Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 934–948

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

HPLC-DAD and HPLC-ESI-MS/MS methods for metabolite profiling of propolis extracts

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ARTICLE INFO

Article history:
Received 26 November 2010
Received in revised form 16 March 2011
Accepted 17 March 2011
Available online 25 March 2011

Keywords:
Propolis
Phenolic acids
Flavonoids
HPLC
Mass spectrometry

ABSTRACT

In this study, the composition of polyphenols (phenolic acids and flavonoids) in propolis extracts was investigated by HPLC-DAD and HPLC-ESI-MS/MS by comparing the performance of ion trap and triple quadrupole mass analyzers. The analyses were carried out on an Ascentis C_{18} column (250 mm × 4.6 mm I.D., 5 μm), with a mobile phase composed by 0.1% formic acid in water and acetonitrile. Overall, the UV spectra, the MS and MS/MS data allowed the identification of 40 compounds. In the case of flavonoids, the triple quadrupole mass analyzer provided more collision energy if compared with the ion trap, originating product ions at best sensitivity.

The HPLC method was validated in agreement with ICH guidelines: the correlation coefficients were >0.998; the limit of detection was in the range 1.6–4.6 μg/ml; the recovery range was 96–105%; the intra- and inter-day %RSD values for retention times and peak areas were found to be <0.3 and 1.9%, respectively.

The developed technique was applied to the analysis of hydroalcoholic extracts of propolis available on the Italian market. Although the chromatographic profile of the analyzed samples was similar, the quantitative analysis indicated that there is a great variability in the amount of the active compounds: the content of total phenolic acids ranged from 0.17 to 16.67 mg/ml and the level of total flavonoids from 2.48 to 41.10 mg/ml. The proposed method can be considered suitable for the phytochemical analysis of propolis extracts used in phytotherapy.

1. Introduction

Propolis, also called bee glue, is a dark-coloured resinous material collected by honeybees (*Apis mellifera* L.) from leaf buds and cracks in the bark of several tree species [1]. Once collected, this material is enriched with salivary and enzymatic secretions [1]. Several pharmacological activities have been attributed to propolis extracts, mainly antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, antiproliferative, immunostimulating, anti-ulcerous etc. [2]. Current applications of propolis include herbal products for cold syndrome and dermatological preparations [3]. In addition, propolis is used to prevent and treat oral inflammations [3]. Most of these preparations contain ethanolic extracts of propolis [3].

The detailed chemical composition of propolis is known to be very complex [1,4]. In propolis from temperate zones, the most important class of biologically active compounds is characterized by polyphenols, including flavonoids, phenolic acids and their esters [1]. In contrast, propolis from tropical areas has shown to contain prenylated phenylpropanoids and non-typical propolis flavonoids (Brazil) or polyisoprenylated benzophenones (Cuba) [5]. The content of polyphenols in "poplar type" propolis extracts may vary as a function of the origin of samples and these differences can affect the biological activity of preparations and therefore their pharmacological effects [1]. In this context, the development of analytical methods for the phytochemical analysis propolis is of great interest.

Several methods have been described in the literature for the analysis of polyphenols in propolis, based on non-separation techniques, such as UV–vis spectrophotometry [1] and NMR [6] or on separation techniques, including GC, HPTLC, HPLC and HPCE [1]. Of these methods, the spectrophotometric ones are considered to be useful especially for the routine control of propolis samples [1,7]. HPLC in combination with spectroscopic and spectrometric detection has significantly improved the analysis of phenolic compounds in natural products derived from bees, providing definitive information for the identification and quantification of these biologically active constituents [3,5,6,8–11]. However, most of these methods have not been validated in agreement with ICH guidelines [12] for comprehensive multi-component analysis of phenolic acids and flavonoids in propolis samples.

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doi:10.1016/j.jpba.2011.03.024

In this context, this paper aims to provide a reliable and fully validated method for the phytochemical analysis of propolis hydroalcoholic extracts by HPLC-DAD and HPLC-ESI-MS/MS with ion trap (IT) and triple quadrupole (TQ) mass analyzers. By using HPLC-ESI-MS/MS, it was possible to obtain the quasi-molecular ions and the MS/MS spectra, which, in combination with retention times and UV data, made the peak identification of target analytes very reliable. The fragmentation patterns of flavonoids and caffeic acids obtained by IT and TQ are discussed in the present work. The practical applicability of the technique was demonstrated by the analysis of propolis extracts representative of the Italian market to provide a reliable phytochemical profiling of their health-promoting secondary metabolites.

2. Experimental

2.1. Chemicals and solvents

Caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, pinocembrin, cinnamic acid, apigenin, kaempferol and galangin were from Sigma–Aldrich–Fluka (Milan, Italy). Isorhamnetin and luteolin were from Roth (Karlsruhe, Germany). Chrysin was from Extrasynthese (Genay, France).

Quercetin-3-methyl-ether, pinobanksin, galangin-5-methyl-ether, quercetin-7-methyl-ether, caffeic acid phenylethyl ester (CAPE) and pinobanksin-3-*O*-acetate were kindly donated by Prof. Dr. Eckhard Wollenweber, Institut für Botanik, Darmstadt, Germany.

HPLC-grade methanol (MeOH), acetonitrile (ACN), formic acid and analytical grade absolute ethanol (EtOH) were from Sigma (Milan, Italy). Water was purified using a Milli-Q Plus185 system from Millipore (Milford, MA, USA).

Propolis hydroalcoholic extracts (i.e. extracts obtained by using aqueous EtOH as the extraction solvent), indicated in the text as PE-1/PE-9, were purchased from local pharmacies and herbal shops in fall 2009. As indicated by the manufacturers in the label claims, the extraction solvent used for these preparations was aqueous EtOH at concentrations ranging from 60% to 90%. The sample PE-1 contained also lemon essential oil. The applied sample-to-solvent ratios were 1:10 (w/v) for PE-2 and PE-6, 2:10 (w/v) for PE-4 and 3:10 (w/v) for PE-9. Samples PE-1 and PE-7 claimed to contain 3.7 and 40 mg/ml of total flavonoids, respectively. Sample PE-3 was classified as a homeopathic preparation. For products PE-5 and PE-8 detailed information on the content was not available. Specific information on the extraction procedures followed by the manufacturers of the analyzed samples was not available. These samples were stored at low temperature, protected from light and humidity, until required for chemical analysis.

For comparison purpose, one sample of raw propolis was collected in spring 2010 from *A. mellifera* hives located in Italian Northern Apennines (Polinago, Modena, Italy). This sample was obtained after honey extraction, by scratching the hive walls and frames, followed by the removal of debris and bees. This raw material was stored at −20 °C until chemical analysis.

2.2. HPLC-UV/DAD conditions

Chromatography was performed using an Agilent Technologies (Waldbronn, Germany) modular model 1100 system, consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment and a diode array detector (DAD). The chromatograms were recorded using an Agilent ChemStation for LC and LC-MS systems (Rev. B.01.03).

The analyses were carried out on an Ascentis C_{18} column (250 mm × 4.6 mm I.D., 5 μm, Supelco, Bellefonte, PA, USA). The mobile phase was composed by (A) 0.1% formic acid in H_2O and (B) ACN. The gradient elution was modified as follows: 0–3 min 25% B, 3–10 min linear gradient from 25% to 30% B, 10–40 min from 30% to 40% B, 40–60 min from 40% to 60% B, 60–80 min from 60% to 90% B, 80–92 min 90% B. The post-running time was 5 min. The flow rate was 1.2 ml/min. The column temperature was set at 30 °C. The sample injection volume was 5 μl. The DAD acquisitions were performed in the range 190–450 nm and chromatograms were integrated at 265 nm (for chrysin and galangin), 290 nm (for cinnamic acid, pinocembrin and pinobanksin), 320 nm (for caffeic acid, *p*-coumaric acid and ferulic acid), 338 nm (for apigenin and luteolin) and 370 nm (for quercetin, isorhamnetin and kaempferol). Two injections were performed for each sample.

2.3. HPLC-ESI-MS and MS/MS conditions

Analyses were performed using two HPLC-ESI-MS/MS systems: (a) an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, an autosampler, a thermostatted column compartment and a 6310A IT mass analyzer with an ESI ion source; (b) an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, an autosampler, a thermostatted column compartment and a 6410B TQ mass analyzer with an ESI ion source. The HPLC column and the applied chromatographic conditions were the same as reported for the HPLC-DAD system. The flow-rate was split 6:1 before the ESI source.

For ESI-MS^2 (IT), the parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 20 psi, the drying gas (N_2) temperature was 350 °C, the drying gas flow was 9 l/min and the skimmer voltage was 40 V. Data were acquired by Agilent 6300 Series Ion Trap LC/MS system software (version 6.2). IT was used in the full-scan positive and negative ion modes in the *m*/*z* range 100–1000. MS^2 spectra were automatically performed with helium as the collision gas by using the SmartFrag function.

For ESI-MS/MS (TQ), the parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 20 psi, the drying gas temperature was 350 °C, the drying gas flow was 9 l/min and the fragmentor voltage was 135 V. Data were acquired by Agilent MassHunter Workstation (Rev. B.02.01). TQ was used in the full-scan positive and negative ion modes in the *m*/*z* range 100–1000 and in the product ion scan (PIS) mode using nitrogen as the collision gas (with a collision energy (CE) of 20 V for phenolic acids and 20–30 V for flavonoids in the positive ion mode; 20 V CE for phenolic acids and 20–40 V CE for flavonoids in the negative ion mode).

2.4. Standard solutions for HPLC-DAD quantification

The stock standard solution of each compound (caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, pinocembrin, cinnamic acid, chrysin, apigenin, kaempferol, isorhamnetin and galangin) was prepared as follows: an accurately weighed amount of pure compound (2–6 mg) was placed into a 10 ml volumetric flask; MeOH was added and the solution was diluted to volume with the same solvent. The external standard calibration curve was generated using five data points, covering the concentration ranges reported in Table 1. Five μl aliquots of each standard solution were used for HPLC analysis. Injections were performed in triplicate for each concentration level. The calibration curve was obtained by plotting the peak area of the compound at each level *versus* the concentration of the sample.

The amount of phenolic acids and flavonoids in propolis samples was determined by using these calibration curves, when the standard was available. All the other constituents identified in propolis samples (phenolic acid and flavonoid derivatives) were quantified

Table 1
Linearity and sensitivity parameters for phenolic acids and flavonoids used as propolis standards.

