Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography–tandem mass spectrometry

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Abstract

A reverse phase LC–DAD–MS method for quantification of phenolic acids and flavonoids in propolis raw materials was developed. The propolis samples from different geographical areas were extracted with ethanol for 2 h at 70 °C and the resulting solutions analyzed on a 5 μm C$_{18}$ symmetry 250 mm × 4.6 mm column. The separation was performed by means of a linear gradient elution and DAD and MS data were acquired in the 200–450 nm and 100–1000 Da range, respectively. The identity of most of the compounds was assessed by comparing their chromatographic and UV behaviour with that of authentic standards. When the standards were not available, the identity was achieved by means of chromatographic and on-line UV data combined with mass spectrometry. European, Chinese and Argentinean propolis are characterized by the presence of phenolic acids and flavonoids and the most abundant were chrysin (2–4%), pinocembrin (2–4%), pinobanksin-acetate (1.6–3%) and galangin (1–2%). Some Brazilian propolis contains mainly artepillin C, different caffeoyl quinic acids and some flavonoids. When considering the total flavonoid content as quality index, we suggest that propolis with a content less than 11% should be considered of low quality, whereas propolis with a content of 11–14%, 14–17% or >17% should be classified as propolis of acceptable, good and high quality, respectively. The reported LC–DAD–MS analysis method may be applied for the phytochemical screening of raw propolis and its commercial formulations.
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1. Introduction

Propolis is a resinous substance collected by honeybees from buds and cracks in the bark of different plants, mainly from poplar, beech, horsechestnut, birch and conifer trees. Bees mix this substance with beeswax and β-glycosidase they secrete during the propolis collection. The resulting material is used by bees to seal the holes in the hives, exclude draught and protect against external invaders. However, bees also take advantage of its biological action. The antibacterial and antifungal properties of propolis are responsible for the lower incidence of bacteria and moulds within the hive. Propolis is a traditional remedy in folk medicine and there is a substantial evidence [1] indicating that propolis has antibacterial [2,3], antiviral, anti-

fungal [4], anti-inflammatory [5], local-anaesthetic, antioxidant [6], immunostimulating [7–8], cariostatic [9], antitumor [10] and anti-*Helicobacter pylori* [11–12] activities.

Propolis usually contains a variety of different chemical compounds, including phenolic acids and esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols, chalcones), terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes and β-steroids. Propolis cannot be used as raw material and it must be purified by extraction to remove the inert material and preserve the polyphenolic fraction. Indeed, this fraction is considered to contribute more to the therapeutic effects than the other components of propolis. Therefore, the analysis of this fraction has received great attention and different procedures are available [13–16]. This paper aims to describe the results obtained in the quality control of a large number of propolis samples collected in different geographical areas. For this purpose, it was developed an LC–DAD–MS method allowing the qualitative and quantitative analysis of phenolic acids and flavonoids

in propolis extract samples. The identity of different compounds was established through their molecular ions (MS) and related ions product (MS/MS).

2. Materials and methods

2.1. Chemicals

Chrysin (C), galangin (G), pinocembrin (P), quercetin (Q), kaempferol (K), isorhamnetin (I), sakuranetin (S), isosakuranetin (iS), apigenin (A), acacetin (Ac), pinocembrin-5,7-dimethylether (P-DME) and chrysin-7-methylether (C-7ME) were from Extrasynthese (Genay, France). Caffeic acid phenylethyl ester (CAPE), caffeic (CA), p-coumaric (pC), ferulic (FA), cinnamic (CiA), isoferulic (iFA) and 3,4-dimethylcaffeic acid (DMCA) were purchased from Sigma (St. Louis, MO, USA). Methanol, acetonitrile and formic acid were from Merck (Darmstadt, Germany). Water was obtained from a MilliQ apparatus (Millipore, Milford, MA). The propolis raw samples were a gift from Specchiasol s.r.l. (Bussolengo, VR, Italy).

2.2. Propolis samples

Samples of propolis were purchased during the last 5 years from the following areas: China (12), Korea (1), Argentina (25), Brazil (9), Chile (7), Uruguay (4), Paraguay (1), France (6), Poland (12), Germany (1), Macedonia (4), Russia (1), Croatia (12), Italy (9), Bulgaria (1) and Canada (2). The propolis samples were stored at −20 °C.

2.3. Sample preparation

The frozen propolis samples (200 g) were finely powdered by a mill and 5 g extracted with ethanol (70 mL) for 2 h at 70 °C. The mixture was cooled, filtered and the solid residue was re-extracted using the same ethanol volume twice. The resulting three solutions (70 mL each) were singularly adjusted to 100 mL, filtered through a 0.22 μm filter, diluted 50-fold with methanol and 10 μL injected in the HPLC system.

