 179 and 135 specific for caffeic acid and, therefore, was quantified via its calibration curve.

Beside the high fraction of caffeic acid derivatives, *p*-coumaric acids derivatives were also highly represented in the tested European propolis samples. Given that it belongs to the same class with the caffeic acid, cross-reactivity may occur, so these natural constituents can also be potential allergens. Here, we detected and identified the *p*-coumaric acid prenyl ester and *p*-coumaric acid cinnamyl ester. Also, additional six new *p*-coumaric derivatives were detected and their molecular formulas were provided. Their fragments of m/z 163 $[C_9H_7O_3]^-$ specific for the phenolic acid and m/z 119 $[C_9H_7O_3-H-CO_2]^-$ as a loss of CO_2 group revealed two already confirmed *p*-coumaric acid derivatives and six

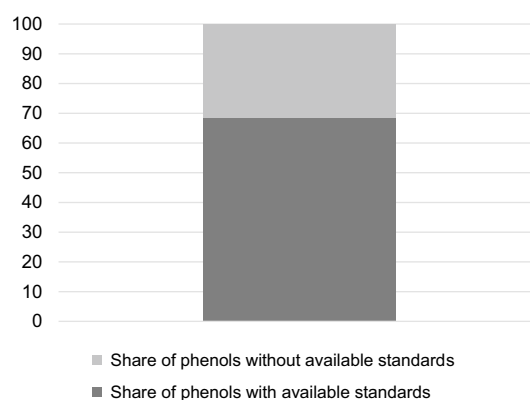


Fig. 1 Ratio between the concentrations of phenols with standards available in this research and phenols and phenolic derivatives without available standards, i.e., mostly the hydroxycinnamic acid and pinobanksin derivatives in propolis samples

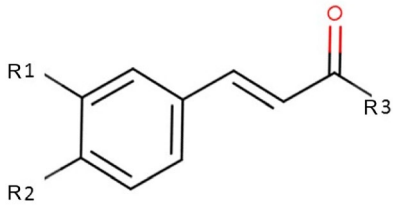
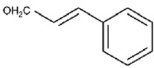
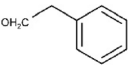
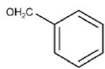
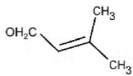
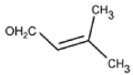
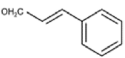
new compounds (Table 2). The *p*-coumaric acid prenyl and cinnamyl esters were confirmed previously in the literature [3, 17], whereas the *p*-coumaric acid prenyl ester was seen in the negative ion mode with the m/z precursor ion of 231.1030 and specific fragments m/z 163, 119, and 118, and *p*-coumaric acid cinnamyl ester in the same conditions with the precursor ion m/z 279.0877 and the fragments m/z 163, 145, and 119. The fragment ion m/z 145 indicates a loss of the H_2O group $[C_9H_7O_3-H-H_2O]^-$. Furthermore, six new possible *p*-coumaric acid derivatives were observed in the propolis sample. They were all detected using the same strategy as for the caffeic acid derivatives (Precursor ion scan) and they all had both characteristic ions for the *p*-coumaric acid: m/z 163 and 119. Figure 1 shows the presence of these hydroxycinnamic acids and their derivatives in the propolis samples in high concentrations—up to 32% (calculated as % of hydroxycinnamic acid derivatives in the sum of all analyzed phenols), and should not be neglected when doing the standardization of propolis. Structures of all detected and identified hydroxycinnamic acids and their ester are presented in Table 4.

Characterization of different propolis extracts

The developed MS method was applied for the identification and quantification of phenolic compounds in different propolis extracts. Namely, three different solvents (water, olive oil, and methanol) with modification of pH, USE time and glycerol addition (5, 10, and 20%) were tested to obtain extracts with a high yield of phenolic compounds but a low share of potential allergens. Finally, PCAs were performed to determine the effect of the extraction procedures on the phenolic composition, especially on potential allergens.

