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Evaluation of allergens in propolis by ultra-performance liquid chromatography/tandem mass spectrometry

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The purified extract of propolis is used as a traditional remedy for the treatment of several diseases. Its beneficial activities are mainly attributed to the polyphenolic fraction. Nevertheless, propolis can cause allergic dermatitis and the sensitization rate in humans is increasing significantly mainly in younger subjects. The aim of this study was to develop and validate a selective and sensitive ultra-performance liquid chromatography tandem mass spectrometry analysis (UPLC/MS/MS) for the evaluation of the amount of caffeic acid and its esters with allergenic action in raw propolis samples and commercial formulations. The separation was carried out on a 1.7 μm C₁₈ BEH Shield column and the detection performed by means of electrospray ionization in negative ion mode with multiple reaction monitoring. The confirmation of formulae of the precursor and product ions was accomplished by injection into a high-resolution system (FTICR-MS) using accurate mass measurements. The error was below 1.4 ppm. The range of the standard curves was 0.5–10 $\mu\text{g/mL}$ and dihydrocaffeic acid was used as internal standard (IS). The lower limit of detection (LLOD) for 3-methyl-2-butenyl- (3M2B), 3-methyl-3-butenyl- (3M3B), 2-methyl-2-butenyl- (2M2B), benzyl- (CABE), phenylethylcaffeic acid (CAPE) and for caffeic acid (CA) and the IS was 0.1 and 0.3 $\mu\text{g/mL}$, respectively. The recoveries were in the range 96–104% and the intra- and inter-day precisions were within 6.2%. In the European ($n = 8$) and Asiatic ($n = 3$) propolis the most abundant allergens were CABE > 3M2B > CAPE > 3M3B > CA > 2M2B. These compounds were not found in the red ($n = 1$) and green ($n = 1$) Brazilian propolis. Hydroalcoholic extracts ($n = 6$) and tablets ($n = 6$) were analyzed by the proposed UPLC/MS/MS method. The results showed that in the commercial products CABE, 3M2B, CAPE and 3M3B were also the most abundant. Copyright © 2011 John Wiley & Sons, Ltd.

Propolis is a complex natural product containing material collected from buds or resin and beeswax produced by honeybees. Its polyphenolic profile depends on the region of collection^[1] and the chemical composition differs substantially between propolis samples from tropical and temperate regions. In fact, in Europe, Asia, North America and some South American regions the composition of propolis is very similar, the main constituents being flavonoids, aromatic acids and their esters.^[2] In contrast, green, red and brown Brazilian propolis contains less flavonoids and more quinic acid esters. The typical compound of green Brazilian propolis is a prenylated *p*-coumaric acid derivative, named artepillin C.^[3]

Propolis seems relatively safe, with a calculated safe dose of about 1.4 mg/kg body weight (BW)/day in humans,^[4] and the purified extract of propolis is used as a traditional remedy for its antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, immunomodulatory, cariostatic, antitumor^[5] and anti-*Helicobacter pylori*^[6] activities. These beneficial activities are mainly attributed to flavonoids and phenolic acids with their esters such as caffeic acid phenethyl ester (CAPE).^[7] Nevertheless, propolis can cause occupational allergic eczematous contact dermatitis mostly in apiarists,^[8] but also in consumers

with an allergic predisposition.^[9] The sensitization rate to propolis in patients suffering from dermatitis has been reported to vary between 1.2 and 6.6%^[10–17] and a significant increasing trend from 1995 (2%) to 2002 (13.7%) in young subjects was reported.^[18] The allergenic action seems to be due to some caffeic acid derivatives^[16] (Fig. 1), and 3-methyl-2-butenyl caffeate (3M2B) seems the most active compound.^[19]

Propolis composition has been evaluated using different analytical methods including micellar electrokinetic chromatography (MEKC),^[20] gas chromatography/mass spectrometry (GC/MS),^[21] nuclear magnetic resonance (NMR),^[22] liquid chromatography (LC)/MS,^[2] electrospray ionization (ESI)-MS^[23] and, recently, a semi-qualitative determination was performed by ambient sonic-spray ionization MS.^[24]

Despite the great deal of available analytical information regarding propolis composition, data concerning the allergenic fraction are scarce. Recently, Aliboni *et al.*^[25] reported the content of two non-prenylated esters with low allergenic activity, benzyl cinnamate and benzyl salicylate, in raw propolis samples. Medana *et al.*^[26] reported the determination of some flavonoids and phenolic acid esters by LC/ESI-MS/MS in propolis samples of different origin, but they evaluated only one prenyl caffeate and its chromatographic peak overlapped benzyl caffeate. The discrimination between different prenyl caffeates and their quantitative determination are important to assess the allergenic potential of propolis. Thus, for the first time we developed and validated a sensitive and simple method using ultra-performance liquid chromatography coupled with