2.4. LC–DAD–MS analysis

The chromatographic system consisted of an Alliance 2695 (Waters, Milford, MA) equipped with a model 2996 (Waters) photodiode array detector and a triple quadrupole mass spectrometer mod. Quattro micro (Micromass, Beverly, MA). A 5 μm C_{18} symmetry column (250 mm × 4.6 mm, waters) was used for the separation at a flow rate of 1.2 mL/min. The column was maintained at 30 °C and the flow rate split 5:1 before ESI source. The separation was performed by means of a linear gradient elution (eluent A, 0.1% formic acid; eluent B, acetonitrile). The gradient was as follows: 20% B for 6 min, 20–30% B in 4 min, 30–40% B in 30 min, 40–60% B in 20 min, 60–90% B in 20 min and 90% B for 10 min. Chromatographic data were acquired in the 200–450 nm range and were integrated at 290 nm. Mass spectrometer operated in negative and positive

full-scan mode in the range 100–1000 Da. The capillary voltage set to 3.0 kV, the cone voltage was 20 V, the source temperature was 130 °C and the desolvating temperature was 350 °C. Data were acquired by Masslinx 4.0 software (Micromass) with Quan-Optimize option for fragmentation study. The calibration curves were obtained from phenolic acids and flavonoids mother solutions prepared by dissolving 20 mg of each standard in 20 mL methanol and the calibration curves were in the range of 2–50 μg/mL.

2.5. Method validation

The LC–DAD–MS method was validated for linearity, limit of quantization and detection, accuracy, peak purity, precision and repeatability. Limit of quantization (S/N ratio of 6) and limit of detection (S/N ratio of 3) were determined by serial dilution of standard solutions. Accuracy (recovery) was evaluated by spiking five propolis raw sample (5 g) with three amounts (2, 10, 20 mg) of the mix standard compounds containing CA, pC, FA, iFA, DMCA, CiA, CAPE, Q, C, P, G, C-7ME and P-DME. The spiked samples were extracted under optimized conditions and recovery rates were calculated for flavonoids and phenolic acids. Peak purity and identity were confirmed by LC–DAD–MS and LC–MS/MS experiments. Precision (intra- and inter-day) of the assay was verified by analyzing propolis samples five times on five consecutive days. Repeatability was confirmed by evaluating standard deviations of the retention times.

3. Results and discussion

3.1. Sample preparation

Three subsequent extractions were applied to extract all the flavonoids and phenolic acids present in the different propolis samples. For the first and second extraction the calculated recovery was about 92.8 ± 2.1% and 7.2 ± 2.1%, respectively. Flavonoids or phenolic acids were not detected in the third extract.

3.2. Method validation

Calibration curves were constructed for each standard at five concentration levels and three independent determinations were performed at each concentration. Regression analysis was employed to determine the linearity of the calibration graphs and the calculated equations are reported in Table 1. The accuracy (recovery) of the extraction for phenolic acids and flavonoids from spiked propolis samples was 98.2 ± 3.1% and 97 ± 2.4%, respectively. The precision of the method was tested by both intra-day ($n = 5$) and inter-day (5 days, $n = 5$) reproducibility and the coefficient of variation was below 5.4%. Limit of quantization (LOQ) and limit of detection (LOD) were 2 and 0.8 μg/mL, respectively. Only for quercetin and galangin the LOD was 1.5 μg/mL. Regarding repeatability, a maximum relative standard deviation of 2% (for chrysin, pinocembrin and galangin) was observed for triplicate injections.

Table 1
Calibration equations ($Y = mX + q$)[a] for the quantization of different polyphenols in propolis extract

Standard	Range (µg/mL)	Slope	Intercept	r
CA	2.5–41	28.9	−2.1	0.998
pC	2.5–45	49.2	−5.3	0.999
FA	2.5–40	25.3	−0.4	0.998
DMCA	2.5–41	27.2	−3.3	0.999
CiA	2.0–42	46.0	−4.4	0.997
Q	3.0–50	9.2	−2.7	0.997
C	2.0–45	17.2	−1.9	0.999
P	2.5–45	29.3	1.1	0.999
G	3.0–30	11.2	−3.6	0.996
CAPE	2.0–40	16.2	−4.2	0.998

[a] $Y = \text{area}/1000$, $X = \mu g/mL$.

3.3. LC–DAD–MS and LC–MS/MS analysis

The polyphenol fraction of propolis is represented by a group of cinnamic acid derivatives and flavonoids, whose structures are given in Fig. 1. This fraction is highly complex and the identity of its components can be correctly assessed by an approach based on HPLC combined with on-line UV detection and MS/MS spectrometry. Otherwise, misidentification is possible. This is

the case of a recent paper based on LC–ESI–MS [13] that reports the presence of naringenin in propolis. Our data suggest that naringenin is not present in propolis and it is possible that in cited paper it was confused with pinobanksin. Both naringenin and pinobanksin have the same molecular weight and UV spectra and similar retention time. Thus, LC–DAD–ESI–MS is inadequate and LC with on-line MS/MS detection is needed to discriminate these compounds. In fact, in tandem mass spectrometry naringenin gives the ion product with $[m/z]^-$ 151, while pinobanksin produces the typical ion product with $[m/z]^-$ 253.