First, two groups of extractions were tested: methanol-based, as they extract the highest amount of phenols and

Table 4 Structures of hydroxycinnamic acid derivatives identified and quantified in propolis samples

Compound			
	R1	R2	R3
hydroxycinnamic acid	H	H	OH
caffeic acid	OH	OH	OH
caffeic acid cinnamyl ester	OH	OH	
caffeic acid phenylethyl ester (CAPE)	OH	OH	
caffeic acid benzyl ester	H	OH	
caffeic acid prenyl ester	H	OH	
dymethyl caffeic acid (DMCA)	OCH ₃	OCH ₃	OH
caffeic acid methyl ester	OH	OH	CH ₃
<i>p</i> -coumaric acid	H	OH	H
<i>p</i> -coumaric acid prenyl ester	H	OH	
<i>p</i> -coumaric acid cinnamyl ester	H	OH	
ferulic acid	OCH ₃	OH	OH

non-methanol extractions as green solution for phenol extraction. For non-methanol methods, water and olive oil were selected with the adjustment of pH and the addition of glycerol, as they are nonhazardous solvents that can be immediately released for human usage. Results clearly showed that there is no one answer to the selection of the extraction procedure that would allow a reduced amount of allergens. Each extraction procedure in the analysis allowed the extraction of the other fraction of hydroxycinnamic derivatives. Therefore, the selection method should be based on the avoidance or aspiration towards specific derivatives. For example, for a lower amount of the caffeic acid prenyl ester (CAPE), i.e., the strongest allergen in propolis, one would prefer the water-based extraction procedure. These

results gave the vision of the composition of propolis extract and possible allergen composition, and can be used as a base in the development of safer propolis-based products.

In Fig. 2, we preformed PCA projections using all analyzed phenols (with previously described MS protocol) as variables. In PCA with methanol-based extracts (Fig. 2a), the first two components account for 86% of the total model variability. As it can be seen, alkaline methanol-based extraction methods (A2 and A3) provide extracts with highly different phenolic constituents than extractions with a lower pH (M2–M5). This finding is attributed to the partition coefficient (logD) of analyzed phenols. Namely, the logD value is the distribution coefficient, i.e., the concentration of all forms (ionized and nonionised) of each phenol in a mixture