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R	Compound	CV	Elab	Product ions
	Benzyl caffeate CABE , (<i>m/z</i>) [−] 269	24	20	134 (100), 133(16), 161(11), 178(8), 179(3), 269(1)
	3-methyl-2-butenyl caffeate 3M2B , (<i>m/z</i>) [−] 247	21	21	134 (100), 178(9), 179(5), 135(4), 161(4), 247(2)
	2-methyl-2-butenyl caffeate 2M2B , (<i>m/z</i>) [−] 247	21	21	134 (100), 178(15), 135(4), 179(4), (161), 247(3)
	Phenylethyl caffeate CAPE , (<i>m/z</i>) [−] 283	21	18	135 (100), 161(31), 134(17), 179(13), 283(1)
	3-methyl-3-butenyl caffeate 3M3B , (<i>m/z</i>) [−] 247	24	21	135 (100), 179(42), 134(19), 161(26), 247(5)
H	Caffeic acid CA , (<i>m/z</i>) [−] 179	15	15	135 (100), 134(12), 179(2)

	dihydrocaffeic acid (IS) DHCA , (<i>m/z</i>) [−] 181	15	15	137 (100), 121(38), 109(21), 135(13), 181(5)
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CV: cone voltage (V), Elab: energy applied during collision (eV)

Figure 1. Chemical structure of IS, caffeic acid and its esters determined in propolis raw samples and commercial preparations. The main product ions obtained in ESI-MS/MS negative ion mode are reported in order of percent relative intensities (in brackets).

tandem mass spectrometry (UPLC/MS/MS) for the determination of caffeic acid and its esters (CAEs) with allergenic action. The proposed method was then subsequently applied to determine these compounds in raw propolis samples from different ethnogeographical origin and commercial preparations containing propolis.

EXPERIMENTAL

Chemicals and reagents

Caffeic acid (CA), 3-methyl-2-butenyl-CA (3M2B), internal standard (dihydrocaffeic acid, DHCA), caffeic acid phenyl ethyl ester (CAPE), dimethylaminopyridine (DMAP) and 1,3-dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methyl caffeate was from Chromadex (Milan, Italy), ethyl caffeate from Waterstone Tech. (Carmel, IN, USA) and ethyl ferulate from Santa

Cruz Tech. (Santa Cruz, CA, USA). Methanol, acetonitrile, ethyl acetate, tetrahydrofuran (THF), HCl, NaCl, NaHCO₃ and formic acid were supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained from a MilliQ apparatus (Millipore, Milford, MA, USA). The raw propolis samples were collected from the following areas: Italy (1–3), Macedonia (4–5), Poland (6), Uruguay (7), France (8), Nepal (9), China (10–11), Brazil (12–13). The propolis samples were stored at −20°C. The commercial products containing propolis were purchased in a local drugstore.

Synthesis of CABE, 2M3B and 3M3B

2-Methyl-3-butenyl-, benzyl- and 3-methyl-3-butenyl caffeate were synthesized as described by Sestili *et al.*^[27] with slight modifications. Briefly, 0.11 mmol DMAP, 0.11 mmol DCC and 1.7 mmol of the alcohol were added to a solution of 1.1 mmol CA in 15 mL of THF at 4°C. The mixture was stirred at room temperature and after 24 h the reaction mixture was

filtered using a Gooch funnel. The solution was dried, the residue dissolved in ethyl acetate and the sample sequentially washed with 0.1 N HCl, NaCl and then NaHCO₃ saturated solutions. The organic solution was evaporated in vacuum, the dry residue suspended in methanol and then centrifuged at 1000 g for 10 min before injecting 200 µL in the semi-preparative LC system. The chromatographic system consisted of an Alliance model 2695 (Waters, Milford, MA, USA) equipped with a diode-array detector model 2998 (Waters) and a 10 µm ODS-2 Partisil 10 (Whatman, Clifton, NJ, USA) column was used for the separation at a flow rate of 2.5 mL/min. The eluents were 0.1% HCOOH (A) and CH₃CN (B) and the linear gradient was as follows: 35% to 70% B in 10 min and then 70% B for 5 min. The peak of the analyte was collected by a WFC II fraction collector (Waters), evaporated to dryness under nitrogen and the residue suspended in methanol.

Sample preparation

Raw propolis

Frozen propolis samples (200 g) were firstly finely ground in a mill, passed through a 500 µm (35 mesh) sieve, and then 1 g of the powder was sonicated with 1 mL IS (2 mg/mL) and 70 mL of ethyl acetate for 10 min. The mixture was centrifuged at 1500 g for 5 min, and the supernatant transferred into a flask, while the solid residue was extracted once as described above. The extracts were dried under nitrogen and the residues dissolved in 100 mL methanol. The solutions obtained were serially diluted and centrifuged at 4000 g for 1 min before the UPLC/MS/MS analysis.

Commercial preparations

The IS methanolic solution (1 mL, 1 mg/mL) was added to the alcoholic extracts (1 mL) and the volume adjusted to 10 mL with methanol. Tablet samples (5 g) were finely ground, and then about 0.5 g of the powder was sonicated with 1 mL IS (1 mg/mL) and 8 mL of methanol for 10 min. The mixture was centrifuged at 1500 g for 5 min, the supernatant transferred into a flask and the volume adjusted to 10 mL with methanol. The solutions obtained were serially diluted and centrifuged at 4000 g for 1 min before the UPLC/MS/MS analysis.