An example of the HPLC profiles at 290 nm of propolis from different regions is shown in Fig. 2. Propolis from South America (A, Argentina) and Europe (C, Italy) are comparable and differ markedly from most Brazilian propolis (B). European propolis is characterized by the presence of a number of phenolic acids (Table 2, peaks 1–5, 9, 19, 22a–24, 31–34, 36, 43, 44) and flavonoids (Table 2, peaks 6–8, 10–18, 20–22, 25–30, 35, 37–42). Most of these compounds are present also in Asiatic (e.g. Chinese) and Argentinean propolis. By contrast, Brazilian propolis has a distinct pattern. In green and silvestre propolis artepillin C (peak 53) and an isoprenylated *p*-coumaric acid derivative (peak 56) [17] are the major constituents accompanied by different caffeoyl quinic acids (Table 2, peaks 45–48)
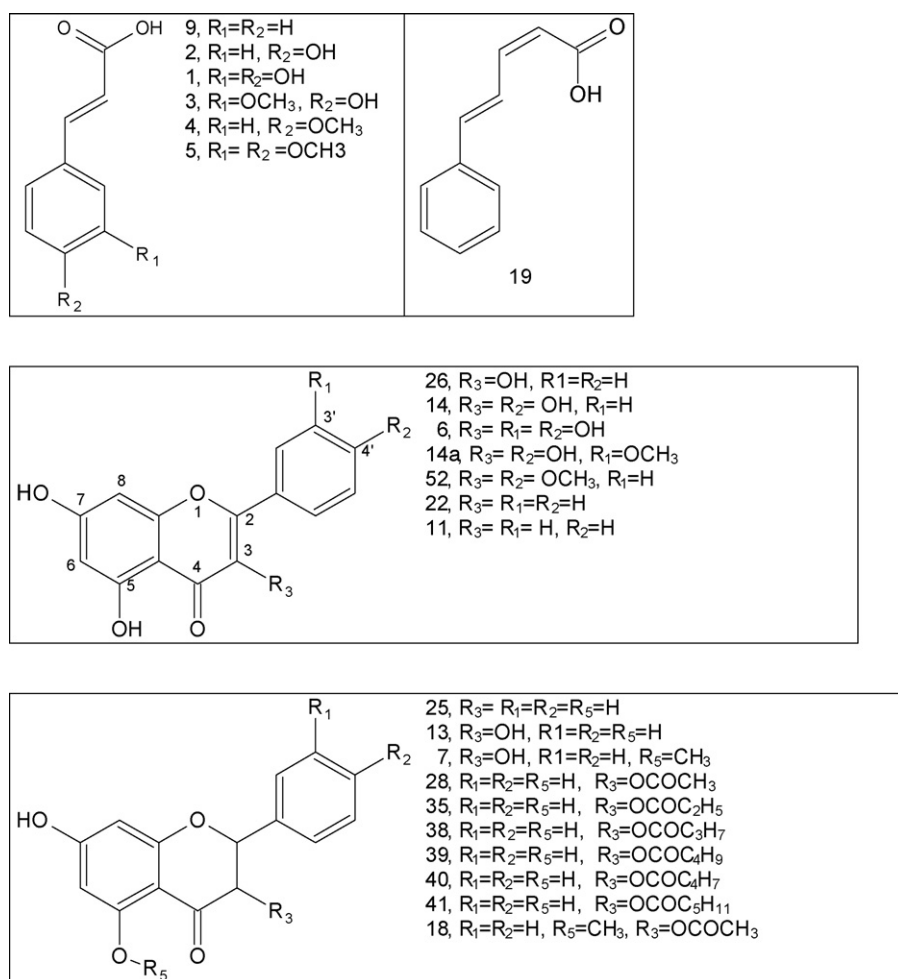