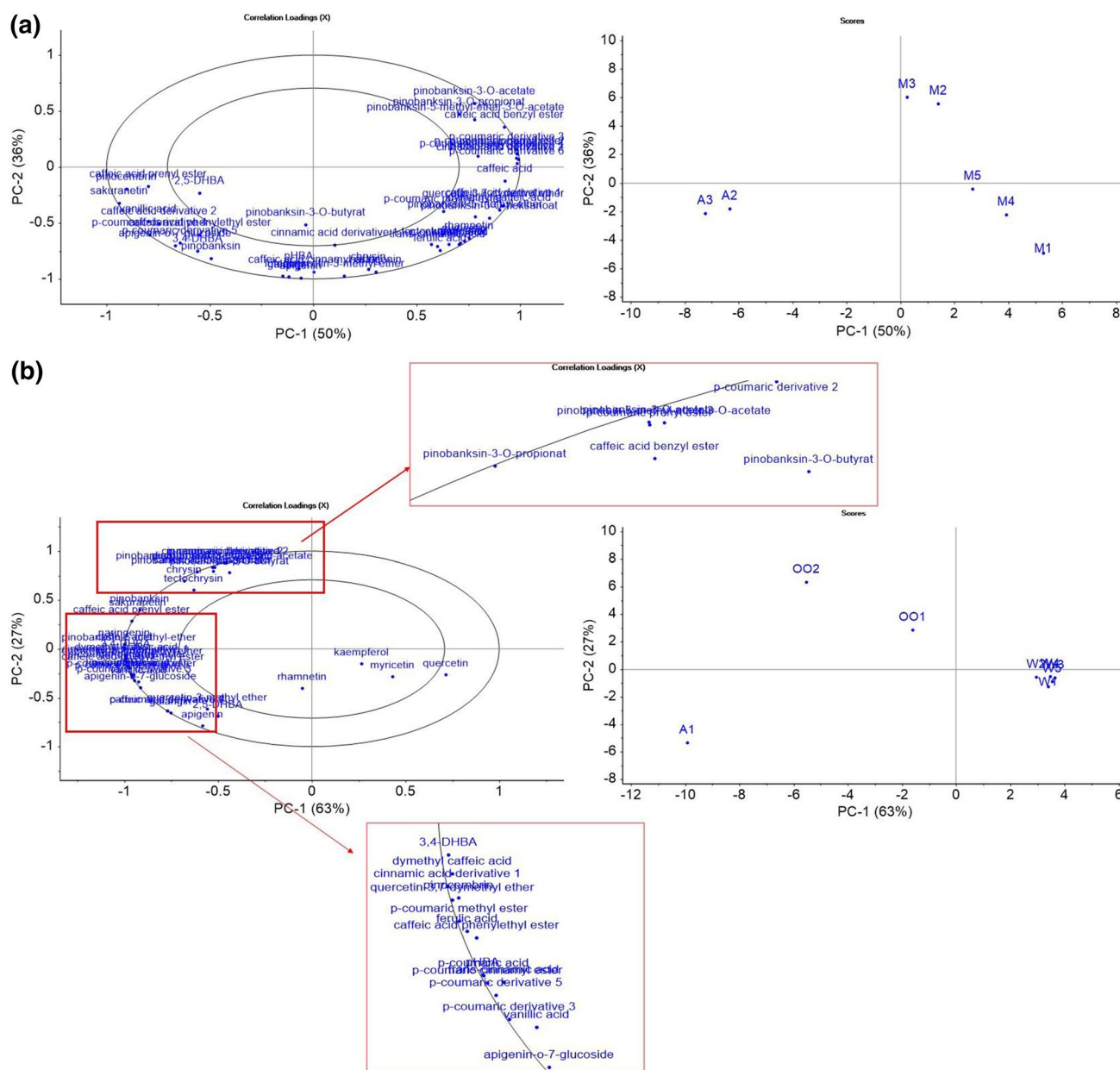


Fig. 2 Distribution of different **a** methanol-based, **b** non-methanol extracts in the space of the principal component 1 and principal component 2 (PC-1 to PC-2), when all analyzed phenols were used as

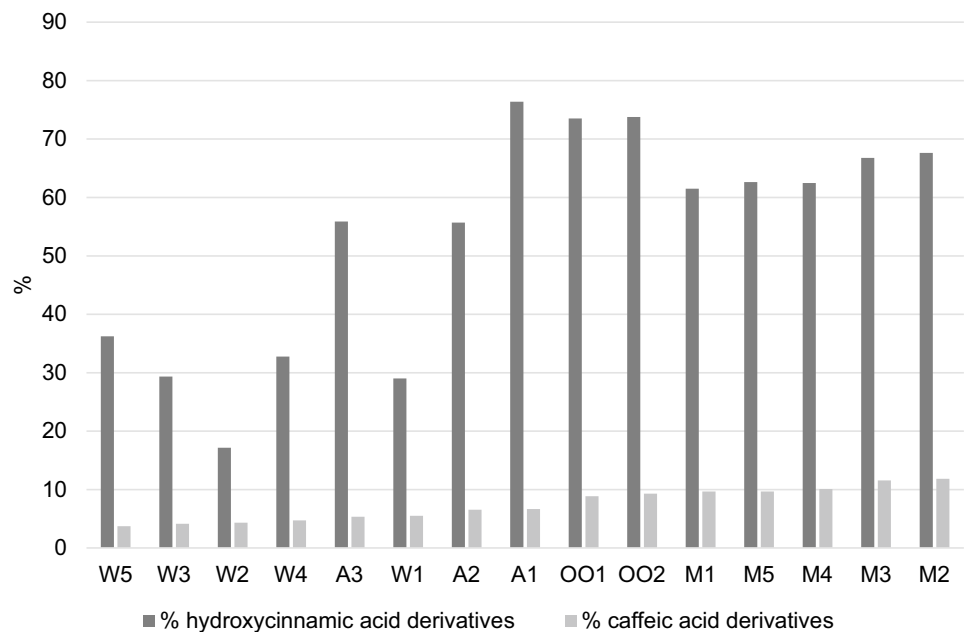
variables. *W* water-based extracts, *OO* olive oil extracts, *A* alkaline extracts, *M* methanol extracts

of two phases: polar and nonpolar. Based on this value, it is possible to determine the lipophilicity of the molecule at each pH value. logD values of most phenols are reduced by increasing the pH of the solution, thus decreasing their lipophilicity. Consequently, the pH increase will cause a higher affinity of the phenolic component to the polar environment so that the phenol at a higher pH will be more soluble in water than methanol or oil. This finding is also a reason why methanol-based extractions with a lower pH had a higher phenolic yield (Online Resource 2).

Furthermore, we analyzed non-methanol extractions (Fig. 2b) and showed that alkaline and oil-based extraction provided the final extracts with a larger amount of phenols than water extractions W1–W5. However, these extractions are not immediately ready for use: alkaline extracts need to be neutralized and an oil-based extract has a limited usage.