UPLC/MS/MS conditions

The chromatographic system consisted of an Acquity UPLC system (Waters) coupled to a Quattro micro triple quadrupole mass spectrometer (Waters). A 1.7 µm C₁₈ BEH Shield column (150 × 2.1 mm, Waters) was used for the separation at a flow rate of 0.45 mL/min. The column was maintained at 40°C and the separation was performed by means of a linear gradient elution (eluent A, 0.05% HCOOH; eluent B, 0.05% HCOOH in acetonitrile). The gradient was as follows: 20 to 30% B in 4 min, 30 to 40% B in 5 min, 40 to 60% B in 3 min, 60 to 90% B in 1 min and then 90% B for 2 min. The capillary voltage was set to 2.7 kV, the cone voltage and the energy applied during fragmentation (Elab) was specific for each compound (see Fig. 1). All mass data were obtained in the negative ion mode. The source temperature was 130°C, the desolvation temperature was 380°C and argon was used at 2.1×10^{-3} mbar to improve fragmentation in the collision cell. Masslynx 4.0 acquired data with the Quan-Optimize option for the fragmentation study.

The fragmentation transitions for the multiple reaction monitoring (MRM) were m/z 179→135 for CA, m/z 247→135 for 3M3B, m/z 247→134 for 3M2B and 2M2B, m/z 269→134 for CABE, m/z 283→135 for CAPE and m/z 181→137 for DHCA, with a dwell time of 0.2 s per transition.

FTICR-MS conditions

All analyses were performed on an APEX II Fourier transform ion cyclotron resonance (FTICR) mass spectrometer equipped with a 4.7 Tesla magnet (Bruker Daltonics, Billerica, MA, USA) and an Apollo ESI source (Bruker Daltonics). ESI and ion transfer conditions were optimized in the range 112–250 Da using a standard solution of NaTFA in acetonitrile.

Samples were introduced into the ESI source via a Cole-Parmer Series 74900 syringe pump at a rate of 120 µL/h, and all mass spectra were acquired in negative ion mode. The electrospray capillary voltage was 3.5 kV, the capillary skimmer was set to 20 V, a countercurrent flow of nitrogen gas (150°C) was employed for the desolvation and argon was used to improve the fragmentation. The scan range was from m/z 100 to 1000 at a resolution of 50,000–120,000.

All experimental event sequences were controlled using Bruker Daltonics XMASS 7.0.8 software.

Preparation of working solutions

The primary stock solutions of CA esters (0.1 mg/mL), CA and DHCA (1 mg/mL) were prepared in methanol and diluted to give working solutions in the range of 0.5–10 µg/mL. All stock solutions and the working solutions were stored at –80°C and –20°C, respectively.

Method validation

Calibration curves were constructed for each standard and internal standard at six concentration levels; four independent determinations were performed at each concentration and regression analysis was carried out to determine the linearity of the calibration graphs. Moreover, dihydrocaffeic acid was used as IS to evaluate the loss of analytes during sample preparation. LLOQ was defined by the lowest injected inter-day concentration whose RSD% resulted to be <20%,^[28] while LLOD was defined by the lowest concentration the assay can differentiate from background levels (signal-to-noise (S/N) ratio >3). The accuracy (matrix effect) of the procedure was determined by a recovery test according to the published method.^[29] Briefly, three raw propolis samples (1 g) were spiked with different amounts of IS, CA and CAEs (1–10 mg). The spiked samples were extracted under optimized conditions, and the recovery rates calculated. Each sample was extracted in triplicate and analyzed in duplicate. The peak identity related to the IS, CA and CAEs was confirmed by co-chromatography with an authentic standard and by MS/MS. Intra- and inter-day precisions of the assay were verified by analyzing spiked samples three times for five consecutive days. Precision was determined by evaluating standard deviations of the amounts and of the retention times. Two analysts evaluating the amounts of the analytes in a raw propolis sample estimated the ruggedness of the proposed UPLC/MS/MS method. Each analyst performed six tests, and standard and extract solutions were injected in triplicate. Robustness was estimated by varying several chromatographic conditions such as flow rate

± 0.05 mL/min, column temperature $\pm 5^\circ\text{C}$, organic strength $\pm 50\%$, cone voltage ± 5 V and capillary voltage ± 0.3 kV. Data were analyzed by one-way analysis of variance (ANOVA) for independent samples considering significant a level of $p < 0.05$.

Stability studies

The powder and extracts of propolis and the standards solutions were stored at 4°C and -20°C for up to 90 days to evaluate their stability. Moreover, standard solutions and propolis extracts were placed in the autosampler at 20°C and their stability evaluated overnight.

UPLC/MS/MS optimization

In order to optimize ESI conditions for IS, CA and CAEs, quadrupole full scans were carried out both in positive and negative ion mode. Precursor ion, capillary, extractor and RF lens voltages were optimized by direct infusion experiments, while product ions, cone voltage and the energy applied during fragmentation (Elab) were evaluated by the Quan-optimize option.

RESULTS AND DISCUSSION

Method validation

The calibration curves for all the analytes were linear in the range of 0.5 – 10 $\mu\text{g/mL}$, and the LLOD was 0.3 $\mu\text{g/mL}$ for the IS and CA and 0.1 $\mu\text{g/mL}$ for CAEs. The mean percentage recovery of the analytes from spiked raw samples of propolis was in the range of 96 – 104% (Table 1). Selectivity was assessed by co-chromatography with an authentic standard and MS/MS evaluation. The intra- and inter-day precisions

($n = 5$) were evaluated analyzing spiked samples in triplicate and the resulting RSD% values were lower than 5.5 and 6.2% , respectively. The RSD% of the retention times was lower than 0.22% . The results on the effect of the external factor on the degree of reproducibility of the UPLC/MS/MS method were compared statistically; there was no significant difference in the evaluation of the amount of CA and CAEs in the analyzed propolis extracts ($p = 0.29$). Regarding robustness, slight variations in buffer composition and pH, flow rate and column temperature did not change the peak shape and resolution. Moreover, moderate variations in cone (± 3 V) and capillary (± 0.5 kV) voltage did not influence significantly the quantification of the analytes. It should be pointed out that increasing cone voltage values drastically reduced the quantitative data, mainly for caffeic acid and the IS. Thus, the proposed methods were found to be very robust and rugged even if careful attention had to be paid to cone voltage values.