Fig. 1. Structures of flavonoids, phenylalkyl acids, phenolic acid and their esters found in propolis from different geographical areas.
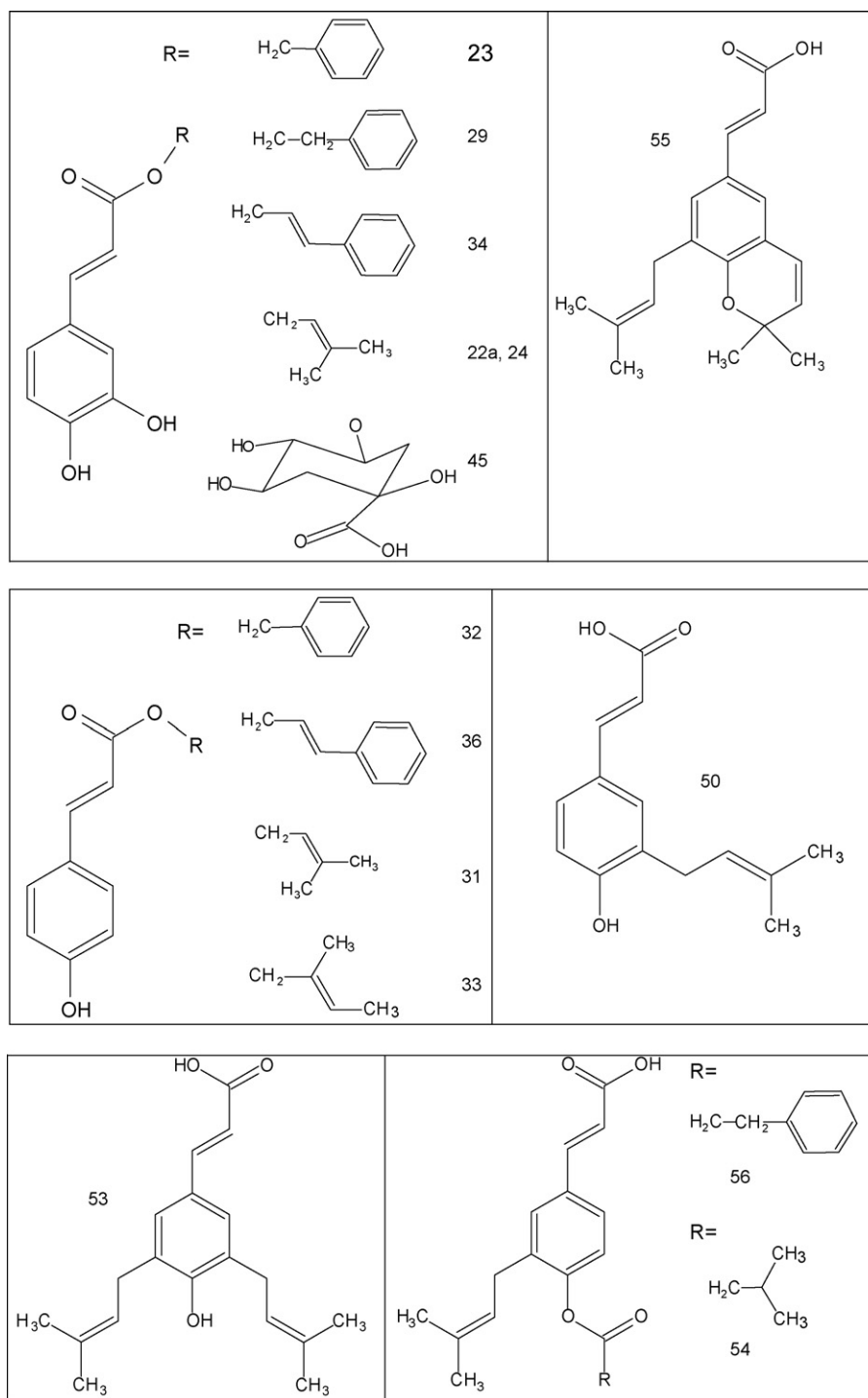
Fig. 1. (*Continude*).

and traces of 4-hydroxy-3-prenylcinnamic acid isoprenyl-*p*-coumarate (peak 50) [18,19], kaempferide (peak 52), a cinnamic acid derivative (peak 54), a chroman derivative (peak 55) and a benzofuran derivative (peak 57). In the Brazilian red propolis the only flavonoids detected were biochanin A and formononetin and their amount was less than 0.2%. The presence of these isoflavonones is also reported by Piccinelli et al. [20].