Compound	Linearity range (μg/ml)	Slope (*a*)	Intercept (*b*)	*r*	LOD (μg/ml)	LOQ (μg/ml)
Caffeic acid	2.68–214.20	22.03 (±0.12)	−32.91 (±1.74)	0.9997	1.60	2.68
p-Coumaric acid	2.74–219.10	27.95 (±0.14)	−41.28 (±1.88)	0.9997	1.64	2.74
Ferulic acid	2.68–214.60	22.30 (±0.11)	−37.34 (±1.18)	0.9997	1.70	2.68
Quercetin	5.34–213.60	15.23 (±0.15)	−27.65 (±2.44)	0.9992	3.20	5.34
Cinnamic acid	2.63–210.80	27.46 (±0.24)	−33.23 (±2.86)	0.9993	1.58	2.63
Apigenin	5.10–203.90	17.28 (±0.17)	−27.13 (±2.34)	0.9992	3.06	5.10
Kaempferol	5.51–220.50	15.21 (±0.19)	−42.90 (±2.65)	0.9987	3.30	5.51
Isorhamnetin	5.10–204.10	10.99 (±0.14)	−29.14 (±1.14)	0.9987	3.06	5.10
Luteolin	5.64–225.70	14.95 (±0.20)	−43.83 (±2.77)	0.9986	3.38	5.64
Chrysin	7.68–307.35	19.93 (±0.28)	−29.20 (±3.80)	0.9985	4.60	7.68
Pinocembrin	7.74–309.75	13.01 (±0.18)	−17.52 (±2.48)	0.9985	4.64	7.74
Galangin	7.58–303.25	16.15 (±0.21)	−69.57 (±2.81)	0.9987	4.54	7.58

Experimental conditions as in Section 2.2. For each curve the equation is $y = ax + b$, where y is the peak area, x the concentration of the analyte (μg/ml), a is the slope, b is the intercept and r the coefficient. Standard error (SE) values are given in parenthesis. The p value was <0.0001 for all calibration curves.

by using the above mentioned calibration curves and their amounts were corrected by using the molecular weight ratio.

2.5. Extraction of phenolics from raw propolis

The frozen sample of raw propolis (30 g) was finely powdered and two extraction procedures were carried out [1]. In both cases, the applied sample-to-solvent ratio was 1:10 (w/v) [1].

The first method was based on the decoction of a weighed amount of sample (1.00 g) with 10 ml of 80% EtOH at 70 °C for 1 h under stirring. After centrifugation for 5 min at 4000 rpm, the supernatant solution was filtered in a vacuum into a 10 ml volumetric flask and the solvent was added to the final volume.

The second method was based on the maceration of a weighed amount of sample (1.00 g) with 10 ml of 80% EtOH for 24 h at room temperature under stirring. After centrifugation for 5 min at 4000 rpm, the supernatant solution was filtered in a vacuum into a 10 ml volumetric flask and the solvent was added to the final volume.

Both extraction procedures were repeated twice. The propolis extracts obtained by decoction and maceration were indicated in the text as PE-10 and PE-11, respectively.

2.6. Sample preparation for HPLC analysis

An aliquot of 500 μl of each commercially available propolis hydroalcoholic extract (PE-1/PE-9) and propolis extract used as reference (PE-10 and PE-11) was properly diluted with MeOH in a volumetric flask, filtered through a 0.45 μm PTFE filter into a HPLC vial and injected in the HPLC system. All sample preparations were carried out in duplicate. The quantification data are therefore the mean of four results.

3. Results and discussion

3.1. Identification of propolis secondary metabolites

The HPLC-DAD analysis of a typical commercial sample of propolis (PE-9) available on the Italian market at 290 nm indicated a very complex composition, as shown in Fig. 1. The corresponding peak identification is described in Tables 2A–2C. Considering the complexity of the sample, the overall chromatographic separation can be considered satisfactory. The only limitation is the separation of caffeic acid phenylethyl ester (CAPE) (peak 28) and pinobanksin-3-*O*-acetate (peak 29): these compounds have the same retention and cannot be separated under the applied chromatographic conditions.

3.1.1. Identification of phenolic acids and derivatives

The MS and MS/MS spectra of propolis phenolic acids obtained by both IT and TQ indicated that the negative ion mode provided higher level of sensitivity if compared with the positive one, allowing the identification of several compounds [5,10], such as caffeic acid (*m*/*z* 179), *p*-coumaric acid (*m*/*z* 163), ferulic acid and isoferulic acid (*m*/*z* 193), 3,4-dimethyl caffeic acid (DMCA) (*m*/*z* 207). All these phenolic acids were prone to fragmentation and shared a common fragmentation pathway, based on the loss of the CO_2 group (−44 u), with both IT and TQ. In the case of ferulic acid, isoferulic acid and DMCA, other fragments due to the loss of CH_3 groups (−15 u) were also commonly observed. Cinnamic acid yielded a diagnostic product ion at *m*/*z* 103 in the negative ion mode, corresponding to the $[M-H-CO_2]^-$ fragment.

Five caffeic acid derivatives were also identified in the analyzed propolis samples [10], including caffeic acid prenyl ester and its isomer (*m*/*z* 247), caffeic acid benzyl ester (*m*/*z* 269), caffeic acid phenylethyl ester (CAPE) (*m*/*z* 283) and caffeic acid cinnamyl ester (*m*/*z* 295). The main fragmentation mechanism for the benzyl and cinnamyl caffeate derivatives in the negative ion mode with both IT and TQ was the homolytic cleavage of the ester moiety with the benzyl and the cinnamyl residues, respectively [11]. The resulting odd electron product ion at *m*/*z* 178, after the loss of a CO_2 molecule, gave the radical product ion at *m*/*z* 134. Regarding the phenylethyl caffeate derivative (CAPE), the mechanism of fragmentation observed with both IT and TQ was the heterolytic breakdown of the bond with the phenylethyl group with the loss of a styrene residue, originating the negative product ion at *m*/*z* 179, which in turn, after the loss of CO_2, gave the ion at *m*/*z* 135. The prenyl caffeate derivative showed the same behaviour of the previous compound, with the loss of an isoprene residue, originating the ion at *m*/*z* 179 and a further fragment at *m*/*z* 135, due to the loss of CO_2. In the MS/MS spectra obtained by TQ in the negative mode, benzyl, phenylethyl and prenyl caffeate derivatives showed also a common negative fragment at *m*/*z* 161, due to the heterolytic cleavage of the C–O ester bond of the quasi-molecular ion.

Four *p*-coumaric acid esters were also confirmed as constituents of Italian propolis [10], including *p*-coumaric prenyl ester and its isomer (*m*/*z* 231), *p*-coumaric benzyl ester (*m*/*z* 253) and *p*-coumaric cinnamyl ester (*m*/*z* 279). In analogy with the caffeate derivatives, the MS/MS spectra of these compounds in the negative mode with both IT and TQ indicated a fragmentation pattern based on the homolytic and heterolytic cleavages of the bonds with the benzyl, cinnamyl and prenyl groups, which generated a radical product ion at *m*/*z* 162 and a negative product ion at *m*/*z* 163, respectively, which, after the loss of a CO_2 molecule, originated further fragments at *m*/*z* 118 and 119, respectively. In particular, the first mechanism was observed for the ben-

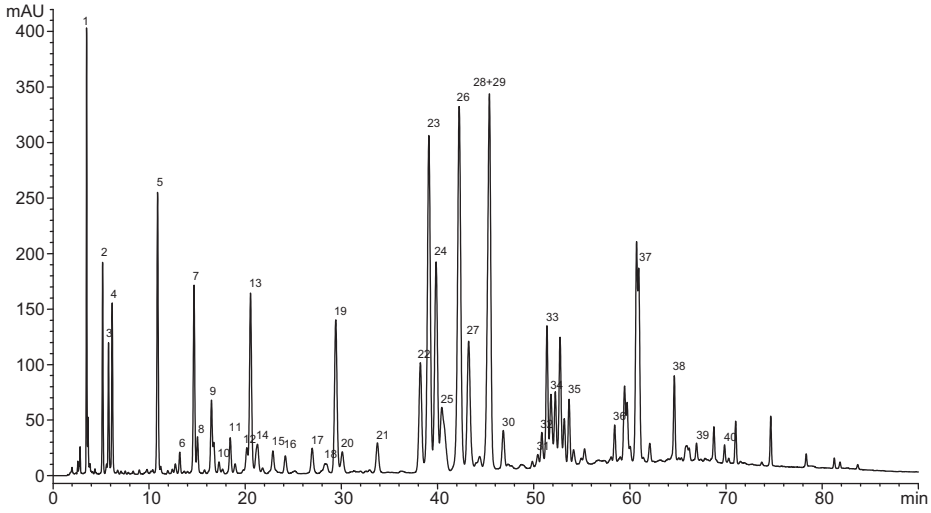


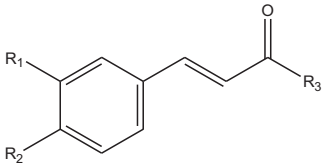
Fig. 1. Chromatogram obtained by HPLC-DAD analysis of a propolis hydroalcoholic extract (PE-9) at 290 nm. For peak identification see Tables 2A–2C. Experimental conditions as in Section 2.2.

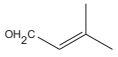
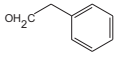
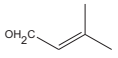
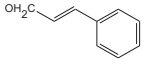
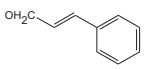
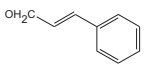
zyl and the cinnamyl coumarate derivatives, while the second pattern occurred in the case of the prenyl coumarate derivative. Another negative product ion at *m*/*z* 145 was obtained for benzyl and prenyl coumarate derivatives, originated from the heterolytic breakdown of the C–O ester bond of the quasi-molecular ion.

3.1.2. Identification of flavonoids and derivatives

All flavonoids occurring in the propolis samples analyzed in the present study showed quasi-molecular ions $[M+H]^+$ and $[M-H]^-$ of good intensity. In this way, it was possible to individuate flavones, flavonols and flavanones and dihydroflavonols, either as free form or as ether or ester derivatives. In particular, the follow-

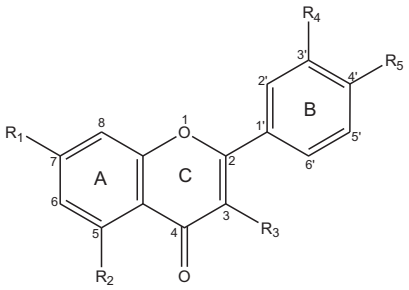
Table 2A
Structures of phenolic acids and derivatives identified in propolis extracts[a].



Compound	Peak number	R_1	R_2	R_3
Caffeic acid	1	OH	OH	OH
p-Coumaric acid	2	H	OH	OH
Ferulic acid	3	OCH_3	OH	OH
Isoferulic acid	4	OH	OCH_3	OH
3,4-Dimethyl-caffeic acid (DMCA)	5	OCH_3	OCH_3	OH
Cinnamic acid	9	H	H	OH
Caffeic acid prenyl ester	22/25	OH	OH	
Caffeic acid benzyl ester	24	OH	OH	
Caffeic acid phenylethyl ester (CAPE)	28	OH	OH	
p-Coumaric prenyl ester	31	H	OH	
p-Coumaric benzyl ester	32	H	OH	
Caffeic acid cinnamyl ester	33	OH	OH	
p-Coumaric cinnamyl ester	36	H	OH	
p-Methoxy cinnamic acid cinnamyl ester	40	H	OCH_3	

[a] Compounds are in order of elution time.

Table 2B
Structures of flavones and flavonols identified in propolis extracts[a].