Stability studies

The means of CAEs recovered from raw propolis samples and commercial formulations stored at 4°C for up to 90 days were consistent ($>94\%$) and caffeic acid did not increase indicating that its esters were not hydrolyzed. Standard solutions stored at -20°C for up to 90 days showed a reduction lower than 2.4% for all the analytes. Standard solutions and propolis extracts in methanol were stable in the autosampler at 20°C overnight (RSD $< 1.9\%$).

UPLC/MS/MS optimization

The negative ESI ion mode was selected as it was more sensitive and the IS, CA and CAEs gave a better fragmentation. Figure 1 shows the chemical structures of these compounds, their main

Table 1. Recovery test of the UPLC/MS/MS assay

Analyte	Added (mg)	Detected (mg)	s.d. (mg)	RSD %	Expected (mg)	Recovery (%)	Error %
CA	0	3.7	0.2	5.9			
	1	4.5	0.2	4.9	4.7	96	-4.3
	5	8.9	0.2	5.1	8.7	102	2.3
	10	13.4	0.3	5.2	13.7	98	-2.2
3M3B	0	9.7	0.3	2.9			
	1	10.9	0.3	3.0	10.7	102	2.0
	5	15.1	0.4	3.2	14.7	103	2.7
	10	19.2	0.3	2.6	19.7	97	-2.5
3M2B	0	11.8	0.5	3.9			
	1	12.5	0.5	4.0	12.8	97	-2.6
	5	16.3	0.5	4.1	16.8	97	-3.2
	10	21.1	0.6	4.3	21.8	97	-3.4
2M2B	0	4.7	0.4	7.7			
	1	5.5	0.3	6.3	5.7	96	-3.5
	5	10.1	0.4	6.6	9.7	104	4.1
	10	14.4	0.3	4.9	14.7	98	-2.0
CABE	0	15.1	0.6	4.1			
	1	16.3	0.6	4.1	16.1	101	1.2
	5	20.7	0.8	4.9	20.1	103	3.0
	10	24.5	0.8	4.9	25.1	98	-2.4
CAPE	0	10.8	0.3	3.1			
	1	11.3	0.3	3.1	11.8	96	-4.3
	5	16.1	0.4	3.2	15.8	102	1.8
	10	20.2	0.4	3.5	20.8	97	-2.9

product ions and the cone voltage value and the energy applied during collision for their production. The collision-induced dissociation (CID) spectrum of CA gave a typical product ion with m/z 135 corresponding to $[M-COO]^-$, while its 3-methyl-3-butenyl ester gave product ions with m/z 179 and 135 corresponding to $[M-prenyl]^-$ and $[M-prenyl-COO]^-$, respectively. In the same way, the CAPE fragmentation gave product ions with m/z 179 and 135 corresponding to $[M-phenylethyl]^-$ and $[M-phenylethyl-COO]^-$, respectively. In contrast, in MS/MS 3M2B and 2M2B yielded typical product ions with m/z 178 and 134 corresponding to $[M-prenyl]^-$ and $[M-prenyl-COO]^-$, respectively. The same product ions were also obtained from CABE fragmentation, and in Figs. 2 and 3 MS² spectra of the

different CAEs and their hypothetical fragmentation pattern are reported, respectively. Thus, 3M2B, 2M2B and CABE gave common product ions resulting from homolytic cleavage with formation of a radical product with m/z 134, and this behavior was presumably related to the stability of the derived prenyl and benzyl radicals. In particular, the benzyl radical derived from CABE fragmentation can rearrange to give a more stable radical ion (Fig. 3). In contrast, CAPE and 3M3B gave a heterolytic cleavage and the main product ion was the ion with m/z 135. Thereby, the different fragmentations depend on the alcohol moiety structure. Specifically, it seems that when the C atom linked to the carboxyl group is allylic the MS/MS fragmentation gives negative radical products (m/z 134 and

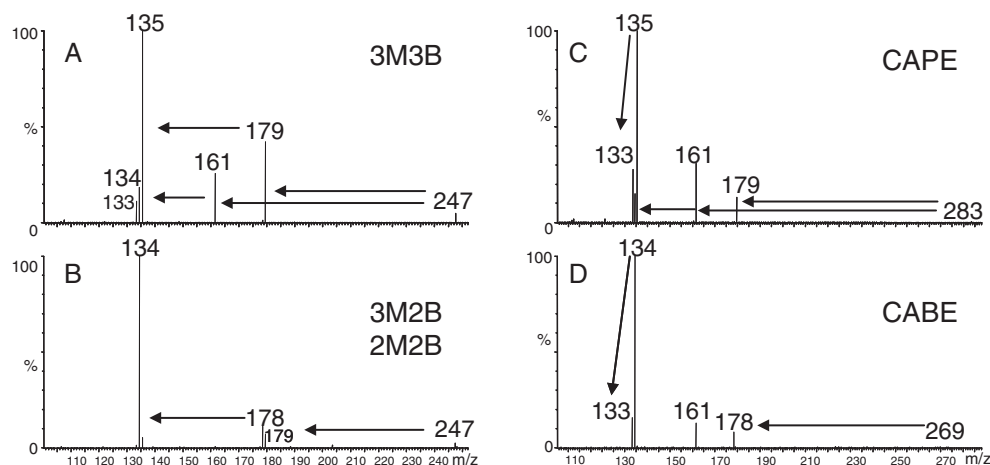


Figure 2. Product ions of 3M3B (A), 3M2B and 2M2B (B), CAPE (C), and CABE (D) obtained in ESI negative mode at lower energy applied during collision.