In Fig. 3, a graphical presentation is divided into the total amounts of caffeic and hydroxycinnamic acids. This is mainly due to the fact that all analyzed hydroxycinnamic acid derivatives are potential allergens, while caffeic acid

Fig. 3 The relationship between the proportion of the analyzed and identified caffeic acid derivatives in propolis extracts (determined as the proportion of the caffeic acid derivatives in the total value of all analyzed phenolic components) and the relationship between the proportion of all hydroxycinnamic acid derivatives analyzed in propolis extracts (determined as the proportion of the hydroxycinnamic acid derivatives in the total value of all analyzed phenolic components). The extracts are lined by ratio of the caffeic acid derivatives in the extract, from the least total amount of caffeic acid derivatives to the highest. *W* water-based extracts, *OO* olive oil extracts, *A* alkaline extracts, *M* methanol extracts

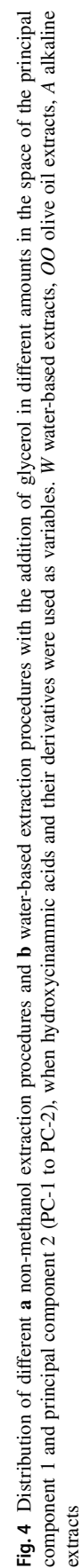


derivatives are confirmed allergens. It can be seen that the extraction M1, which provides the highest phenolic yield, does not extract the largest amounts of the caffeic acid derivatives. However, other methanol-based extracts (M2–M5) contain a higher amount of caffeic acid derivatives, followed by oil-based extracts (OO1 and OO2). On the other hand, it is evident that the largest fraction of all hydroxycinnamic acids and their derivatives, i.e., potential allergens contains the aqueous extract at higher pH values. Thus, it can be concluded that allergens have a different lipophilicity and each extraction procedure can extract a different propolis allergens fraction. However, known and confirmed propolis allergens, i.e., caffeic acid derivatives are mostly lipophilic molecules for which the extraction procedure using solvents with reduced polarity is better suited.

Additionally, non-methanol extraction procedures were analyzed by PCA using just hydroxycinnamic acid derivatives as variables. If we look at Fig. 4, PC1 and PC2 make up to 99% of the model's variability, suggesting a high diversity among the analyzed extracts. Oil extractions (OO1 and OO2) are grouped in the negative part of PC1 and in the positive part of the PC2 axis, suggesting a similar composition of the extract. Water extracts W1–W5 are narrowly grouped in the positive part of the PC1 axis and the negative part of the PC2 axis, indicating a high similarity between extracts (relative to OO and A1 extracts). To determine the influence of glycerol addition, only water-based extraction procedures with the same pH value and USE time but different glycerol concentrations were tested (Fig. 4b). It was interesting to note

that the 5% glycerol extraction provided the final extract with a low amount of potential allergens. Water extraction without glycerol addition provided an extract with a higher amount of caffeic acid prenyl and benzyl esters, while other hydroxycinnamic acids and derivatives were more extracted with W4 and W5 extraction procedures. Overall, glycerol addition in the non-methanol extraction increased the share of hydroxycinnamic acid derivatives in the final product. However, at the same time, the 20% glycerol (W5) provides the final extract with a higher phenolic content (Online Resource 2). On the other hand, olive oil demonstrated to be a better solvent for polyphenol extraction than just water with glycerol. However, it provided the final extract with the largest share of hydroxycinnamic acid derivatives (Fig. 3), but could be immediately used as a capsule filling or as part of different beauty products.

The demonstrated approach may be useful for an appropriate detection of specific components and isolation of targeted fractions in different industrial applications. For example, propolis extracts used in the beauty industry should contain less allergens; however, if there is a need for a propolis extract in the drug industry, some of the allergens are desirable due to strong pharmacological activity that they provide. This research has shown that the phenol composition changes significantly with the type of extraction, and a desirable composition may be obtained by means of a modulation of the green extraction procedures although the allergen problem is not entirely solved.



Conclusions

The LC–QQQ study allowed an analysis of the propolis complete phenolic content by comparing the retention time and MS/MS patterns with commercially available standards and literature data. Furthermore, unknown phenols were detected with the Precursor and Product ion scan modes on the LC–QQQ instrument with information on specific MS/MS patterns of available standards. Additionally, few different extraction procedures were tested to monitor the extraction efficiency of all caffeinates or hydroxycinnamate derivatives. That way, we provide insight into how to optimize the extraction in a way that the final product has as few allergens as possible, while at the same time, keeping all polyphenols with beneficial effects in high concentrations. Still, in some cases, these known allergens can have a strong pharmacological and biological activity, so that the question of their need in the final extract depends on the final application. The developed LC–QQQ quantitative and LC–QTOF confirmatory methods provided a perception of how the extraction method can influence the final propolis composition and offers the basis for further focused optimization of the extraction process. Despite the problem of the exact identification of all detected compounds, these results suggest that it is possible to monitor all cinnamates in propolis samples without an available reference standard.