Compound	Peak number	R_1	R_2	R_3	R_4	R_5
Quercetin	6	OH	OH	OH	OH	OH
Quercetin-3-methyl-ether	8	OH	OH	OCH_3	OH	OH
Chrysin-5-methyl-ether	10	OH	OCH_3	H	H	H
Apigenin	11	OH	OH	H	H	OH
Kaempferol	12	OH	OH	OH	H	OH
Isorhamnetin	14	OH	OH	OH	OCH_3	OH
Galangin-5-methyl-ether	17	OH	OCH_3	OH	H	H
Quercetin-7-methyl-ether	20	OCH_3	OH	OH	OH	OH
Chrysin	23	OH	OH	H	H	H
Galangin	27	OH	OH	OH	H	H

[a] Compounds are in order of elution time.

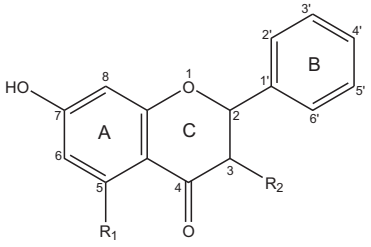
ing compounds were initially hypothesized on the basis of their molecular weight (MW): apigenin (MW 270) and chrysin (MW 254) among flavones; quercetin (MW 302), kaempferol (MW 286), isorhamnetin (MW 316) and galangin (MW 270) among flavonols; pinobanksin (MW 272) and pinocembrin (MW 256) among dihydroflavonols and flavanones, respectively.

MS/MS spectra were therefore recorded to study the fragmentation pathways of the different classes of flavonoids. The collision energies were optimized with both IT and TQ mass analyzers in order to acquire spectra with a good fragmentation degree from the precursor ions and thus obtain as much structural information as possible. As previously described by Medana et al. [11], in this study the TQ mass analyzer provided more collision energy if compared with IT, originating product ions at best sensitivity.

Neutral losses commonly described to occur for these compounds, such as H_2O (−18 u), CO (−28 u) and C_2H_2O (−42 u) in the positive mode and CO, C_2H_2O and CO_2 in the negative mode or the successive loss of these small groups, were frequently observed [13–15]. Methylated or methoxylated flavonoids presented also product ion s characterized by the loss of CH_3 (−15 u) both in the positive and in the negative mode and CH_3OH (−32 u) in the positive mode [13–15]. In some cases, a direct cleavage of the bond between the B- and C-rings, resulting in a [M-ring B] fragment, was observed in the negative mode [13,15].

The most useful fragmentations in terms of flavonoid identification are those that require the cleavage of two C–C bonds of the C-ring, due to retro-Diels-Alder (RDA) reactions, resulting in structurally informative ^{i,j}A and ^{i,j}B ions [13–15]. The following positions were involved in the RDA reactions of the main flavonoid classes present in propolis extracts in the positive mode: 1/3 and 0/4 for flavones and flavanones; 1/3 and 0/2 for flavonols; 1/2 and 1/3 for dihydroflavonols. The $[^{1,3}A]^+$ ion, which was observed for all flavonoid groups and usually occurred at m/z 153 for un-substituted compounds, was generally the fragment most readily formed and often represented the most abundant product ion [13,14,16].

Table 2C
Structures of flavanones and dihydroflavonols identified in propolis extracts[a].



Compound	Peak number	R_1	R_2
Pinobanksin-5-methyl-ether	7	OCH_3	OH
Pinobanksin	13	OH	OH
Pinobanksin-5-methyl-ether-3-*O*-acetate	18	OCH_3	$OCOCH_3$
Pinocembrin	26	OH	H
Pinobanksin-3-*O*-acetate	29	OH	$OCOCH_3$
Pinobanksin-3-*O*-propionate	35	OH	$OCOC_2H_5$
Pinobanksin-3-*O*-butyrate[b]	37	OH	$OCOC_3H_7$
Pinobanksin-3-*O*-pentanoate[b]	38	OH	$OCOC_4H_9$
Pinobanksin-3-*O*-hexanoate[b]	39	OH	$OCOC_5H_{11}$

[a] Compounds are in order of elution time.
[b] Or positional isomers.

In the negative mode, different mechanisms and structures have been proposed for RDA reactions of flavonoids [15], involving the following positions: 1/3 and 1/4 for flavones, flavanones and dihydroflavonols; 1/2 and 1/3 for flavonols. The $[^{1,3}A]^-$ ions of flavones, flavonols and flavanones were found at *m/z* 151 in the negative mode. In the case of quercetin and its derivatives, the ions at *m/z* 151 were attributed to $[^{1,2}A–CO]^-$ fragments, which exhibit the same structure of the $[^{1,3}A]^-$ ions, but are obtained from a different fragmentation pathway [15].

By following all the fragmentation pathways previously described for the target analytes in the positive [13,14,16] and the negative [15] ion modes, the structure of the aglycones initially hypothesized on the basis of their quasi-molecular ions was finally confirmed.

Regarding the methyl derivatives of flavones (chrysin and luteolin) and flavonols (quercetin and galangin), in most cases their fragmentation patterns in both the negative and the positive ion modes suggested that the methyl substituents are linked to the γ-benzopyrone moiety, but the exact position could not be discriminated by MS/MS analysis. In the case of quercetin-3-methyl ether, quecetin-7-methyl ether and galangin-5-methyl-ether, the availability of the reference standards allowed to unambiguously assign the corresponding chromatographic peaks. In the case of chrysin and luteolin methyl derivatives, whose standards were not available, the MS/MS fragmentation pattern indicated that the methyl group is located in the A-ring, either at C_5 or C_7. In accordance with the literature [5,10], the C_5 derivatives of flavonoids tend to elute before the corresponding aglycones under RP-HPLC conditions. This consideration was found to occur in the case of chrysin methyl derivative (t_R = 17.3 min), which eluted earlier in comparison with the corresponding aglycone (t_R = 39.1 min); thus, the methyl group of the chrysin derivative was finally located at C_5. In the case of luteolin, the HPLC analysis demonstrated that its methyl derivative eluted after (t_R = 22.9 min) its aglycone (t_R = 12.7 min). Therefore, the methyl group of the luteolin derivative was supposed to be located at C_7.

Among dihydroflavonols, pinobanksin esters deserve a specific comment. The first fragmentation observed for these compounds was the loss of the acyl group, yielding fragments at *m/z* 273 in the positive ion mode and *m/z* 271 in the negative ion mode, corresponding to [M–acyl] ions, which in turn produced the ions at *m/z* 255 and 253, accounting for the fragments [M–acyl–H_2O]. All the subsequent fragmentation steps of pinobanksin esters followed the pathways proposed for flavones both in the positive and the negative ion modes [14,15]. Very frequently, the loss of CO from the flavone moiety originated the product ions at *m/z* 227 in the positive mode, accounting for the [M+H–acyl–H_2O–CO] ions. In this way, pinobanskin-3-*O*-acetate, propionate, butyrate (or isomer), pentanoate (or isomer) and hexanoate (or isomer) were identified. A similar trend was also observed for pinobanksin ethers, such as pinobanksin-5-methyl-ether and pinobansksin-5-methyl-ether-3-*O*-acetate, as described by Gardana et al. [10].

Tables 3–6 describe the MS and MS/MS data obtained by the HPLC analysis of a representative sample of propolis extract (PE-9) by using IT and TQ mass analyzers in the positive and the negative ion modes.

3.2. HPLC-DAD method validation

HPLC-DAD was finally preferred for quantitative analysis of phenolics in propolis samples, in view of the higher availability and use of this equipment in the phytochemical analysis of natural products. The method validation was performed to show compliance with international requirements for analytical techniques for the quality control of pharmaceuticals (ICH guidelines) [12].

The validation parameters of each calibration curve (slope (*a*), intercept (*b*), standard error of slope, standard error of intercept, correlation coefficient (*r*), limit of detection (LOD) and quantification (LOQ)) are shown in Table 1. Good linearity was observed for the analytes between peak areas and concentrations over the range tested ($r > 0.998$). The LOD and LOQ values were in the range 1.6–4.6 μg/ml and 2.6–7.7 μg/ml, respectively. These results indicate that the proposed HPLC method is sufficiently sensitive for the determination of phenolic acids and flavonoids in propolis samples.

The accuracy of the analytical procedure was evaluated using the recovery test. The percentage recovery values that were obtained by comparing the results from samples and fortified samples were found to be in the range 96–105%. Considering the results of the recovery test, this method can be considered accurate.

The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections of a standard solution containing pure standards of phenolic acids and flavonoids available in this study. The low values of intra- and inter-day %RSD values for both retention times (%RSD < 0.3) and peak areas (%RSD < 1.9) indicate the high precision of the chromatographic system.

Specificity was tested by using the HPLC method to analyze a commercial sample containing a hydroalcoholic propolis extract (PE-1) in combination with other plant extracts (lemon essential oil). The chromatogram obtained from this product showed that the HPLC method can discriminate propolis components from the constituents of other plant extracts. Furthermore, peak purity tests were performed using the diode array detector to demonstrate that the analyte chromatographic peak was pure and not attributable to more than one component, with the exception of caffeic acid phenylethyl ester (CAPE) and pinobanksin-3-*O*-acetate.

Stability was tested with a propolis extract (PE-9) that was stored in amber glass flasks at 4 °C and at room temperature (about 25 °C) and analyzed every 12 h. The analytes in solution did not show any appreciable change in the chromatographic profile over 72 h. No degradation products were detected.

The validation data indicated that the proposed HPLC method provides good linearity, sensitivity, accuracy, precision and specificity and highlighted its suitability for the analysis of phenolic acids and flavonoids in propolis samples.

3.3. Content of phenolic acids and flavonoids in propolis extracts

The developed RP-HPLC method was applied to the analysis of phenolics in propolis extracts available on the Italian market. Qualitative and quantitative data are reported in Table 7.

All the analyzed samples displayed a common phytochemical profile, based on the presence of five classes of phytochemicals, including phenolic acids, flavones, flavonols, flavanones and dihydroflavonols. However, there was a great variability in the concentrations of the active constituents among the commercial samples on sale on the Italian market. The preparations indicated as PE-7 and PE-9 contained higher amounts of total phenolics (51.09 ± 1.22 and 54.14 ± 2.21 mg/ml, respectively), whereas PE-1 contained lower levels (2.65 ± 0.02 mg/ml). In particular, the sample labelled as PE-7 displayed a higher level of all the five classes of active compounds previously described, while sample PE-9 contained a higher level of phenolic acids (16.67 ± 0.68 mg/ml). All the other samples contained medium level concentration of total phenolics, from 9.61 ± 0.60 mg/ml in sample PE-2 to 33.38 ± 0.87 mg/ml in sample PE-4. The values of total flavonoids for samples PE-1 and PE-7 were of the same order of magnitude of their label claims.

The standard preparation of propolis hydroalcoholic extracts is usually based on maceration of the raw material with the extraction solvent (usually EtOH-H_2O) for a long period of time or on

Table 3
HPLC-ESI-MS^2 (IT) data obtained for the analysis of propolis constituents in the positive ion mode.