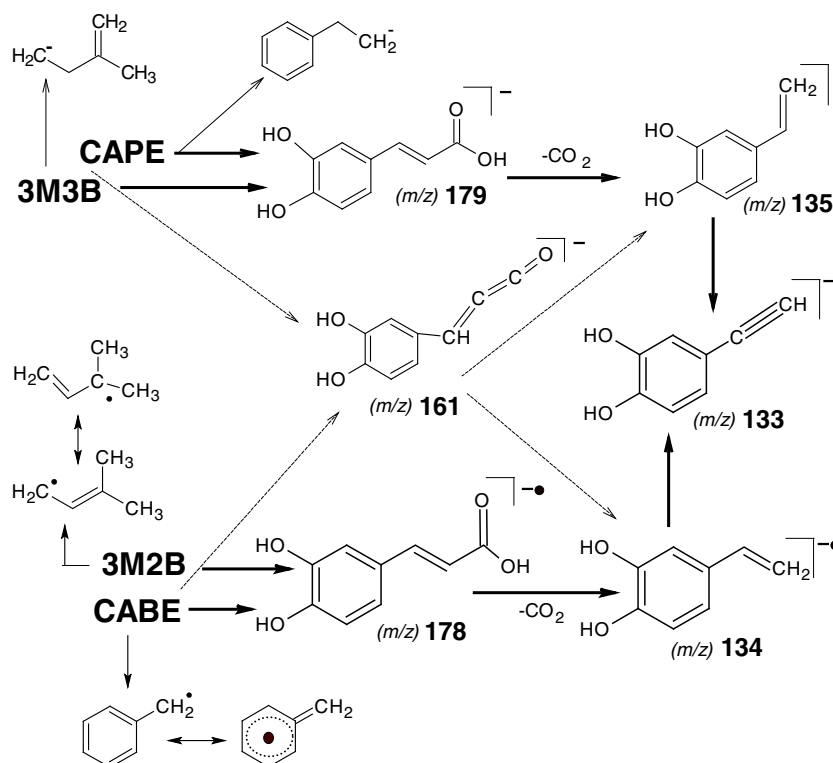


Figure 3. Hypothetical fragmentation pattern of the different caffeic acid esters.

178), while negative product ions (m/z 135, 179) are obtained if the allyl group is not present.

The MS/MS spectra of CAEs, obtained at lower Elab, showed also the presence of product ions with m/z 161, which could be the result of H_2O elimination from the deprotonated caffeoyl group. On the contrary, the product ion with m/z 161 was not present in the MS/MS spectrum of CA. Thus, it seems that this fragment is derived from the cleavage of the ester bond (Fig. 3).

Methyl-, ethyl caffeate and ethyl ferulate were evaluated for their potential use as IS but for different reasons they were not used. Methyl and ethyl caffeate showed a chromatographic behavior, a deprotonated molecule and a fragmentation pattern similar to ferulic- and 3,4-dimethylcaffeic acid, respectively, two compounds present in several propolis samples.^[2] Regarding ethyl ferulate, although it gave typical product ions, it was not possible to employ it because it is a compound naturally found in some European propolis samples (data not shown).

FTICR-MS

High-resolution collision-induced dissociation (CID) was employed to confirm the identity of the analytes and their product ions in the negative mode using ESI-FTICR-MS. The resolution over all fragment ions was from 60.000 to 90.000. In the MS^2 spectra of 3M2B and 3M2B, the $[M-H]^-$ ion at m/z 247 gave mainly product ions at m/z 178 and 134, and a lower amount of product ions at m/z 179 and 135. The MS^2 spectra of CAFE showed similar fragmentation features, confirming that these CAEs underwent homolytic cleavage with formation of a radical product. Thus, the FTICR-MS data confirm the results obtained using the triple quadrupole system.

The FTICR-MS/MS spectra of 3M3B and CAPE showed ions corresponding to the deprotonated molecule $[M-H]^-$, the loss of the acyl moiety (m/z 179) and the loss of acyl and CO_2 (m/z 135) as characteristic fragment ions. These CAEs gave also a deprotonated molecule at m/z 161, resulting from water elimination from the deprotonated caffeoyl group. On the contrary, the product ion at m/z 161 was not found in the MS^2 spectrum of 3M2B, 2M2B, CAFE and CA. Table 2 shows the

compounds identified through FTICR-MS/MS experiments along with accurate mass, molecular formula, error (in ppm) between the mass found and the accurate mass of CA and CAEs, and the MS/MS ions.