The identity of most compounds was assessed by comparing their chromatographic and UV behaviour with that of standards. When the standards were not available, the identity was achieved from chromatographic and on-line UV data combined with mass spectrometry. Indeed, the chromatographic and UV behaviour ensure the distinction between different classes of polyphenols. In fact, under the applied reversed phase chromatographic conditions, flavonoids of the same class elute differently depend-
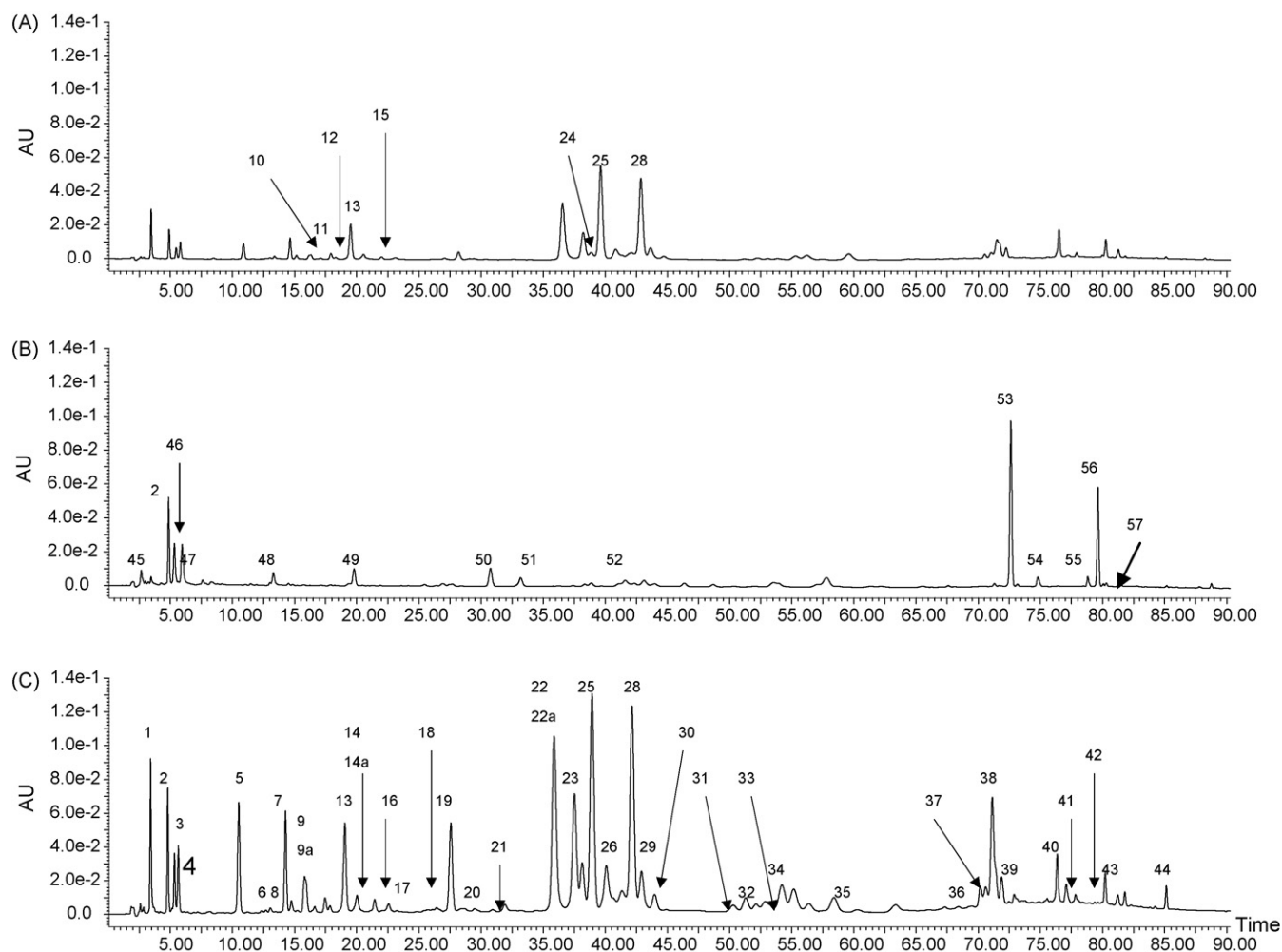
Fig. 2. Typical HPLC chromatograms at 290 nm of ethanolic propolis extracts from South America (A: Argentina, B: Brazil) and Europe (C, Italy). See Fig. 1 for peak number attribution.

ing on the degree of hydroxylation and methylation. Increased hydroxylation reduces retention times, whereas methylation increases retention times as a function of the methoxyl/hydroxyl ratio, excepting for methyl substitution at C_5. Furthermore, flavanonols (i.e., pinobanksin), flavones (i.e., chrysin) and flavanones (i.e., pinocembrin) elute earlier than related flavonols (i.e., galangin).

Concerning the UV behaviour, cinnamic acid derivatives yielded typical UV spectra with maxima in the 275–325 nm regions. By contrast, flavonols and flavones were distinguished by their UV spectra with maxima at 265/358 and 268/310 nm, respectively. Both these classes can be easily differentiated from flavanonols (UV$_{max}$ 290 nm) and flavanones (UV$_{max}$ 292 nm), which in turn cannot be discriminated.

From the molecular masses it was possible to select possible candidates and among these the specific one was established on the basis of its ions product. MS/MS was carried out in both positive and negative mode, as the fragmentation differed for each group of phenolic compounds. Cinnamic acid yielded in the positive mode peak ions $[M–H_2O]^+$ and in the negative mode peak ions $[M–COOH]^-$. Conversely, cinnamic acid esters yielded

fragments mainly in the negative mode. For example, caffeic acid benzylester (peak 23) produced the ions with $[m/z]^-$ 179 (caffeic acid) $[m/z]^-$ 161 (caffeic acid–H_2O) and $[m/z]^-$ 135 (caffeic acid–COOH).