Peak number	Compound	UV λ_{max} (nm)	$[M+H]^+$	MS^2 fragment identification	Identification method
1	Caffeic acid	298,324	181	$[M+H-H_2O]^+ = 163$, $[M+H-H_2O-CO]^+ = 135$	a,b,c
2	*p*-Coumaric acid	298,310	165	$[M+H-H_2O]^+ = 147$, $[M+H-H_2O-CO]^+ = 119$	a,b,c
3	Ferulic acid	298,324	195	$[M+H-H_2O]^+ = 177$, $[M+H-H_2O-CH_3OH]^+ = 145$, $[M+H-H_2O-CO-CH_3OH]^+ = 117$	a,b,c
4	Isoferulic acid	296,321	195	$[M+H-H_2O]^+ = 177$, $[M+H-H_2O-CH_3OH]^+ = 145$, $[M+H-H_2O-CO-CH_3OH]^+ = 117$	b,c
5	3,4-Dimethyl-caffeic acid (DMCA)	296,322	209	$[M+H-H_2O]^+ = 191$, $[M+H-H_2O-CO]^+ = 163$, $[M+H-H_2O-CO-2CH_3]^+ = 133$	b,c
6	Quercetin	256,372	303	$[M+H-H_2O]^+ = 285$, $[M+H-H_2O-CO]^+ = 257$, $[M+H-H_2O-2CO]^+ = 229$, $[^{0,2}A]^+ = 165$, $[^{1,3}A]^+ = 153$, $[^{1,3}B-2H]^+ = 149$	a,b,c
7	Pinobanksin-5-methyl-ether	288,318sh	287	$[M+H-H_2O]^+ = 269$, $[M+H-H_2O-CO]^+ = 241$, $[^{1,3}A-CH_3]^+ = 152$, $[^{1,2}B]^+ = 91$	b,c
8	Quercetin-3-methyl-ether	256,358	317	$[M+H-CH_3]^+ = 302$, $[^{0,2}A]^+ = 165$, $[^{1,3}A]^+ = 153$, $[^{0,2}A-CO]^+$ or $[^{0,2}B]^+ = 137$	a,b,c
9	Cinnamic acid	278	149	–	a,c
10	Chrysin-5-methyl-ether	264,314	269	$[M+H-CH_3]^+ = 254$, $[^{1,3}A]^+ = 167$	b,c
11	Apigenin	267,338	271	$[M+H-H_2O]^+ = 253$, $[^{1,3}A]^+ = 153$, $[^{1,3}B]^+ = 119$	a,b,c
12	Kaempferol	266,366	287	$[^{0,2}A]^+ = 165$, $[^{1,3}A]^+ = 153$, $[^{0,2}B]^+ = 121$	a,b,c
13	Pinobanksin	291,330sh	273	$[M+H-H_2O]^+ = 255$, $[M+H-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	a,b,c
14	Isorhamnetin	255,372	317	$[M+H-CH_3]^+ = 302$, $[M+H-CH_3OH]^+ = 285$, $[M+H-2CO-C_4H_4O_2]^+ = 177$, $[^{1,3}A]^+ = 153$	a,b,c
15	Luteolin-methyl-ether	266,350	301	$[M+H-CH_3]^+ = 286$, $[M+H-2C_2H_2O]^+ = 217$	b,c
16	Quercetin-dimethyl-ether	254,356	331	$[M+H-CH_3]^+ = 316$, $[M+H-2CH_3]^+ = 301$, $[M+H-CH_3OH]^+ = 299$	b,c
17	Galangin-5-methyl-ether	260,302sh,352	285	$[M+H-CH_3]^+ = 270$, $[M+H-H_2O-CO]^+ = 239$, $[^{1,3}A]^+ = 167$	a,b,c
18	Pinobanksin-5-methyl-ether-3-*O*-acetate	288,326	329	$[M+H-acetate]^+ = 287$, $[M+H-acetate-H_2O-CO]^+ = 241$	b,c
19	Cinnamilidenacetic acid	312	175	$[M+H-H_2O]^+ = 157$, $[M+H-H_2O-CO]^+ = 129$	b,c
20	Quercetin-7-methyl-ether	256,372	317	$[M+H-CH_3]^+ = 302$, $[M+H-H_2O-CO]^+ = 271$, $[M+H-H_2O-2CO]^+ = 243$, $[^{0,2}A]^+ = 179$, $[^{1,3}A]^+ = 167$	a,b,c
21	Quercetin-dimethyl-ether	256,357	331	$[M+H-CH_3]^+ = 316$, $[M-CH_3OH]^+ = 299$	b,c
22	Caffeic acid prenyl ester	298,326	249	$[C_9H_7O_3]^+ = 163$	b,c
23	Chrysin	268,314sh	255	$[M+H-H_2O-CO]^+ = 209$, $[^{1,3}A]^+ = 153$, $[^{0,4}B-H_2O]^+ = 129$	a,b,c
24	Caffeic acid benzyl ester	298,328	271	–	c
25	Caffeic acid prenyl ester	296,326	249	–	c
26	Pinocembrin	290,330sh	257	$[M+H-C_2H_2O]^+ = 215$, $[^{1,3}A]^+ = 153$, $[^{0,4}B-H_2O]^+ = 131$, $[^{0,4}B-H_2O-CO]^+ = 103$	a,b,c
27	Galangin	260,308sh,360	271	$[^{0,2}A]^+ = 165$, $[^{1,3}A]^+ = 153$, $[^{0,2}B]^+ = 105$	a,b,c
28	Caffeic acid phenylethyl ester (CAPE)	298,328	285	$[C_9H_7O_3]^+ = 163$, $[C_8H_9]^+ = 105$	a,b,c
29	Pinobanksin-3-*O*-acetate	294,332sh	315	$[M+H-acetate]^+ = 273$, $[M+H-acetate-H_2O]^+ = 255$, $[M+H-acetate-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	a,b,c
30	Methoxy-chrysin	266,310sh,340sh	285	$[M+H-CH_3]^+ = 270$, $[M+H-CO]^+ = 257$, $[M+H-CH_3-CO]^+ = 242$	b,c
31	*p*-Coumaric prenyl ester	294,310	233	$[C_9H_7O_2]^+ = 147$	b,c
32	*p*-Coumaric benzyl ester	298,312	255	–	c
33	Caffeic acid cinnamyl ester	297,326	297	–	c
34	*p*-Coumaric prenyl ester	296,324	233	$[C_9H_7O_2]^+ = 147$	b,c
35	Pinobanksin-3-*O*-propionate	292,330sh	329	$[M+H-propionate]^+ = 273$, $[M+H-propionate-H_2O]^+ = 255$, $[M+H-propionate-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	b,c
36	*p*-Coumaric cinnamyl ester	296,310	281	–	c
37	Pinobanksin-3-*O*-butyrate[a]	268,310sh	343	$[M+H-butyrate]^+ = 273$, $[M+H-butyrate-H_2O]^+ = 255$, $[M+H-butyrate-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	b,c
38	Pinobanksin-3-*O*-pentanoate[a]	292,332sh	357	$[M+H-pentanoate]^+ = 273$, $[M+H-pentanoate-H_2O]^+ = 255$, $[M+H-pentanoate-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	b,c
39	Pinobanksin-3-*O*-hexanoate[a]	282	371	$[M+H-hexanoate]^+ = 273$, $[M+H-hexanoate-H_2O]^+ = 255$, $[M+H-hexanoate-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	b,c
40	*p*-Methoxy cinnamic acid cinnamyl ester	278	295	149	c

Experimental conditions as in Section 2.3. a: Confirmed with standard, b: confirmed with MS^2 fragmentation, c: confirmed with references.

[a] Or positional isomers.

Table 4
HPLC-ESI-MS/MS (TQ) data obtained for the analysis of propolis constituents in the positive ion mode.

Peak number	Compound	UV λ_{max} (nm)	$[M+H]^+$	MS/MS fragment identification	Identification method
1	Caffeic acid	298,324	181	$[M+H-H_2O]^+ = 163$, $[M+H-H_2O-CO]^+ = 135$	a,b,c
2	*p*-Coumaric acid	298,310	165	$[M+H-H_2O]^+ = 147$, $[M+H-H_2O-CO]^+ = 119$	a,b,c
3	Ferulic acid	298,324	195	$[M+H-H_2O]^+ = 177$, $[M+H-H_2O-CO]^+ = 149$, $[M+H-H_2O-CH_3OH]^+ = 145$, $[M+H-H_2O-CO-CH_3]^+ = 134$, $[M+H-H_2O-CO-CH_3OH]^+ = 117$	a,b,c
4	Isoferulic acid	296,321	195	$[M+H-H_2O]^+ = 177$, $[M+H-H_2O-CO]^+ = 149$, $[M+H-H_2O-CH_3OH]^+ = 145$, $[M+H-H_2O-CO-CH_3]^+ = 134$, $[M+H-H_2O-CO-CH_3OH]^+ = 117$	b,c
5	3,4-Dimethyl-caffeic acid (DMCA)	296,322	209	$[M+H-H_2O]^+ = 191$, $[M+H-H_2O-CH_3]^+ = 176$, $[M+H-H_2O-CO]^+ = 163$, $[M+H-H_2O-CO-CH_3]^+ = 148$, $[M+H-H_2O-CO-2CH_3]^+ = 133$	b,c
6	Quercetin	256,372	303	$[M+H-H_2O]^+ = 285$, $[M+H-H_2O-CO]^+ = 257$, $[M+H-2CO]^+ = 247$, $[M+H-H_2O-2CO]^+ = 229$, $[^{0,2}A]^+ = 165$, $[^{1,3}A]^+ = 153$, $[^{0,2}A-CO]^+$ or $[^{0,2}B]^+ = 137$, $[^{1,3}B-2H-CO]^+ = 121$, $[^{1,3}A-C_2H_2O]^+ = 111$	a,b,c
7	Pinobanksin-5-methyl-ether	288,318sh	287	$[M+H-H_2O-CO]^+ = 241$, $[M+H-H_2O-CO-CH_3]^+ = 226$, $[^{1,3}A]^+ = 167$, $[^{1,2}B]^+ = 91$	b,c
8	Quercetin-3-methyl-ether	256,358	317	$[M+H-CH_3]^+ = 302$, $[M+H-CH_3OH]^+ = 285$, $[^{0,2}A-CO]^+$ or $[^{0,2}B]^+ = 137$	a,b,c
9	Cinnamic acid	278	149	–	a,c
10	Chrysin-5-methyl-ether	264,314	269	$[M+H-CH_3]^+ = 254$, $[M+H-CH_3-CO]^+ = 226$, $[^{1,3}A-CH_3]^+ = 152$	b,c
11	Apigenin	267,338	271	$[^{1,3}A]^+ = 153$, $[^{0,4}B-H_2O]^+ = 145$, $[^{1,3}B]^+ = 119$, $[^{1,3}B-CO]^+ = 91$	a,b,c
12	Kaempferol	266,366	287	$[M+H-H_2O-CO]^+ = 241$, $[M+H-2CO]^+ = 231$, $[M+H-H_2O-2CO]^+ = 213$, $[^{0,2}A]^+ = 165$, $[^{1,3}A]^+ = 153$, $[M+H-2CO-C_4H_4O_2]^+ = 147$, $[^{0,2}A-CO]^+ = 137$, $[^{0,2}B]^+ = 121$, $[^{1,3}A-C_2H_2O]^+ = 111$	a,b,c
13	Pinobanksin	291,330sh	273	$[M+H-H_2O]^+ = 255$, $[M+H-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$, $[^{1,2}B]^+ = 91$	a,b,c
14	Isorhamnetin	255,372	317	$[M+H-CH_3]^+ = 302$, $[M+H-CH_3OH]^+ = 285$, $[M+H-CH_3OH-CO]^+ = 257$, $[M+H-2CO-C_4H_4O_2]^+ = 177$, $[^{0,2}A]^+ = 165$, $[^{1,3}A]^+ = 153$	a,b,c
15	Luteolin-methyl-ether	266,350	301	$[M+H-CH_3]^+ = 286$, $[M+H-CH_3-CO]^+ = 258$	b,c
16	Quercetin-dimethyl-ether	254,356	331	$[M+H-CH_3]^+ = 316$, $[M+H-2CH_3]^+ = 301$	b,c
17	Galangin-5-methyl-ether	260,302sh,352	285	$[M+H-CH_3]^+ = 270$	a,b,c
18	Pinobanksin-5-methyl-ether-3-*O*-acetate	288,326	329	$[M+H-acetate]^+ = 287$, $[M+H-acetate-H_2O]^+ = 269$, $[M+H-acetate-H_2O-CO]^+ = 241$, $[M+H-acetate-H_2O-CO-CH_3]^+ = 226$, $[^{1,3}A]^+ = 167$, $[^{1,2}B]^+ = 91$	b,c