Amount of CA and CAEs in propolis samples

A good separation of the analytes was achieved with a C_{18} BEH Shield sub-2 μm column and gradient mode elution, and Fig. 4 shows the chromatograms of a raw propolis extract acquired in MRM mode. Benzyl caffeate ($0.85 \pm 0.39\%$), 3M2B ($0.64 \pm 0.33\%$), CAPE ($0.61 \pm 0.29\%$) and 3M3B ($0.54 \pm 0.38\%$) were the most abundant CAEs found in the analysis of European and Asiatic raw propolis samples (Table 3), respectively. In these samples lower amounts of 2M2B ($0.2 \pm 0.1\%$) were also present. On the contrary, these compounds were not found in the red and green Brazilian raw propolis samples. Commercial hydroalcoholic extracts and tablets were also analyzed by the proposed UPLC/MS/MS method. The results obtained showed that CAFE, 3M2B, CAPE and 3M3B were the most abundant CAEs in all of them, indicating that the tested commercial products were prepared using European or Asiatic raw propolis samples.

CONCLUSIONS

A sensitive, specific and reproducible UPLC/MS/MS method for the quantitative determination of caffeic acid and CAEs with allergenic action in propolis samples has been developed and validated. The amounts of these compounds was successfully determined in raw propolis and commercial preparations. The results obtained showed that in all the analyzed extracts the most abundant caffeates were benzyl-, 3-methyl-2-butenyl-, phenylethyl- and 3-methyl-3-butenyl ester, respectively. Despite being bioactive compounds with both toxicological and pharmacological effects, they are not actually exploited as markers of propolis quality – the latter is normally based only on the content of flavonoids. Thus, it is advisable that

Table 2. Precursor ion and fragments of high-resolution ESI CID for caffeic acid and its esters

Compound	$[M-H]^-$	Product ion (m/z)	Calculated (m/z)	Error (ppm)	MF
3M2B	247.09758	178.02719	178.02716	0.2	$C_9H_6O_4$
		134.03744	134.03732	0.9	$C_8H_6O_2$
3M2B	247.09751	178.02726	178.02716	0.6	$C_9H_6O_4$
		134.03739	134.03732	0.5	$C_8H_6O_2$
CAFE	269.08193	178.02731	178.02716	0.8	$C_9H_6O_4$
		134.03736	134.03732	0.3	$C_8H_6O_2$
3M3B	247.09764	179.03515	179.03498	0.9	$C_9H_7O_4$
		135.04529	135.04515	1.0	$C_8H_7O_2$
		161.02453	161.02442	0.7	$C_9H_5O_3$
		133.02969	133.02950	1.4	$C_8H_5O_2$
CAPE	283.09758	179.03498	179.03493	0.3	$C_9H_7O_4$
		135.04523	135.04515	0.6	$C_8H_7O_2$
		161.02459	161.02442	1.1	$C_9H_5O_3$
		133.02968	133.02950	1.4	$C_8H_5O_2$
CA	179.03504	135.04522	135.04515	0.5	$C_8H_7O_2$

MF: molecular formula $[M-H]^-$.

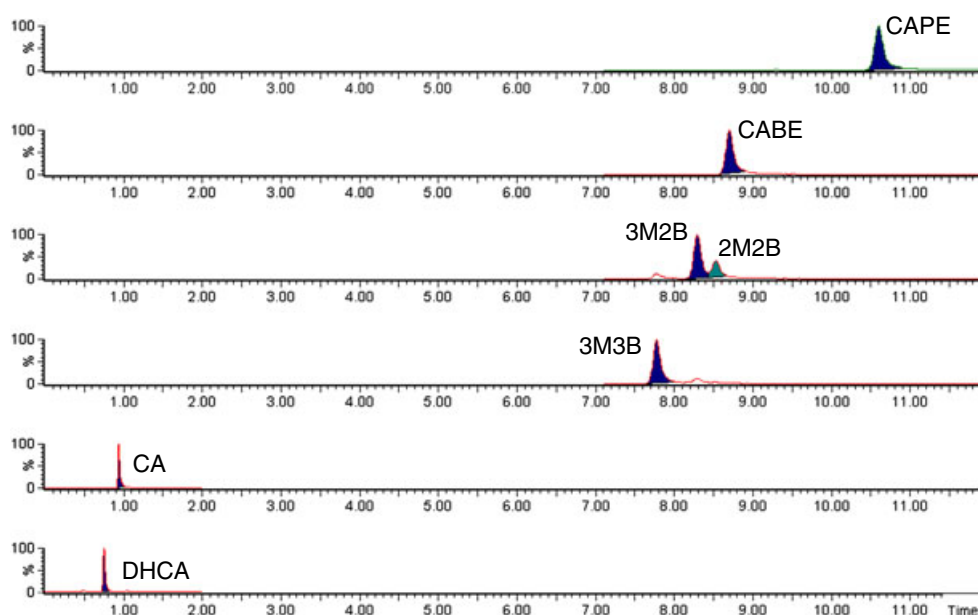


Figure 4. Typical UPLC/MS/MS chromatogram (MRM mode) of a raw propolis extract (sample 1). m/z 181→137 for IS (DHCA); 179→135 for CA; 247→135 for 3M2B; 247→134 for 3M2 and 2M2B; 283→135 for CAPE; and 269→134 for CABB.