Flavonols, flavanones and flavones produced a negative ion with $[m/z]^-$ 151 and a positive ion with $[m/z]^+$ 153, if lacking in substituents in the A ring. The fragment with $[m/z]^-$ 151 is a common ion product for different flavonoids ant it results from a retro Diels-Alder reaction of the A ring. Flavanonols, like pinobanksin, behaved differently. They gave an ion with $[m/z]^-$ 253 corresponding to $[M–H_2O]^-$. The loss of water is due to the presence of a hydroxyl group at C_3 of the C ring. Analogously, pinobanksin-5-methylether acetate (peak 18) yielded under low collision energy as a main fragment the ion with $[m/z]^-$ 285 accounting for $[M–acetate]^-$ accompanied by minor ions with $[m/z]^-$ 267 arising from $[M–acetate–H_2O]^-$ and $[m/z]^-$ 253 $[M–acetate–H_2O–CH_3]^-$ (Fig. 3). At higher collision energy only the fragment with $[m/z]^-$ 253 was produced. A similar fragmentation pattern was observed for pinobanksin-esters, as exemplified for pinobanksin-3-propionate (Fig. 4, peak 35). Peak 8 deserves a specific comment. Its UV spectrum refers

Table 2
Compounds identified in different propolis samples and their RT, UV, MS and MS/MS characteristics