Acknowledgements We greatly appreciate the granted access to equipment owned by the University of Rijeka within the project “Research Infrastructure for Campus-based Laboratories at University of Rijeka”, financed by the European Regional Development Fund (ERDF). We also acknowledge the University of Rijeka research support 13.11.1.1.11.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

References

- Sforzin JM (2016) biological properties and therapeutic applications of propolis. *Phytother Res* 30:894–905. <https://doi.org/10.1002/ptr.5605>
- Falcão SI, Vale N, Gomes P et al (2013) Phenolic profiling of portuguese propolis by LC–MS spectrometry: uncommon propolis rich in flavonoid glycosides. *Phytochem Anal* 24:309–318. <https://doi.org/10.1002/pca.2412>
- Falcão SI, Vilas-Boas M, Estevinho LM et al (2010) Phenolic characterization of Northeast Portuguese propolis: usual and unusual compounds. *Anal Bioanal Chem* 396:887–897. <https://doi.org/10.1007/s00216-009-3232-8>
- Ristivojević P, Trifković J, Gašić U et al (2015) Ultrahigh-performance liquid chromatography and mass spectrometry (UHPLC–LTQ/Orbitrap/MS/MS) study of phenolic profile of Serbian poplar type propolis. *Phytochem Anal* 26:127–136. <https://doi.org/10.1002/pca.2544>
- Gardana C, Barbieri A, Simonetti P, Guglielmetti S (2012) Bio-transformation strategy to reduce allergens in propolis. *Appl Environ Microbiol* 78:4654–4658. <https://doi.org/10.1128/AEM.00811-12>
- Gardana C, Simonetti P (2011) Evaluation of allergens in propolis by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 25:1675–1682. <https://doi.org/10.1002/rcm.5025>
- Oldoni TLC, Oliveira SC, Andolfatto S et al (2015) Chemical characterization and optimization of the extraction process of bioactive compounds from propolis produced by selected bees *Apis mellifera*. *J Braz Chem Soc* 26:2054–2062. <https://doi.org/10.5935/0103-5053.20150186>
- Hegazi AG, Abd El Hady FK, Abd Allah FA (2000) Chemical composition and antimicrobial activity of European propolis. *Z Naturforsch C* 55:70–75
- Carvalho R, Silva-carvalho R, Baltazar F, Almeida-aguiar C (2015) Propolis : a complex natural product with a plethora of biological activities that can be explored for drug development propolis : a complex natural product with a plethora of biological activities that can be. *Evid-based Complement Altern Med*. <https://doi.org/10.1155/2015/206439>
- Murtaza G, Karim S, Akram M (2014) Caffeic acid phenethyl ester and therapeutic potentials. *Biomed Res Int*. <https://doi.org/10.1155/2014/145342>
- Sforzin JM, Bankova V (2011) Propolis: is there a potential for the development of new drugs? *J Ethnopharmacol* 133:253–260. <https://doi.org/10.1016/j.jep.2010.10.032>
- Oryan A, Alemzadeh E, Moshiri A (2018) Potential role of propolis in wound healing: biological properties and therapeutic activities. *Biomed Pharmacother* 98:469–483. <https://doi.org/10.1016/j.biopha.2017.12.069>
- Zabaioni N, Fouache A, Trousson A et al (2017) Biological properties of propolis extracts: something new from an ancient product. *Chem Phys Lipids* 207:214–222. <https://doi.org/10.1016/j.chemphyslip.2017.04.005>
- Rasouli H, Farzei MH, Khodarahmi R (2017) Polyphenols and their benefits: a review. *Int J Food Prop* 10942912(2017):1354017. <https://doi.org/10.1080/10942912.2017.1354017>
- Sačić L, Peršurić Ž, Fornal E et al (2019) Targeted and untargeted LC–MS polyphenolic profiling and chemometric analysis of propolis from different regions of Croatia. *J Pharm Biomed Anal* 165:162–172. <https://doi.org/10.1016/j.jpba.2018.11.061>
- ICH harmonised tripartite guideline, Validation of analytical procedures: text and methodology Q2(R1) (2015) International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf. Accessed 3 Sept 2018
- Falcão SI, Tomás A, Vale N et al (2013) Phenolic quantification and botanical origin of Portuguese propolis. *Ind Crops Prod* 49:805–812. <https://doi.org/10.1016/j.indcrop.2013.07.021>
- Fabre N, Rustan I, de Hoffmann E et al (2001) Determination of flavone, flavanol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J Am Soc Mass Spectrom* 12:707–715. [https://doi.org/10.1016/S1044-0305\(01\)00226-4](https://doi.org/10.1016/S1044-0305(01)00226-4)
- Tsimogiannis D, Samiotaki M, Panayotou G, Oreopoulou V (2007) Characterization of flavonoid subgroups and hydroxy substitution by HPLC–MS/MS. *Molecules* 12:593–606
- Pinheiro P, Justino G (2012) Structural analysis of flavonoids and related compounds—a review of spectroscopic applications. In:

- Phytochemicals—a global perspective of their role in nutrition and health. InTech
21. Volpi N, Bergonzini G (2006) Analysis of flavonoids from propolis by on-line HPLC–electrospray mass spectrometry. *J Pharm Biomed Anal* 42:354–361. <https://doi.org/10.1016/j.jpba.2006.04.017>
 22. Medana C, Carbone F, Aigotti R et al (2008) Selective analysis of phenolic compounds in propolis by HPLC-MS/MS. *Phytochem Anal* 19:32–39. <https://doi.org/10.1002/pca.1010>
 23. Gardana C, Scaglianti M, Pietta P, Simonetti P (2007) Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography–tandem mass spectrometry. *J Pharm Biomed Anal* 45:390–399. <https://doi.org/10.1016/j.jpba.2007.06.022>
 24. Pellati F, Orlandini G, Pinetti D, Benvenuti S (2011) HPLC-DAD and HPLC-ESI-MS/MS methods for metabolite profiling of propolis extracts. *J Pharm Biomed Anal* 55:934–948. <https://doi.org/10.1016/j.jpba.2011.03.024>
 25. Justesen U (2001) Collision-induced fragmentation of deprotonated methoxylated flavonoids, obtained by electrospray ionization mass spectrometry. *J Mass Spectrom* 36:169–178. <https://doi.org/10.1002/jms.118>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

LC-QQQ and LC-QTOF MS methods for comprehensive detection of potential allergens in various propolis extracts

Author names and affiliations:

Lara Saftić^{a,b}, Željka Peršurić^a, Sandra Kraljević Pavelić^{a*}

^aDepartment of Biotechnology, Centre for high-throughput technologies, University of Rijeka, Radmile Matejčić 2, 51000 Rijeka, Croatia

^bJuraj Dobrila University of Pula, Zagrebačka 30, 52100 Pula, Croatia

*Correspondence:

University of Rijeka, Department of Biotechnology, Centre for high-throughput technologies, Radmile Matejčić 2, 51000 Rijeka, Croatia, e-mail: sandrakp@biotech.uniri.hr, tel: +385 51 584 550

Online Resource 1 List of detected phenolic compounds in propolis samples and their LC-QTOF parameters and tentative identification

Tentative identification of phenolic compound	Teoretical m/z	Molecular formula	Ion mode	Fragmento r/V	m/z precursor ion	m/z qualifiers	Teoretical m-H	Error/ppm	Fragmentation pattern	Ident ificat ion*	Reference
caffeic acid cinnamyl ester	296.1049	C ₁₈ H ₁₆ O ₄	-	300	295.2300	178 134 112	295.0916	-2.32	178[C ₉ H ₆ O ₄ -H] ⁻ 134[C ₉ H ₆ O ₄ -H-CO ₂] ⁻	2	[2, 24]
caffeic acid phenylethyl ester	284.1049	C ₁₇ H ₁₆ O ₄	-	150	283.0973	179 161 135	283.0976	0.09	179[C ₉ H ₇ O ₄ -H] ⁻ 161[C ₉ H ₅ O ₃ -H] ⁻ 135[C ₉ H ₇ O ₄ -H-CO ₂] ⁻	2	[4, 23, 24]
caffeic acid benzyl ester	270.0892	C ₁₆ H ₁₄ O ₄	-	300	269.0846	178 161 134	269.0813	0.75	178[C ₉ H ₆ O ₄ -H] ⁻ 134[C ₉ H ₆ O ₄ -H-CO ₂] ⁻	2	[4, 23, 24]
caffeic acid prenyl ester	248.1049	C ₁₄ H ₁₆ O ₄	-	150	247.0978	179 161 135 112	247.0976	0.63	179[C ₉ H ₇ O ₄ -H] ⁻ 161[C ₉ H ₅ O ₃ -H] ⁻ 135[C ₉ H ₇ O ₄ -H-CO ₂] ⁻	2	[23, 24]
dymethyl caffeic acid	208.0736	C ₁₁ H ₁₂ O ₄	+	150	209.0810	179 163 141 133 120 119	209.0808	0.96	179[M-H-2CH ₃] ⁻ 163[M-H-CO ₂] ⁻	2	[4, 23]
caffeic acid methyl ester	196.0736	C ₁₀ H ₁₂ O ₄	+	150	197.0805	178 177 134 89	197.0808	1.97	178[C ₉ H ₆ O ₄ -H] ⁻ 134[C ₉ H ₆ O ₄ -H-CO ₂] ⁻	2	
caffeic acid	458.1213	C ₂₃ H ₂₂ O ₁₀	-	300	457.1153	179	457.1140	-2.29	179[C ₉ H ₇ O ₄ -H] ⁻	2	