Table 4 (*Continued*)

Peak number	Compound	UV λ_{max} (nm)	$[M+H]^+$	MS/MS fragment identification	Identification method
19	Cinnamilidenacetic acid	312	175	$[M+H-H_2O]^+ = 157$, $[M+H-H_2O-CO]^+ = 129$	b,c
20	Quercetin-7-methyl-ether	256,372	317	$[M+H-CH_3]^+ = 302$, $[M+H-H_2O-CO]^+ = 271$, $[M+H-2CO]^+ = 261$, $[M+H-H_2O-2CO]^+ = 243$, $[^{0,2}A]^+ = 179$, $[^{1,3}A]^+ = 167$, $[^{0,2}A-CO]^+ = 151$, $[^{0,2}B]^+ = 137$, $[^{0,2}A-2CO]^+ = 123$	a,b,c
21	Quercetin-dimethyl-ether	256,357	331	$[M+H-CH_3]^+ = 316$, $[M+H-CH_3OH]^+ = 299$	b,c
22	Caffeic acid prenyl ester	298,326	249	$[C_9H_7O_3]^+ = 163$, $[C_8H_7O_2]^+ = 135$	b,c
23	Chrysin	268,314sh	255	$[M+H-H_2O-CO]^+ = 209$, $[^{1,3}A]^+ = 153$, $[^{0,4}B]^+ = 147$, $[^{0,4}B-H_2O]^+ = 129$, $[^{1,3}B]^+ = 103$	a,b,c
24	Caffeic acid benzyl ester	298,328	271	$[C_9H_7O_3]^+ = 163$, $[C_8H_7O_2]^+ = 135$	b,c
25	Caffeic acid prenyl ester	296,326	249	–	c
26	Pinocembrin	290,330sh	257	$[^{1,3}A]^+ = 153$, $[^{0,4}B-H_2O]^+ = 131$, $[^{0,4}B-H_2O-CO]^+ = 103$	a,b,c
27	Galangin	260,308sh,360	271	$[M+H-2CO]^+ = 215$, $[M+H-H_2O-2CO]^+ = 197$, $[^{0,2}A]^+ = 165$, $[^{1,3}A]^+ = 153$, $[M+H-2CO-C_4H_4O_2]^+ = 131$, $[^{0,2}B]^+ = 105$, $[^{0,2}B-CO]^+ = 77$	a,b,c
28	Caffeic acid phenylethyl ester (CAPE)	298,328	285	–	a,c
29	Pinobanksin-3-*O*-acetate	294,332sh	315	$[M+H-acetate]^+ = 273$, $[M+H-acetate-H_2O]^+ = 255$, $[M+H-acetate-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	a,b,c
30	Methoxy-chrysin	266,310sh,340sh	285	$[M+H-CH_3]^+ = 270$	b,c
31	*p*-Coumaric prenyl ester	294,310	233	$[C_9H_7O_2]^+ = 147$, $[C_8H_7O]^+ = 119$	b,c
32	*p*-Coumaric benzyl ester	298,312	255	–	c
33	Caffeic acid cinnamyl ester	297,326	297	–	c
34	*p*-Coumaric prenyl ester	296,324	233	$[C_8H_7O]^+ = 119$	b,c
35	Pinobanksin-3-*O*-propionate	292,330sh	329	$[M+H-propionate]^+ = 273$, $[M+H-propionate-H_2O]^+ = 255$, $[M+H-propionate-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	b,c
36	*p*-Coumaric cinnamyl ester	296,310	281	–	c
37	Pinobanksin-3-*O*-butyrate[a]	268,310sh	343	$[M+H-butyrate]^+ = 273$, $[M+H-butyrate-H_2O]^+ = 255$, $[M+H-butyrate-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	b,c
38	Pinobanksin-3-*O*-pentanoate[a]	292,332sh	357	$[M+H-pentanoate-H_2O]^+ = 255$, $[M+H-pentanoate-H_2O-CO]^+ = 227$, $[^{1,3}A]^+ = 153$	b,c
39	Pinobanksin-3-*O*-hexanoate[a]	282	371	–	c
40	*p*-Methoxy cinnamic acid cinnamyl ester	278	295	–	c

Experimental conditions as in Section 2.3. a: Confirmed with standard, b: confirmed with MS/MS fragmentation, c: confirmed with references.

[a] Or positional isomers.

Table 5
HPLC-ESI-MS^2 (IT) data obtained for the analysis of propolis constituents in the negative ion mode.

Peak number	Compound	UV λ_{max} (nm)	$[M-H]^-$	MS^2 fragment identification	Identification method
1	Caffeic acid	298,324	179	$[M-H-CO_2]^-$ = 135	a,b,c
2	*p*-Coumaric acid	298,310	163	$[M-H-CO_2]^-$ = 119	a,b,c
3	Ferulic acid	298,324	193	$[M-H-CO_2]^-$ = 149, $[M-H-CO_2-CH_3]^-$ = 134	a,b,c
4	Isoferulic acid	296,321	193	$[M-H-CO_2]^-$ = 149, $[M-H-CO_2-CH_3]^-$ = 134	b,c
5	3,4-Dimethyl-caffeic acid (DMCA)	296,322	207	$[M-H-CO_2]^-$ = 163, $[M-H-CO_2-2CH_3]^-$ = 133	b,c
6	Quercetin	256,372	301	$[M-H-CO_2]^-$ = 257, [M−H–ring B]$^-$ = 193, $[^{1,2}A]^-$ = 179, $[^{1,2}A-CO]^-$ = 151, $[^{1,2}B]^-$ = 121, $[^{1,2}A-CO-CO_2]^-$ = 107	a,b,c
7	Pinobanksin-5-methyl-ether	288,318sh	285	$[M-H-H_2O]^-$ = 267, $[M-H_2O-CH_3]^-$ = 253, $[M-H-H_2O-CO]^-$ = 239, $[M-H-H_2O-2CO_2]^-$ = 179, $[^{1,3}A]^-$ = 165, $[^{1,4}A]^-$ = 139	b,c
8	Quercetin-3-methyl-ether	256,358	315	$[M-H-CH_3]^-$ = 300, $[M-H-CO_2]^-$ = 271, $[M-H-CH_3-CO_2-CO]^-$ = 228, $[^{1,2}A-CO]^-$ = 151	a,b,c
9	Cinnamic acid	278	147	$[M-H-CO_2]^-$ = 103	a,b,c
10	Chrysin-5-methyl-ether	264,314	267	$[M-H-CH_3]^-$ = 252, $[M-H-CH_3-CO]^-$ = 224, $[M-H-CH_3-CO_2]^-$ = 180	b,c
11	Apigenin	267,338	269	$[M-H-CO_2]^-$ = 225, $[M-H-CO_2-CO]^-$ = 197, $[^{1,3}A]^-$ = 151, $[^{1,4}B+2H]^-$ = 149, $[^{1,3}B]^-$ = 117	a,b,c
12	Kaempferol	266,366	285	$[M-H-CO]^-$ = 257, $[M-H-CO_2]^-$ = 241, $[^{1,3}A]^-$ = 151, $[^{1,3}B]^-$ = 133	a,b,c
13	Pinobanksin	291,330sh	271	$[M-H-H_2O]^-$ = 253, $[M-H-CO]^-$ = 243, $[M-H-H_2O-2CO_2]^-$ = 165, $[^{1,3}A]^-$ = 151, $[^{1,3}A-CO_2]^-$ = 107	a,b,c
14	Isorhamnetin	255,372	315	$[M-H-CH_3]^-$ = 300, $[^{1,2}A-CO]^-$ = 151	a,b,c
15	Luteolin-methyl-ether	266,350	299	$[M-H-CH_3]^-$ = 284, $[M-H-CH_3-CO]^-$ = 256, $[M-H-CO_2]^-$ = 255, $[^{1,3}A-CH_3]^-$ = 151	b,c
16	Quercetin-dimethyl-ether	254,356	329	$[M-H-CH_3]^-$ = 314, $[M-H-2CH_3]^-$ = 299, $[M-H-CO_2]^-$ = 285, $[M-H-C_2H_2O-CO_2]^-$ = 243	b,c
17	Galangin-5-methyl-ether	260,302sh,352	283	$[M-H-CH_3]^-$ = 268, $[M-H-CO_2]^-$ = 239	a,b,c
18	Pinobanksin-5-methyl-ether-3-*O*-acetate	288,326	327	$[M-acetate]^-$ = 285, $[M-acetate-H_2O]^-$ = 267, $[M-acetate-H_2O-CH_3]^-$ = 252, $[M-acetate-H_2O-CO-CH_3]^-$ = 224	b,c
19	Cinnamilidenacetic acid	312	173	–	c
20	Quercetin-7-methyl-ether	256,372	315	$[M-H-CH_3]^-$ = 300, $[M-H-CO_2]^-$ = 271, $[M-H-CH_3-CO_2]^-$ = 256, [M−H–ring B]$^-$ = 206, $[^{1,2}A]^-$ = 193, $[^{1,2}A-CO]^-$ = 165	a,b,c

Table 5 (*Continued*)