| Peak	Compound	RT	UV$_{max}$	M_W	$(m/z)^-$ Ms^2	$(m/z)^+$ Ms^2
1	Caffeic acid	3.4	324	180	135	163
2	p-Coumaric	4.8	310	164	119	147
3	Ferulic acid	5.3	324	194	149	177
4	Isoferulic	5.6	324	194	149	177
5	3,4-Dimethyl-caffeic acid (DMCA)	10.5	324	208	163	191
6	Quercetin	13	358	302	151	153
7	Pinobanksin-5-methyl-ether	14.3	290	286	267, 253, 139	91, 153
8	Quercetin-3-methylether	14.7	257, 357	316	151	153
9	Cinnamic acid	15.8	279	148	103	131
9a	Coumaric acid methyl ester	15.8	311	178	163, 119	147
10	Chrysin-5-methyl-ether	16.6	265, 311sh	268	253, 151	153
11	Apigenin	17.5	265, 336	270	151, 139	153
12	Pinocembrin-5-methyl-ether	17.9	290	270	255, 165	
13	Pinobanksin	19	290	272	253	153
14	Kaempferol	20.1	265, 364	286	151	153
14a	Isorhamnetin	20.1	254, 367	316	151	153
15	Luteolin-5-methyl-ether	21.4	268, 350	300	285, 151	153
16	Quercetin-5,7-dimethyl-ether	22.6	254, 357	330	315, 165	
17	Galangin-5-ME	25.7	260, 302sh, 354	284	165, 151	
18	Pinobanksin-5-methyl-ether-3-acetate	26.4	290	328	285, 253	
19	Cinnamilidenacetic acid	27.6	311	174	129	157
20	Quercetin-7-methyl-ether	28.2	257, 357	316	165	167
21	Quercetin-7-methyl-X-methyl-ether	31.9	257, 357	330	165	167
22	Chrysin	35.9	265, 311sh	254	151	153
22a	Caffeic acid isoprenyl ester	35.9		248	179, 135	
23	Caffeic acid benzyl ester	37.5	324	270	179, 135	
24	Caffeic acid isoprenyl ester	38.2	324	248	179, 135	
25	Pinocembrin	39	290	256	151	153
26	Galangin	40	354	270	151	153
27	Pinobanksin-5,7-dimethylether	41.5	292	300	285, 253, 139	
28	Pinobanksin-3-O-acetate	42.1	293	314	271, 253	
29	CAPE	42.9	325	284	179, 135	
30	Methoxy-Chrysin	44	264, 311sh	284	269	
31	p-Coumaric-methyl-butenyl ester	50.3	311	232	163, 119	
32	p-Coumaric benzyl ester	51.3	311	254	163, 119	
33	p-Coumaric-methyl-butenyl ester	52.9	311	232	163, 119	