Table 3. Amounts^a of caffeic acid and CAEs with allergenic action found in raw propolis samples from different geographic areas (1–13), commercial hydroalcoholic extracts (A–F) and tablets (I–VI). The raw propolis samples were from Italy (1–3), Macedonia (4–5), Poland (6), Uruguay (7), France (8), Nepal (9) and China (10–11). The samples 12 and 13 were red and green Brazilian propolis, respectively

Sample	mg/g						Total %	RSD %
	CA	3M3B	3M2B	2M2B	CABB	CAPE		
1	5.50	6.70	7.60	2.40	9.00	7.00	3.82	2.88
2	6.10	8.10	9.20	2.30	9.60	8.60	4.39	3.19
3	2.40	5.40	7.90	1.10	10.90	4.70	3.24	3.40
4	7.20	8.40	9.80	3.40	13.00	8.10	4.99	4.21
5	5.30	8.10	9.10	2.10	8.70	9.00	4.23	3.30
6	2.80	2.20	3.70	1.20	3.70	2.90	1.65	4.85
7	1.70	2.80	2.80	1.10	10.50	4.70	2.36	4.66
8	2.90	5.50	6.30	2.60	11.20	8.90	3.74	3.48
9	0.12	0.32	0.44	N.F.	1.00	0.80	0.27	7.40
10	7.63	9.30	10.54	3.33	12.49	9.71	5.30	3.02
11	2.10	2.40	2.70	0.40	3.50	2.60	1.37	5.84
12	N.F.	N.F.	N.F.	N.F.	N.F.	N.F.		
13	N.F.	N.F.	N.F.	N.F.	N.F.	N.F.		
I	0.09	0.10	0.12	0.04	0.14	0.11	0.06	8.38
II	0.79	0.97	1.10	0.35	1.30	1.01	0.55	5.44
III	0.13	0.14	0.17	0.02	0.23	0.15	0.08	8.10
IV	0.14	0.15	0.17	0.02	0.26	0.17	0.09	8.72
V	0.17	0.21	0.24	0.04	0.38	0.23	0.13	7.06
VI	0.08	0.09	0.12	0.01	0.15	0.11	0.06	8.74
Sample	mg/mL						Total %	RSD %
	CA	3M3B	3M2B	2M2B	CABB	CAPE		
A	0.57	0.69	0.78	0.25	0.93	0.72	0.39	5.08
B	0.41	0.41	0.52	0.11	0.71	0.52	0.27	7.84
C	1.45	1.61	2.15	0.29	2.71	1.84	1.01	4.98
D	0.66	0.82	0.89	0.11	1.41	1.10	0.50	6.01
E	0.54	0.62	0.73	0.12	1.11	0.69	0.38	5.26
F	0.11	0.17	0.18	N.F.	0.24	0.19	0.09	7.87

^aValues are expressed as mean of four determinations. N.F. not found.

analytical control of propolis should be based on determination of CAEs as a further marker, not only of quality, but also of the safety of the derived products. In particular, the 3-methyl-2-butenyl caffeate (3M2B) quantification should be taken into account since it seems to be the most allergenic compound and is available as a pure reference standard. We strongly recommend that its amount should be made easily visible on the external packaging so that consumers can choose the product with the lowest content of allergenic compound.