Peak number	Compound	UV λ_{max} (nm)	$[M-H]^-$	MS^2 fragment identification	Identification method
21	Quercetin-dimethyl-ether	256,357	329	$[M-H-CH_3]^-$ = 314, $[M-H-2CH_3]^-$ = 299, $[M-H-2CH_3-CO]^-$ = 271	b,c
22	Caffeic acid prenyl ester	298,326	247	$[C_9H_7O_4]^-$ = 179, $[C_8H_7O_2]^-$ = 135	b,c
23	Chrysin	268,314sh	253	$[M-H-CO_2]^-$ = 209, $[M-H-C_2H_2O-CO_2]^-$ = 167, $[^{1,3}A]^-$ = 151, $[M-H-C_3O_2-C_2H_2O]^-$ = 143	a,b,c
24	Caffeic acid benzyl ester	298,328	269	$[C_9H_6O_4]^-$ = 178, $[C_8H_6O_2]^-$ = 134	b,c
25	Caffeic acid prenyl ester	296,326	247	$[C_9H_7O_4]^-$ = 179, $[C_8H_7O_2]^-$ = 135	b,c
26	Pinocembrin	290,330sh	255	$[M-H-C_2H_2O]^-$ = 213, $[M-H-C_3O_2]^-$ = 187, $[^{1,3}A]^-$ = 151, $[M-H-C_3O_2-C_2H_2O]^-$ = 145, 136, $[^{1,4}A]^-$ = 125, $[^{1,3}B]^-$ = 103	a,b,c
27	Galangin	260,308sh,360	269	$[M-H-C_2H_2O]^-$ = 227, $[M-H-CO_2-CO]^-$ = 197, $[M-H-C_2H_2O-CO_2]^-$ = 183, $[^{1,3}A]^-$ or $[^{1,2}A-CO]^-$ = 151	a,b,c
28	Caffeic acid phenylethyl ester (CAPE)	298,328	283	$[C_9H_7O_4]^-$ = 179, $[C_8H_7O_2]^-$ = 135	a,b,c
29	Pinobanksin-3-*O*-acetate	294,332sh	313	$[M-acetate]^-$ = 271, $[M-acetate-H_2O]^-$ = 253, $[M-acetate-H_2O-CO_2]^-$ = 209, $[M-acetate-H_2O-C_3O_2]^-$ = 185	a,b,c
30	Methoxy-chrysin	266,310sh,340sh	283	$[M-H-CH_3]^-$ = 268, $[M-H-CO_2]^-$ = 239, $[M-H-CO_2-CO]^-$ = 211	b,c
31	*p*-Coumaric prenyl ester	294,310	231	$[C_9H_7O_3]^-$ = 163, $[C_8H_7O]^-$ = 119	b,c
32	*p*-Coumaric benzyl ester	298,312	253	$[C_9H_6O_3]^-$ = 162, $[C_9H_5O_2]^-$ = 145, $[C_8H_6O]^-$ = 118	b,c
33	Caffeic acid cinnamyl ester	297,326	295	$[C_9H_6O_4]^-$ = 178, $[C_8H_6O_2]^-$ = 134	b,c
34	*p*-Coumaric prenyl ester	296,324	231	–	c
35	Pinobanksin-3-*O*-propionate	292,330sh	327	$[M-propionate]^-$ = 271, $[M-propionate-H_2O]^-$ = 253, $[M-propionate-H_2O-C_2H_2O]^-$ = 211	b,c
36	*p*-Coumaric cinnamyl ester	296,310	279	$[C_9H_6O_3]^-$ = 162, $[C_8H_6O]^-$ = 118	b,c
37	Pinobanksin-3-*O*-butyrate[a]	268,310sh	341	$[M-butyrate]^-$ = 271, $[M-butyrate-H_2O]^-$ = 253	b,c
38	Pinobanksin-3-*O*-pentanoate[a]	292,332sh	355	$[M-pentanoate]^-$ = 271, $[M-pentanoate-H_2O]^-$ = 253	b,c
39	Pinobanksin-3-*O*-hexanoate[a]	282	369	$[M-hexanoate]^-$ = 271, $[M-hexanoate-H_2O]^-$ = 253	b,c
40	*p*-Methoxy cinnamic acid cinnamyl ester	278	293	–	c

Experimental conditions as in Section 2.3. a: Confirmed with standard, b: confirmed with MS^2 fragmentation, c: confirmed with references.

[a] Or positional isomers.

Table 6
HPLC-ESI-MS/MS (TQ) data obtained for the analysis of propolis constituents in the negative ion mode.

Peak number	Compound	UV λ_{max} (nm)	$[M-H]^-$	MS/MS fragment identification	Identification method
1	Caffeic acid	298,324	179	$[M-H-CO_2]^-$ = 135	a,b,c
2	*p*-Coumaric acid	298,310	163	$[M-H-CO_2]^-$ = 119	a,b,c
3	Ferulic acid	298,324	193	$[M-H-CH_3]^-$ = 178, $[M-H-CO_2]^-$ = 149, $[M-H-CO_2-CH_3]^-$ = 134	a,b,c
4	Isoferulic acid	296,321	193	$[M-H-CH_3]^-$ = 178, $[M-H-CO_2-CH_3]^-$ = 134	b,c
5	3,4-Dimethyl-caffeic acid (DMCA)	296,322	207	–	c
6	Quercetin	256,372	301	$[M-H-CO]^-$ = 273, $[M-H-2CO]^-$ = 245, $[M-H-CO_2-CO]^-$ = 229, [M−H–ring B]$^-$ = 193, $[^{1,2}A]^-$ = 179,$[^{1,2}A-CO]^-$ = 151, $[^{1,2}B]^-$ = 121,$[^{1,2}A-CO-CO_2]^-$ = 107	a,b,c
7	Pinobanksin-5-methyl-ether	288,318sh	285	$[M-H-H_2O]^-$ = 267, $[M-H-H_2O-CH_3]^-$ = 252, $[M-H_2O-CO]^-$ = 239, $[M-H-H_2O-CH_3-CO]^-$ = 224, [M−H-ring B]$^-$ = 208, $[M-H-H_2O-CO-CO_2]^-$ = 195, $[M-H-H_2O-CH_3-CO_2]^-$ = 180, $[^{1,3}A]^-$ = 165, $[M-H-H_2O-CH_3-CO_2-CO]^-$ = 152, $[M-H-H_2O-CH_3-2CO_2]^-$ = 136	b,c
8	Quercetin-3-methyl-ether	256,358	315	$[M-H-CH_3]^-$ = 300, $[M-H-CO_2]^-$ = 271, $[M-H-CO_2-CO]^-$ = 243	a,b,c
9	Cinnamic acid	278	147	$[M-H-CO_2]^-$ = 103	a,b,c
10	Chrysin-5-methyl-ether	264,314	267	$[M-H-CH_3]^-$ = 252, $[M-H-CH_3-CO]^-$ = 224, $[M-H-CO_2-CO]^-$ = 195, $[M-H-CH_3-CO-CO_2]^-$ = 180	b,c
11	Apigenin	267,338	269	$[M-H-C_2H_2O]^-$ = 227, $[M-H-2CO_2]^-$ = 181, $[^{1,3}A]^-$ = 151, $[^{1,4}B+2H]^-$ = 149, $[^{1,3}B]^-$ = 117, $[^{1,3}A-CO_2]^-$ = 107	a,b,c
12	Kaempferol	266,366	285	$[M-H-2CO]^-$ = 229, $[^{1,3}A]^-$ = 151	a,b,c
13	Pinobanksin	291,330sh	271	$[M-H-H_2O]^-$ = 253, $[M-H-H_2O-CO]^-$ = 225, $[M-H-H_2O-CO_2]^-$ = 209, $[M-H-H_2O-C_3O_2]^-$ = 185, $[^{1,3}A]^-$ = 151, $[^{1,4}B+2H]^-$ = 133, $[^{1,4}A]^-$ = 125, $[^{1,3}A-CO_2]^-$ = 107	a,b,c
14	Isorhamnetin	255,372	315	$[M-H-CH_3]^-$ = 300, $[^{1,2}A-CO]^-$ = 151	a,b,c
15	Luteolin-methyl-ether	266,350	299	$[M-H-CH_3]^-$ = 284, $[M-H-CO_2]^-$ = 255, $[M-H-CO_2-CO]^-$ = 227, $[M-H-2CO_2]^-$ = 211	b,c
16	Quercetin-dimethyl-ether	254,356	329	$[M-H-CH_3]^-$ = 314, $[M-H-2CH_3]^-$ = 299, $[M-H-CO_2]^-$ = 285, $[M-H-2CH_3-CO]$ = 271, $[M-H-CO_2-CO]^-$ = 257, $[M-H-C_2H_2O-CO_2]^-$ = 243, $[M-H-2CH_3-CO-CO_2]$ = 227	b,c
17	Galangin-5-methyl-ether	260,302sh,352	283	$[M-H-CH_3]^-$ = 268, $[M-H-CO_2]^-$ = 239, $[M-H-CO_2-CO]^-$ = 211	a,b,c
18	Pinobanksin-5-methyl-ether-3-*O*-acetate	288,326	327	[M−acetate]$^-$ = 285, [M−acetate–H_2O]$^-$ = 267, $[M-acetate-H_2O-CH_3]^-$ = 252, $[M-acetate-H_2O-CO]^-$ = 239, $[M-acetate-H_2O-CO-CH_3]^-$ = 224, $[M-acetate-H_2O-CO-CO_2]^-$ = 195, $[M-acetate-H_2O-CH_3-CO-CO_2]^-$ = 180, $[^{1,3}A]^-$ = 165, $[^{1,4}A]^-$ = 139	b,c
19	Cinnamilidenacetic acid	312	173	–	c
20	Quercetin-7-methyl-ether	256,372	315	$[M-H-CH_3]^-$ = 300, $[^{1,2}A]^-$ = 193, $[^{1,2}A-CO]^-$ = 165, $[^{1,2}B]^-$ = 121	a,b,c
21	Quercetin–dimethyl-ether	256,357	329	$[M-H-CH_3]^-$ = 314, $[M-H-2CH_3]^-$ = 299, $[M-H-2CH_3-CO]^-$ = 271, $[M-H-C_2H_2O-CO_2]^-$ = 243, $[M-H-2CH_3-CO-CO_2]$ = 227	b,c

Table 6 (*Continued*)