derivative 1						161 135			161[C ₉ H ₅ O ₃ -H] ⁻ 135[C ₉ H ₇ O ₄ -H-CO ₂] ⁻		
caffeic acid derivative 2	416.1107	C ₂₁ H ₂₀ O ₉	-	300	415.1036	253 179 163 135	415.1036	-0.35	179[C ₉ H ₇ O ₄ -H] ⁻ 135[C ₉ H ₇ O ₄ -H-CO ₂] ⁻	3	
cinnamic acid derivative 1	388.1158	C ₂₀ H ₂₀ O ₈	-	150	387.1086	327 193 149 137	387.1085	-1.45	193[C ₁₀ H ₁₀ O ₄ -H] ⁻ 149[C ₁₀ H ₁₀ O ₄ -H-CO ₂] ⁻	3	
cinnamic acid derivative 2	376.1158	C ₁₉ H ₂₀ O ₈	-	300	375.1080	284 179 163 145 135	375.1085	1.62	179[C ₉ H ₈ O ₄ -H] ⁻ 135[C ₉ H ₇ O ₄ -H-CO ₂] ⁻	3	
<i>p</i> -coumaric acid prenyl ester	232.1099	C ₁₄ H ₁₆ O ₃	-	300	231.1030	163 119 118	231.1027	0.58	163[C ₉ H ₇ O ₃ -H] ⁻ 119[C ₉ H ₇ O ₃ -H-CO ₂] ⁻	2	[2, 24]
<i>p</i> -coumaric acid cinnamyl ester	280.0947	C ₁₄ H ₁₆ O ₆	-	300	279.0877	163 14 119	279.0874	1.17	163[C ₉ H ₇ O ₃ -H] ⁻ 145[C ₉ H ₅ O ₂ -H] ⁻ 119[C ₉ H ₇ O ₃ -H-CO ₂] ⁻	2	[24]
<i>p</i> -coumaric acid derivative 1	330.1103	C ₁₈ H ₁₈ O ₆	-	300	329.1024	311 267 163 145 119	329.1031	3.58	163[C ₉ H ₇ O ₃ -H] ⁻ 145[C ₉ H ₅ O ₂ -H] ⁻ 119[C ₉ H ₇ O ₃ -H-CO ₂] ⁻	3	
<i>p</i> -coumaric acid derivative 2	360.1209	C ₁₉ H ₂₀ O ₇	-	300	359.1138	195 163 145 119	359.1136	-0.23	163[C ₉ H ₇ O ₃ -H] ⁻ 145[C ₉ H ₅ O ₂ -H] ⁻ 119[C ₉ H ₇ O ₃ -H-CO ₂] ⁻	3	
<i>p</i> -coumaric acid derivative 3	280.1099	C ₁₈ H ₁₆ O ₃	-	300	279.1026	163 14 119	279.1027	0.25	163[C ₉ H ₇ O ₃ -H] ⁻ 145[C ₉ H ₅ O ₂ -H] ⁻ 119[C ₉ H ₇ O ₃ -H-CO ₂] ⁻	3	
<i>p</i> -coumaric acid derivative 4	384.1136	C ₂₁ H ₂₀ O ₇	-	300	383.1137	219 163 145 119	383.1136	-0.30	163[C ₉ H ₇ O ₃ -H] ⁻ 145[C ₉ H ₅ O ₂ -H] ⁻ 119[C ₉ H ₇ O ₃ -H-CO ₂] ⁻	3	
<i>p</i> -coumaric acid	358.0980	C ₁₉ H ₁₈ O ₇	-	300	357.0979	329	357.0980	0.22	163[C ₉ H ₇ O ₃ -H] ⁻	3	