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REFERENCES

- V. S. Bankova, S. L. De Castro, M. C. Marcucci. Propolis: recent advances in chemistry and plant origin. *Apidologie* **2000**, *31*, 3.
- C. Gardana, M. Scaglianti, P. G. Pietta, P. Simonetti. Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2007**, *45*, 390.
- M. C. Marcucci, F. Ferreres, A. R. Custodio, M. Ferreira, V. S. Bankova, C. Garcia-Viguera, W. A. Bretz. Evaluation of phenolic compounds in Brazilian propolis from different geographic regions. *Z. Naturforsch.* **2000**, *55*, 76.
- G. A. Burdock. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem. Toxicol.* **1998**, *36*, 347.
- J. M. Sforzin, V. S. Bankova. Propolis: is there a potential for the development of new drugs? *J. Ethnopharmacol.* **2011**, *133*, 253.
- M. M. Abdel-Latif, H. J. Windle, B. S. Homasany, K. Sabra, D. Kelleher. Caffeic acid phenethyl ester modulates Helicobacter pylori-induced nuclear factor-kappa B and activator protein-1 expression in gastric epithelial cells. *Br. J. Pharm.* **2005**, *146*, 1139.
- I. Jasprica, A. Mornar, Z. Debeljak, A. Smolčić-Bubalo, M. Medić-Sarić, L. Mayer, Z. Romić, K. Bučan, T. Balog, S. Sobocanec, V. Sverko. In vivo study of propolis supplementation effects on antioxidative status and red blood cells. *J. Ethnopharmacol.* **2007**, *110*, 548.
- O. Gulbahar, G. Ozturk, N. Erdem, A. C. Kazandi, A. Kokuludag. In vivo study of propolis supplementation effects on antioxidative status and red blood cells. *Ann. Allergy Asthma Immunol.* **2005**, *94*, 509.
- S. G. Fernandez, E. A. Aleman, B. E. García Figueroa, E. G. Fagoaga, J. M. O. Rivera, A. I. Purroy. Direct and airborne contact dermatitis from propolis in beekeepers. *Contact Dermatitis.* **2004**, *50*, 320.
- E. Rudzki, P. Rebandel, E. Jaworski. Comparison of the eliciting properties of 3 different propolis samples. *Dermatitis* **1998**, *39*, 142.
- B. M. Hausen, P. Evers, H. Stuwe, W. Konig, E. Wollenweber. Propolis allergy (IV). Studies with further sensitizers from propolis and constituents common to propolis, poplar buds and balsam of Peru. *Contact Dermatitis.* **1992**, *26*, 34.
- J. Machackova. The incidence of allergy to propolis in 605 consecutive patients patch tested in Prague. *Contact Dermatitis.* **1988**, *18*, 210.
- S. Wohrl, W. Hemmer, M. Focke, M. Gotz, R. Jarisch. The significance of fragrance mix, balsam of Peru, colophony and propolis as screening tools in the detection of fragrance allergy. *Br. J. Dermatol.* **2001**, *145*, 268.
- S. Rajpara, M. Wilkinson, C. King, D. Gawkrödger, J. S. English, B. N. Statham, C. Green, J. E. Sansom, M. M. Chowdhury, H. L. Horne, A. D. Ormerod. The importance of propolis in patch testing—a multicentre survey. *Contact Dermatitis.* **2009**, *61*, 287.
- U. Jappe, A. Schnuch, W. Uter. Rosacea and contact allergy to cosmetics and topical medicaments-retrospective analysis of multicentre surveillance data 1995–2002. *Contact Dermatitis.* **2005**, *52*, 96.
- B. M. Hausen. Evaluation of the main contact allergens in propolis (1995 to 2005). *Dermatitis* **2005**, *16*, 127.
- T. Hasan, T. Rantanen, K. Alanko, R. J. Harvima, R. Jolanki, K. Kalimo, A. Lahti, K. Lammintausta, A. I. Lauerman, A. Laukkanen, T. Luukkaala, R. Rieki, K. Turjanmaa, E. Varjonen, A. M. Vuorela. Patch test reactions to cosmetic allergens in 1995–1997 and 2000–2002 in Finland – a multicentre study. *Contact Dermatitis.* **2005**, *53*, 40.
- F. Giusti, R. Maglietta, P. Pepe, S. Seidenari. Sensitization to propolis in 1255 children undergoing patch testing. *Contact Dermatitis.* **2004**, *51*, 255.
- M. C. Acciai, M. Ginanneschi, S. Bracci, A. Sertoli. Studies of the sensitizing properties of propolis. *Contact Dermatitis.* **1990**, *23*, 274.
- L. Jiang, G. Fang, Y. Zhang, G. Cao, S. Wang. Analysis of flavonoids in propolis and Ginkgo biloba by micellar electrokinetic capillary chromatography. *J. Agric. Food Chem.* **2008**, *56*, 11571.
- I. Márquez Hernández, O. Cuesta-Rubio, M. Campo Fernández, A. Rosado Pérez, R. Montes de Oca Porto, A. L. Piccinelli, L. Rastrelli. Studies on the constituents of yellow Cuban propolis: GC-MS determination of triterpenoids and flavonoids. *J. Agric. Food Chem.* **2010**, *58*, 4725.
- M. L. Castro, A. M. do Nascimento, M. Ikegaki, C. M. Costa-Neto, S. M. Alencar, P. L. Rosalen. Identification of a bioactive compound isolated from Brazilian propolis type 6. *Bioorg. Med. Chem.* **2009**, *17*, 5332.
- A. C. Sawaya, D. M. Tomazela, I. B. S. Cunha, V. S. Bankova, M. C. Marcucci, A. R. Custodio, M. N. Eberlin. Electrospray ionization mass spectrometry fingerprinting of propolis. *Analyst* **2004**, *129*, 739.
- A. C. Sawaya, P. V. Abdelnur, M. N. Eberlin, S. Kumazawa, M. R. Ahn, K. S. Bang, N. Nagaraja, V. S. Bankova, H. Afrouzan. Fingerprinting of propolis by easy ambient sonicspray ionization mass spectrometry. *Talanta* **2010**, *81*, 100.
- A. Aliboni, A. D'andrea, P. Massanisso. Propolis specimens from different locations of central Italy: chemical profiling and gas chromatography-mass spectrometry (GC-MS) quantitative analysis of the allergenic esters benzyl cinnamate and benzyl salicylate. *J. Agric. Food Chem.* **2011**, *59*, 282.
- C. Medana, F. Carbone, R. Aigotti, G. Appendino, C. Baiocchi. Selective analysis of phenolic compounds in propolis by HPLC-MS/MS. *Phytochem. Anal.* **2008**, *19*, 32.
- P. Sestili, G. Diamantini, A. Bedini, L. Cerioni, I. Tommasini, G. Tarzia, O. Cantoni. Plant-derived phenolic compounds prevent the DNA single-strand breakage and cytotoxicity induced by tert-butylhydroperoxide via an iron-chelating mechanism. *Biochem. J.* **2002**, *364*, 121.
- V. P. Shah, K. K. Midha, J. W. Findlay, H. M. Hill, J. D. Hulse, I. J. McGilveray, G. McKay, K. J. Miller, R. N. Patnaik, M. L. Powell, A. Tonelli, C. T. Viswanathan, A. Yacobi. Bioanalytical method validation – a revisit with a decade of progress. *Pharm. Res.* **2000**, *17*, 1551.
- B. K. Matuszewski, M. L. Constanzer, C. M. Chavez-Eng. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* **2003**, *75*, 3019.