Peak number	Compound	UV λ_{max} (nm)	$[M-H]^-$	MS/MS fragment identification	Identification method
22	Caffeic acid prenyl ester	298,326	247	$[C_9H_7O_4]^-$ = 179, $[C_9H_5O_3]^-$ = 161, $[C_8H_7O_2]^-$ = 135	b,c
23	Chrysin	268,314sh	253	$[M-H-CO_2]^-$ = 209, $[M-H-CO_2-CO]^-$ = 181, $[M-H-C_2H_2O-CO_2]^-$ = 167, $[M-H-2CO_2]^-$ = 165, $[^{1,3}A]^-$ = 151, $[^{1,3}A-CO_2]^-$ = 107, 145, $[M-H-C_3O_2-C_2H_2O]^-$ = 143, 119	a,b,c
24	Caffeic acid benzyl ester	298,328	269	$[C_9H_6O_4]^-$ = 178, $[C_9H_5O_3]^-$ = 161, $[C_8H_6O_2]^-$ = 134	b,c
25	Caffeic acid prenyl ester	296,326	247	$[C_9H_7O_4]^-$ = 179, $[C_9H_5O_3]^-$ = 161, $[C_8H_7O_2]^-$ = 135	b,c
26	Pinocembrin	290,330sh	255	$[M-H-C_2H_2O]^-$ = 213, $[M-H-C_3O_2]^-$ = 187, 171, 164, $[^{1,3}A]^-$ = 151, $[M-H-C_3O_2-C_2H_2O]^-$ = 145, 136, $[^{1,3}A-CO_2]^-$ = 107	a,b,c
27	Galangin	260,308sh,360	269	–	a,c
28	Caffeic acid phenylethyl ester (CAPE)	298,328	283	$[C_9H_7O_4]^-$ = 179, $[C_9H_5O_3]^-$ = 161, $[C_8H_7O_2]^-$ = 135	a,b,c
29	Pinobanksin-3-*O*-acetate	294,332sh	313	$[M-acetate]^-$ = 271, $[M-acetate-H_2O]^-$ = 253, $[M-acetate-H_2O-CO_2]^-$ = 209, $[M-acetate-H_2O-CO_2-CO]^-$ = 181, $[M-acetate-H_2O-2CO_2]^-$ = 165, $[M-acetate-H_2O-C_3O_2-C_2H_2O]^-$ = 143, $[^{1,3}A]^-$ = 151, $[^{1,3}A-CO_2]^-$ = 107	a,b,c
30	Methoxy-chrysin	266,310sh,340sh	283	$[M-H-CH_3]^-$ = 268, $[M-H-CO_2]^-$ = 239, $[M-H-CO_2-CO]^-$ = 211, $[M-H-2CO_2]^-$ = 195	b,c
31	*p*-Coumaric prenyl ester	294,310	231	$[C_9H_7O_3]^-$ = 163, $[C_9H_5O_2]^-$ = 145, $[C_8H_7O]^-$ = 119	b,c
32	*p*-Coumaric benzyl ester	298,312	253	$[C_9H_6O_3]^-$ = 162, $[C_9H_5O_2]^-$ = 145, $[C_8H_6O]^-$ = 118	b,c
33	Caffeic acid cinnamyl ester	297,326	295	$[C_9H_6O_4]^-$ = 178, $[C_8H_6O_2]^-$ = 134	b,c
34	*p*-Coumaric prenyl ester	296,324	231	–	c
35	Pinobanksin-3-*O*-propionate	292,330sh	327	$[M-propionate-H_2O]^-$ = 253, $[M-propionate-H_2O-CO]^-$ = 225, $[M-propionate-H_2O-CO_2]^-$ = 209, $[M-propionate-H_2O-CO_2-CO]^-$ = 181, $[M-propionate-H_2O-2CO_2]^-$ = 165, $[M-propionate-H_2O-C_3O_2-C_2H_2O]^-$ = 143, $[^{1,3}A]^-$ = 151, $[^{1,3}B]^-$ = 101	b,c
36	*p*-Coumaric cinnamyl ester	296,310	279	–	c
37	Pinobanksin-3-*O*-butyrate[a]	268,310sh	341	$[M-butyrate-H_2O]^-$ = 253, $[M-butyrate-H_2O-CO_2]^-$ = 209, $[M-butyrate-H_2O-CO_2-CO]^-$ = 181, $[M-butyrate-H_2O-2CO_2]^-$ = 165, $[^{1,3}A]^-$ = 151, $[^{1,3}A-CO_2]^-$ = 107	b,c
38	Pinobanksin-3-*O*-pentanoate[a]	292,332sh	355	$[M-pentanoate-H_2O]^-$ = 253, $[M-pentanoate-H_2O-CO_2]^-$ = 209, $[M-pentanoate-H_2O-CO_2-CO]^-$ = 181, $[M-pentanoate-H_2O-2CO_2]^-$ = 165, $[M-pentanoate-H_2O-C_3O_2-C_2H_2O]^-$ = 143, $[^{1,3}A-CO_2]^-$ = 107, $[^{1,3}B]^-$ = 101	b,c
39	Pinobanksin–3-*O*-hexanoate[a]	282	369	$[M-hexanoate]^-$ = 271, $[M-hexanoate-H_2O]^-$ = 253, $[M-hexanoate-H_2O-CO_2]^-$ = 209, $[^{1,3}A]^-$ = 151, $[M-hexanoate-H_2O-C_3O_2-C_2H_2O]^-$ = 143	b,c
40	*p*-Methoxy cinnamic acid cinnamyl ester	278	293	–	c

Experimental conditions as in Section 2.3. a: Confirmed with standard, b: confirmed with MS/MS fragmentation, c: confirmed with references.

[a] Or positional isomers.

Table 7
Content of phenolic acids and flavonoids determined in commercial (PE-1/PE-9) and lab-made propolis extracts (PE-10 and PE-11) by HPLC-DAD (data are expressed as mg/ml).[a]