derivative 5						226 163 145 119			145[C ₉ H ₅ O ₂ -H] ⁻ 119[C ₉ H ₇ O ₃ -H-CO ₂] ⁻		
<i>p</i> -coumaric acid derivative 6	442.1264	C ₂₃ H ₂₂ O ₉	-	300	441.1201	179 163 145 135 119	441.1191	2.25	163[C ₉ H ₇ O ₃ -H] ⁻ 145[C ₉ H ₅ O ₂ -H] ⁻ 119[C ₉ H ₇ O ₃ -H-CO ₂] ⁻	3	
pinobanksin-5- methyl-ether-3- O-acetate	328.0947	C ₁₇ H ₁₆ O ₆	-	300	327.0875	285 271 253	327.0874	0.50	285[M-H-acetate] ⁻ 271[M-H-methyl- acetate] ⁻ 253[M-H-methyl- acetate-H ₂ O] ⁻	2	[23, 24]
pinobanksin-3- O-acetate	314.079	C ₁₇ H ₁₄ O ₆	-	300	313.0754	271 253	313.0718	0.55	271[M-H-acetate] ⁻ 253[M-H-acetate-H ₂ O] ⁻	2	[4, 23, 24]
pinobanksin-3- O-butyrate	342.1103	C ₁₉ H ₁₈ O ₆	-	150	341.1033	271 253	341.1031	0.55	271[M-H-butyrate] ⁻ 253[M-H-butyrate - H ₂ O] ⁻	2	[4, 23, 24]
pinobanksin-3- O-propionat	328.0947	C ₁₈ H ₁₆ O ₆	-	300	327.0901	271 253 225 147	327.0874	0.50	271[M-H-propionate] ⁻ 253[M-H-propionate- H ₂ O] ⁻ 225[M-H-propionate- H ₂ O-CO] ⁻	2	[2, 4, 23, 24]
pinobanksin-5- methyl-ether	286.0841	C ₁₆ H ₁₄ O ₅	-	300	285.0769	271 253 249 224	285.0768	-0.31	271[M-H-CH ₃] ⁻ 253[M-H-CH ₃ -H ₂ O] ⁻ 224[M-H-CH ₃ -H ₂ O- CO] ⁻	2	[2, 4, 23, 24]
pinobanksin-3- O-heksanoat	368.1260	C ₂₁ H ₂₀ O ₆	-	150	367.2850	271 253	367.1187	-0.17	271[M-H-heksanoate] ⁻ 253[M-H-heksanoate- H ₂ O] ⁻	2	[2, 4, 23, 24]
pinobanksin-3- o-pentanoate or 2- methylbutyrate	356.1190	C ₂₀ H ₁₉ O ₆	-	300	355.1119	271 253 225	355.1187	0.27	271[M-H-pentanoate] ⁻ 253[M-H-pentanoate- H ₂ O] ⁻ 225[M-H-pentanoate- H ₂ O-CO] ⁻	2	[2, 4, 23, 24]
quercetin-3,7- dimethyl ether	330.0740	C ₁₇ H ₁₄ O ₇	-	150	329.0661	314 299	329.0667	1.65	314[M-H-CH ₃] ⁻ 299[M-H-2CH ₃] ⁻	2	[2, 24]

						285 271 151				271[M-H-2CH ₃ -CO] ⁻ 151[^{1,2} A-CO] ⁻		
quercetin-3- methyl ether	316.0583	C ₁₆ H ₁₂ O ₇	-	150	315.0499	301 271 255 151	315.0510	3.05		301[M-H-CH ₃] ⁻ 271[M-H-CH ₃ -CO] ⁻ 151[^{1,2} A-CO] ⁻	2	[2, 23, 24]

* Identifying a compound by means of standard confirmation (1), by means of MS/MS spectra comparison with the literature (2), by means of diagnostic fragments not reported in the literature (3)

Online Resource 2 The efficiency of extraction procedures with respect to the M1 (100% methanol) extraction which provided the highest phenolic yield (calculated as a sum of concentrations of all quantified phenols). W stands for water based extractions, OO for olive oil extractions, A for alkaline extractions and M for methanol extractions.