Peak number	Compound name	t_R (min)	PE-1	PE-2	PE-3	PE-4	PE-5	PE-6	PE-7	PE-8	PE-9	PE-10	PE-11
1	Caffeic acid	3.5	<LOD	0.04[b]	0.24 ± 0.01	0.81 ± 0.02	0.02[b]	0.38 ± 0.01	0.04[b]	0.47 ± 0.01	1.19 ± 0.05	0.76 ± 0.05	0.78 ± 0.01
2	*p*-Coumaric acid	5.2	<LOD	0.03[b]	1.12 ± 0.06	0.34 ± 0.01	0.02[b]	1.35 ± 0.04	0.09[b]	0.22 ± 0.01	0.48 ± 0.02	0.21 ± 0.02	0.21 ± 0.01
3	Ferulic acid	5.8	<LOD	0.02[b]	0.32 ± 0.02	0.31 ± 0.01	0.02[b]	0.44 ± 0.01	0.10[b]	0.15[b]	0.56 ± 0.02	0.23 ± 0.02	0.23[b]
4	Isoferulic acid	6.1	0.01[b]	0.29 ± 0.02	0.20 ± 0.01	0.44 ± 0.01	0.73 ± 0.04	0.36 ± 0.01	1.69 ± 0.04	0.23 ± 0.01	0.64 ± 0.03	0.30 ± 0.02	0.31 ± 0.01
5	3,4-Dimethyl-caffeic acid (DMCA)	10.9	0.03[b]	0.44 ± 0.03	0.20 ± 0.01	1.25 ± 0.03	1.16 ± 0.06	0.34 ± 0.01	2.50 ± 0.06	0.35 ± 0.01	1.75 ± 0.07	1.19 ± 0.08	1.22 ± 0.03
6	Quercetin	13.2	0.11[b]	0.05[b]	0.07[b]	0.30 ± 0.01	0.08[b]	0.08[b]	0.23[b]	0.21 ± 0.01	0.42 ± 0.02	0.28 ± 0.02	0.29[b]
7	Pinobanksin-5-methyl-ether	14.7	0.01[b]	0.20 ± 0.01	0.49 ± 0.03	1.15 ± 0.03	<LOD	0.64 ± 0.02	<LOD	0.61 ± 0.02	1.73 ± 0.07	1.31 ± 0.10	1.36 ± 0.02
8	Quercetin-3-methyl-ether	15.0	0.02[b]	0.09[b]	0.06[b]	0.33 ± 0.01	0.11[b]	0.08[b]	0.34 ± 0.01	0.19 ± 0.01	0.48 ± 0.02	0.38 ± 0.03	0.39 ± 0.01
9	Cinnamic acid	16.5	0.02[b]	0.40 ± 0.03	0.59 ± 0.03	0.17[b]	0.99 ± 0.05	0.64 ± 0.05	2.27 ± 0.06	0.06[b]	0.34 ± 0.01	0.09[b]	0.09[b]
10	Chrysin-5-methyl-ether	17.3	0.02[b]	0.05[b]	0.03[b]	0.12[b]	0.07[b]	0.03[b]	0.25[b]	0.05[b]	0.16 ± 0.01	0.14 ± 0.01	0.14[b]
11	Apigenin	18.4	0.03[b]	0.10 ± 0.01	0.11 ± 0.01	0.31 ± 0.01	0.14 ± 0.01	0.15[b]	0.44 ± 0.01	0.24 ± 0.01	0.44 ± 0.02	0.25 ± 0.02	0.26[b]
12	Kaempferol	20.2	0.03[b]	0.12 ± 0.01	0.17 ± 0.01	0.39 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.55 ± 0.02	0.24 ± 0.01	0.51 ± 0.02	0.28 ± 0.02	0.29[b]
13	Pinobanksin	20.5	0.05[b]	0.57 ± 0.04	0.57 ± 0.03	1.15 ± 0.03	0.53 ± 0.02	0.76 ± 0.02	2.00 ± 0.04	1.30 ± 0.04	2.12 ± 0.09	0.75 ± 0.06	0.79 ± 0.01
14	Isorhamnetin	21.3	0.03[b]	0.09[b]	<LOD	0.53 ± 0.01	0.14 ± 0.01	<LOD	0.41 ± 0.01	0.22 ± 0.01	0.78 ± 0.04	0.53 ± 0.04	0.55 ± 0.01
15	Luteolin-methyl-ether	22.9	0.04[b]	0.14 ± 0.01	0.10[b]	0.30 ± 0.01	0.17 ± 0.01	0.13[b]	0.57 ± 0.01	0.14[b]	0.40 ± 0.02	0.28 ± 0.02	0.29 ± 0.01
16	Quercetin-dimethyl-ether	24.2	0.04[b]	0.11 ± 0.01	0.07[b]	0.25 ± 0.01	0.18 ± 0.01	0.05[b]	0.52 ± 0.01	0.10[b]	0.35 ± 0.03	0.29 ± 0.02	0.30±0.01
17	Galangin-5-methyl-ether	27.0	0.08[b]	0.23 ± 0.01	0.14 ± 0.01	0.35 ± 0.01	0.34 ± 0.01	0.16[b]	1.22 ± 0.03	0.15[b]	0.51 ± 0.02	0.34 ± 0.02	0.36[b]
18	Pinobanksin-5-methyl-ether-3-*O*-acetate	28.3	0.01[b]	<LOD	0.04[b]	0.20 ± 0.01	0.06[b]	0.06[b]	0.19 ± 0.01	0.07[b]	0.28 ± 0.01	0.21 ± 0.02	0.21[b]
19	Cinnamilidenacetic acid	29.4	0.06[b]	0.36 ± 0.02	0.16 ± 0.01	0.79 ± 0.02	0.56 ± 0.03	0.24 ± 0.01	1.99 ± 0.03	0.23 ± 0.01	1.16 ± 0.05	0.54 ± 0.03	0.56 ± 0.01
20	Quercetin-7-methyl-ether	30.1	0.02[b]	0.08[b]	0.07[b]	0.42 ± 0.01	0.15 ± 0.01	0.09[b]	0.42 ± 0.01	0.19 ± 0.01	0.59 ± 0.02	0.41 ± 0.03	0.43 ± 0.01
21	Quercetin-dimethyl-ether	33.7	0.05[b]	0.14 ± 0.01	0.05[b]	0.56 ± 0.01	0.21 ± 0.01	0.06[b]	0.67 ± 0.01	0.13[b]	0.75 ± 0.03	0.60 ± 0.05	0.62 ± 0.01
22	Caffeic acid prenyl ester	38.2	0.01[b]	0.18 ± 0.01	0.21 ± 0.01	1.64 ± 0.04	0.78 ± 0.04	0.34 ± 0.01	1.10 ± 0.02	0.45 ± 0.01	1.84 ± 0.08	1.07 ± 0.07	1.10 ± 0.02
23	Chrysin	39.1	0.52[b]	1.42 ± 0.09	1.14 ± 0.07	3.31 ± 0.09	2.28 ± 0.11	1.58 ± 0.04	7.50 ± 0.17	2.79 ± 0.08	6.51 ± 0.26	4.38 ± 0.34	4.56 ± 0.06
24	Caffeic acid benzyl ester	39.8	0.01[b]	0.11[b]	1.17 ± 0.07	3.02 ± 0.08	0.25 ± 0.01	1.78 ± 0.05	0.46 ± 0.01	1.44 ± 0.04	4.05 ± 0.17	2.60 ± 0.18	2.69 ± 0.04
25	Caffeic acid prenyl ester	40.4	0.02[b]	0.09[b]	0.12 ± 0.01	1.03 ± 0.03	0.14 ± 0.01	0.14[b]	0.48 ± 0.01	0.24 ± 0.01	1.07 ± 0.05	0.86 ± 0.06	0.90 ± 0.02
26	Pinocembrin	42.2	0.43[b]	1.64 ± 0.10	1.58 ± 0.09	4.32 ± 0.11	3.51 ± 0.22	2.05 ± 0.07	8.60 ± 0.21	3.21 ± 0.10	6.26 ± 0.25	2.81 ± 0.21	2.91 ± 0.05
27	Galangin	43.2	0.54 ± 0.01	1.26 ± 0.08	1.18 ± 0.07	2.20 ± 0.06	2.76 ± 0.14	1.54 ± 0.04	7.54 ± 0.16	1.31 ± 0.04	3.20 ± 0.13	1.87 ± 0.14	1.96 ± 0.03
28	Caffeic acid phenylethyl ester (CAPE)[c]	45.4	<LOD	0.06 ± 0.01	0.13 ± 0.01	0.90 ± 0.03	<LOD	0.24 ± 0.01	0.26 ± 0.03	0.32 ± 0.02	1.44 ± 0.07	1.06 ± 0.07	1.13 ± 0.05
29	Pinobanksin-3-*O*-acetate[c]	45.4	0.04[b]	0.54 ± 0.04	1.66 ± 0.10	2.07 ± 0.05	1.15 ± 0.06	2.53 ± 0.05	1.84 ± 0.05	2.99 ± 0.08	6.10 ± 0.34	3.66 ± 0.36	3.47 ± 0.11
30	Methoxy-chrysin	46.8	0.09[b]	0.24 ± 0.02	0.17 ± 0.01	0.41 ± 0.01	0.37 ± 0.02	0.24 ± 0.01	1.30 ± 0.02	0.20 ± 0.01	0.79 ± 0.03	0.61 ± 0.05	0.64 ± 0.01
31	*p*-Coumaric prenyl ester	50.4	0.02[b]	0.10 ± 0.01	0.03[b]	0.12[b]	0.31 ± 0.01	0.06 ± 0.01	0.65 ± 0.02	0.04[b]	0.14[b]	0.11 ± 0.01	0.12[b]
32	*p*-Coumaric benzyl ester	50.8	0.01[b]	0.06[b]	0.80 ± 0.04	0.18[b]	0.06[b]	1.01 ± 0.03	0.27 ± 0.02	0.12[b]	0.39 ± 0.02	0.18 ± 0.01	0.19[b]
33	Caffeic acid cinnamyl ester	51.4	0.01[b]	0.09 ± 0.01	0.47 ± 0.03	0.27 ± 0.01	<LOD	0.77 ± 0.03	0.48 ± 0.05	0.40 ± 0.01	1.87 ± 0.08	0.71 ± 0.04	0.73 ± 0.01
34	*p*-Coumaric prenyl ester	51.8	0.03[b]	0.21 ± 0.01	0.06[b]	0.66 ± 0.02	0.75 ± 0.03	0.10 ± 0.01	1.40 ± 0.05	0.14[b]	0.86 ± 0.03	0.60 ± 0.04	0.63 ± 0.01
35	Pinobanksin-3-*O*-propionate	53.7	0.03[b]	0.14 ± 0.01	0.19 ± 0.02	0.69 ± 0.02	0.36 ± 0.03	0.27 ± 0.01	1.79 ± 0.01	0.42 ± 0.01	0.96 ± 0.04	1.05 ± 0.09	1.07 ± 0.04
36	*p*-Coumaric cinnamyl ester	58.4	0.01[b]	0.09[b]	0.92 ± 0.05	0.22 ± 0.01	0.13 ± 0.01	1.12 ± 0.04	0.46 ± 0.01	0.12[b]	0.39 ± 0.02	0.20 ± 0.01	0.21[b]
37	Pinobanksin-3-*O*-butyrate[d]	60.9	0.19 ± 0.01	0.31 ± 0.04	0.64 ± 0.04	1.80 ± 0.04	1.06 ± 0.17	0.81 ± 0.03	2.79 ± 0.06	1.01 ± 0.02	2.68 ± 0.11	0.94 ± 0.08	0.94 ± 0.02
38	Pinobanksin-3-*O*-pentanoate[d]	64.6	0.06[b]	0.17 ± 0.02	0.36 ± 0.03	0.85 ± 0.03	0.56 ± 0.05	0.45 ± 0.02	1.40 ± 0.01	0.54 ± 0.02	1.22 ± 0.06	1.05 ± 0.09	1.08 ± 0.05
39	Pinobanksin-3-*O*-hexanoate[d]	66.9	0.05 ± 0.01	0.12 ± 0.02	0.14 ± 0.02	0.19 ± 0.01	0.23 ± 0.03	0.16 ± 0.02	1.46 ± 0.20	0.11 ± 0.03	0.22 ± 0.08	<LOD	<LOD
40	*p*-Methoxy cinnamic acid cinnamyl ester	69.9	0.03[b]	0.14 ± 0.01	0.09 ± 0.01	0.13[b]	0.58 ± 0.03	0.04 ± 0.01	1.39 ± 0.04	0.10[b]	0.22 ± 0.01	0.11 ± 0.01	0.11 ± 0.02
–	Total phenolic acids	–	0.17[b]	1.80 ± 0.08	5.99 ± 0.32	11.19 ± 0.30	4.36 ± 0.20	8.42 ± 0.25	9.99 ± 0.25	4.69 ± 0.15	16.67 ± 0.68	10.09 ± 0.67	10.46 ± 0.22
–	Total flavones	–	0.69[b]	1.95 ± 0.12	1.54 ± 0.08	4.45 ± 0.12	3.02 ± 0.14	2.13 ± 0.06	10.06 ± 0.21	3.42 ± 0.10	8.31 ± 0.33	5.66 ± 0.43	5.89 ± 0.08
–	Total flavonols	–	0.93 ± 0.01	2.16 ± 0.13	1.80 ± 0.10	5.33 ± 0.14	4.18 ± 0.20	2.27 ± 0.06	11.90 ± 0.24	2.73 ± 0.08	7.59 ± 0.32	4.46 ± 0.33	4.64 ± 0.07
–	Total flavanones	–	0.43[b]	1.64 ± 0.10	1.58 ± 0.09	4.32 ± 0.11	3.51 ± 0.22	2.05 ± 0.07	8.60 ± 0.21	3.21 ± 0.10	6.26 ± 0.25	2.81±0.21	2.91 ± 0.05
–	Total dihydroflavonols	–	0.43 ± 0.01	2.06 ± 0.17	4.09 ± 0.26	8.10 ± 0.20	3.95 ± 0.35	5.69 ± 0.13	10.54 ± 0.34	7.05 ± 0.22	15.32 ± 0.63	8.97 ± 0.77	8.92 ± 0.25
–	Total flavonoids	–	2.48 ± 0.02	7.81 ± 0.51	9.02 ± 0.53	22.19 ± 0.57	14.66 ± 0.91	12.14 ± 0.31	41.10 ± 0.99	16.42 ± 0.49	37.47 ± 1.53	21.90 ± 1.75	22.36 ± 0.44
–	Total phenolics	–	2.65 ± 0.02	9.61 ± 0.60	15.01 ± 0.85	33.38 ± 0.87	19.02 ± 1.11	20.56 ± 0.56	51.09 ± 1.22	21.11 ± 0.64	54.14 ± 2.21	31.99 ± 2.42	32.82 ± 0.65

Experimental conditions as in Section 2.2.

[a] Data are expressed as mean ($n = 4$) ± SD.

[b] SD < 0.005.

[c] Peaks are overlapped. Peak integration was tentatively performed following the UV–vis spectra of the analytes.

[d] Or positional isomers.

hot extraction to speed up the process [1]. In this study, reference propolis hydroalcoholic extracts were prepared by using both decoction and maceration as the extraction procedures, with a sample-to-solvent ratio of 1:10 (w/v) [1] and 80% EtOH as the extraction solvent [1]. These conditions are conventionally used in the extraction of raw propolis [1]. The results of the HPLC analysis of these reference samples indicated that the decoction extraction at 70 °C for 1 h (PE-10) provided the same yield of phenolics if compared with maceration at room temperature for 24 h (PE-11). Therefore, decoction extraction, thus being a more aggressive treatment, can be considered as a more suitable procedure for rapid extraction of phenolics from propolis raw material, without causing thermal degradation of the active compounds. The comparison of the reference propolis extracts with those commercially available indicated the same qualitative composition; regarding quantitative analysis, the lab-made hydroalcoholic extracts displayed a medium level content of phenolics (31.99 ± 2.42 and 32.82 ± 0.65 mg/ml for PE-10 and PE-11, respectively) if compared with those commercially available. The amounts of phenolic acids and flavonoids in PE-10 and PE-11 extracts were higher if compared with samples PE-2 and PE-6, which were obtained by using the same sample-to-solvent ratio (1:10 (w/v)), but they were found to be in good agreement with those of sample PE-4, which was prepared by using a double sample-to-solvent ratio (2:10 (w/v)). Commercial sample PE-9 was prepared by using a sample-to-solvent ratio of 3:10 (w/v) and this can explain its higher content of phenolics.

In all the analyzed samples, the most abundant flavonoids were found to be chrysin, galangin, pinocembrin and pinobanksin (and its esters). Regarding phenolic acids, caffeic acid derivatives were found to be present in higher amount, followed by *p*-coumaric acid derivatives and finally by ferulic and isoferulic acids. These constituents are typical for propolis from temperate zones, having *Populus* spp. as plant source [4,7].

4. Conclusion

The proposed HPLC method, based on the use of UV, MS and MS/MS data, allowed the identification and quantification of 40 compounds, including phenolic acids and flavonoids, in hydroalcoholic extracts of propolis on sale on the Italian market. Under the applied conditions, the TQ mass analyzer provided a higher fragmentation degree of the target analytes in comparison with the IT and, therefore, more structural information.

The method validation indicated that this technique represents a reliable tool for the analysis of the target analytes in propolis extracts. The results of the analysis of real matrices indicated a great variability in the content of the secondary metabolites in the products on sale in Italy, highlighting the importance of the development and validation of suitable methods for the phytochemical analysis of propolis extracts used in phytotherapy. In this context, the developed method can be considered very useful for a reliable metabolite profiling of polyphenols in propolis extracts.

Acknowledgments

The authors acknowledge the “Fondazione Cassa di Risparmio di Modena” for funding the HPLC-ESI-IT and HPLC-ESI-TQ systems at the Centro Interdipartimentale Grandi Strumenti (CIGS) of the University of Modena and Reggio Emilia.

The authors are particularly grateful to Prof. Dr. Eckhard Wollenweber (Institut für Botanik, Darmstadt, Germany) for kindly providing several compounds used to confirm the identity of propolis constituents.

The authors are also grateful to Dr. Alessandro Biancardi (Alchimia Natura, Modena, Italy) for providing the raw propolis used as reference.

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