34	Caffeic acid cinnamyl ester	54.2	325	296	179, 135	
35	Pinobanksin-3-O-propionate	58.4	292	328	253, 271	
36	p-Coumaric cinnamyl ester	70.1	311	280	163, 119	
37	Chrysin-5,7-dimethyl ether	70.7	265, 311sh	282	267, 165	
38	Pinobanksin-3-O-(butyrate or isobutyrate)	71.4	290, 330sh	342	253, 271	
39	Pinobanksin-3-O-pentenoate	72	293	354	253, 271	
40	Pinobanksin-3-O-(pentanoate or 2-methyl-butyrate)	76.4	293	356	253, 271	
41	Pinobanksin-3-O-hexanoate	77.1	292	370	253, 271	
42	Pinobanksin-3-O-hexanoate	79	292	370	253, 271	
43	p-Methoxy-cinnamic acid cinnamyl ester	80.2	279	294	177, 133	149
44	p-Coumaric acid ester	85.1	311	344	163, 119	147
45	Chlorogenic acid	2.7	325	354	179, 135, 191	163
46	Dicaffeoyl quinic acid	5.3	325	516	179, 135, 191	163
47	Dicaffeoyl quinic acid	5.9	325	516	179, 135, 191	163
48	Tricaffeoyl quinic acid	13.3	325	678	179, 135, 191	163
49	Methoxy-pinobanksin	19.8	290	302	283, 269	285, 257
50	Drupanin (3-prenyl-p-coumaric acid)	30.8	311	232	187, 133	215, 177
51	Unknown	33.1	236, 315	316	315	243, 225
52	Kaempferide	41.6	264, 363	300	285, 163, 151, 107	229, 153, 69
53	Artepillin C	72.6	311	300	255, 199, 185, 145	245, 227, 189, 69
54	3-Prenyl-4-(2-methylpropionyl-oxy)-cinnamic acid	74.8	279.5	316	271	
55	3-(2-2-Dimehy-3,4-dehydro-8-prenyl-1-benzopyran-6-yl)-2-propenoic acid	78.8	310	298	253, 149	243, 147
56	3-Prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid	79.6	279.5	364	319, 187, 149, 131	
57	Benzofuran derivative	81.3	225, 275sh	302		191, 149
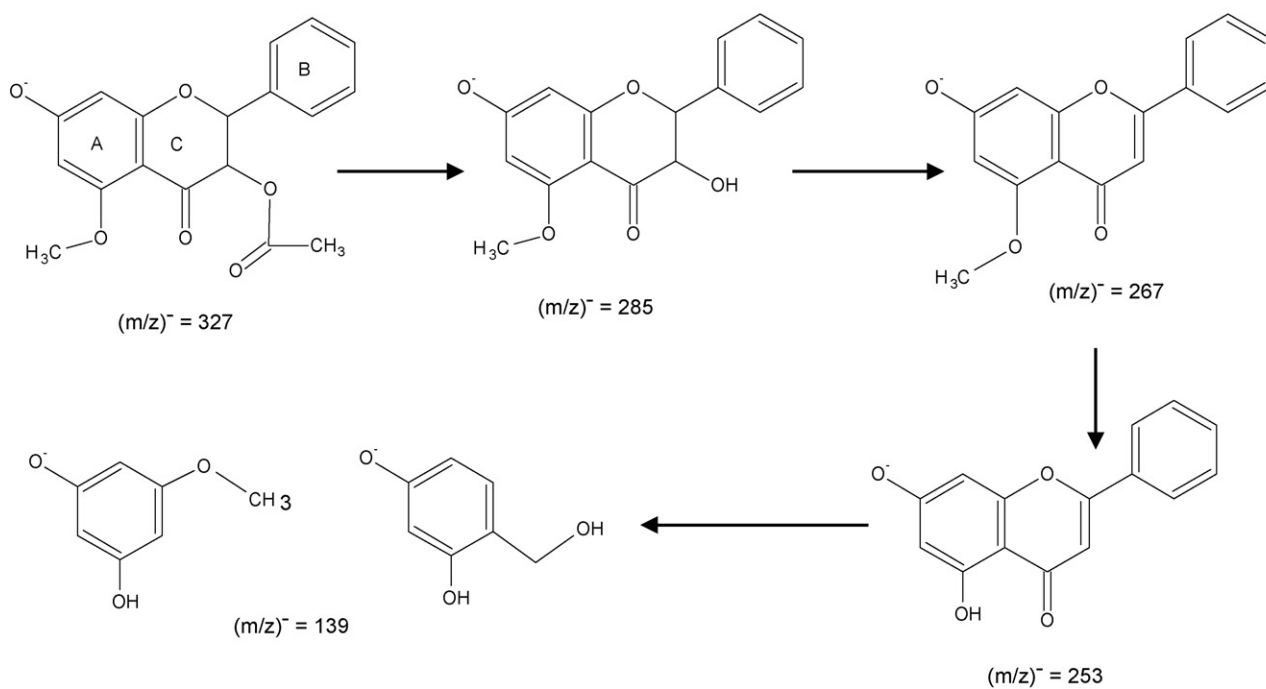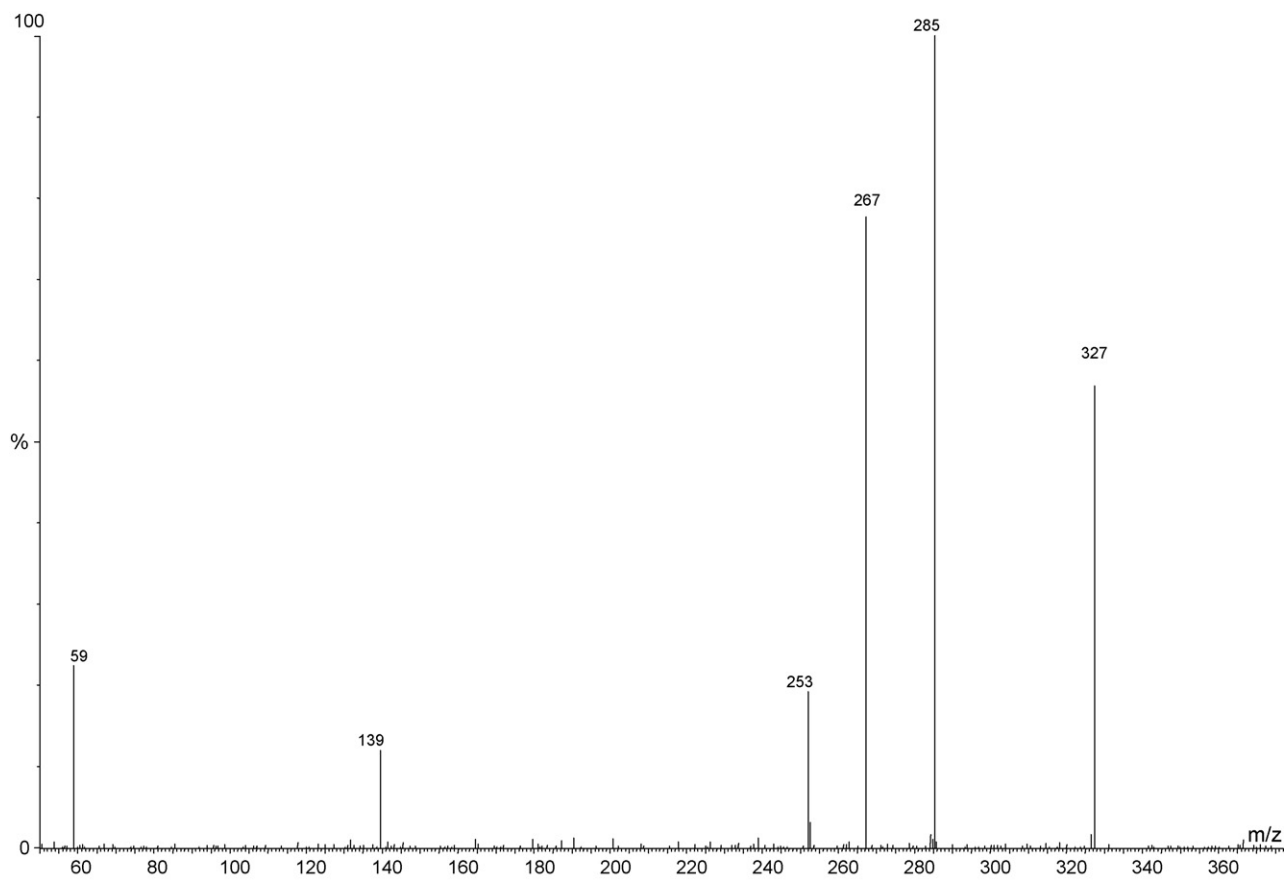
Fig. 3. Pinobanksin-5-methylether acetate (M_W 328, peak 18) fragmentation pattern at lower collision energy (10 eV). The ion product with $[m/z]^-$ 59 is the acetate moiety.
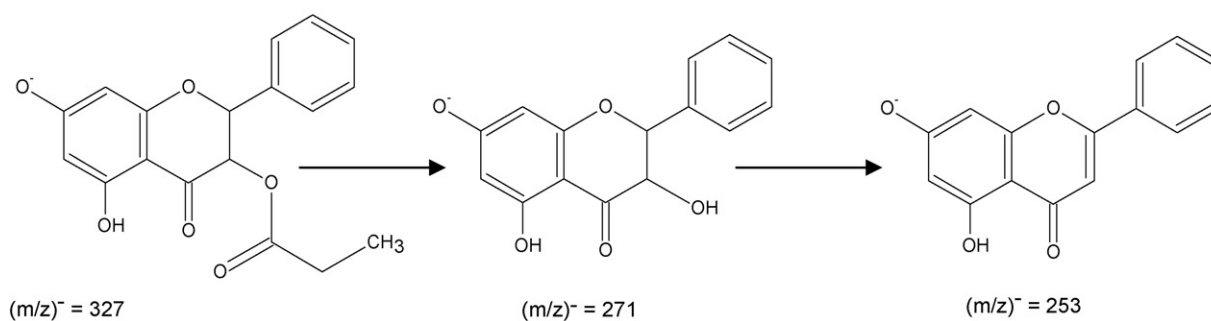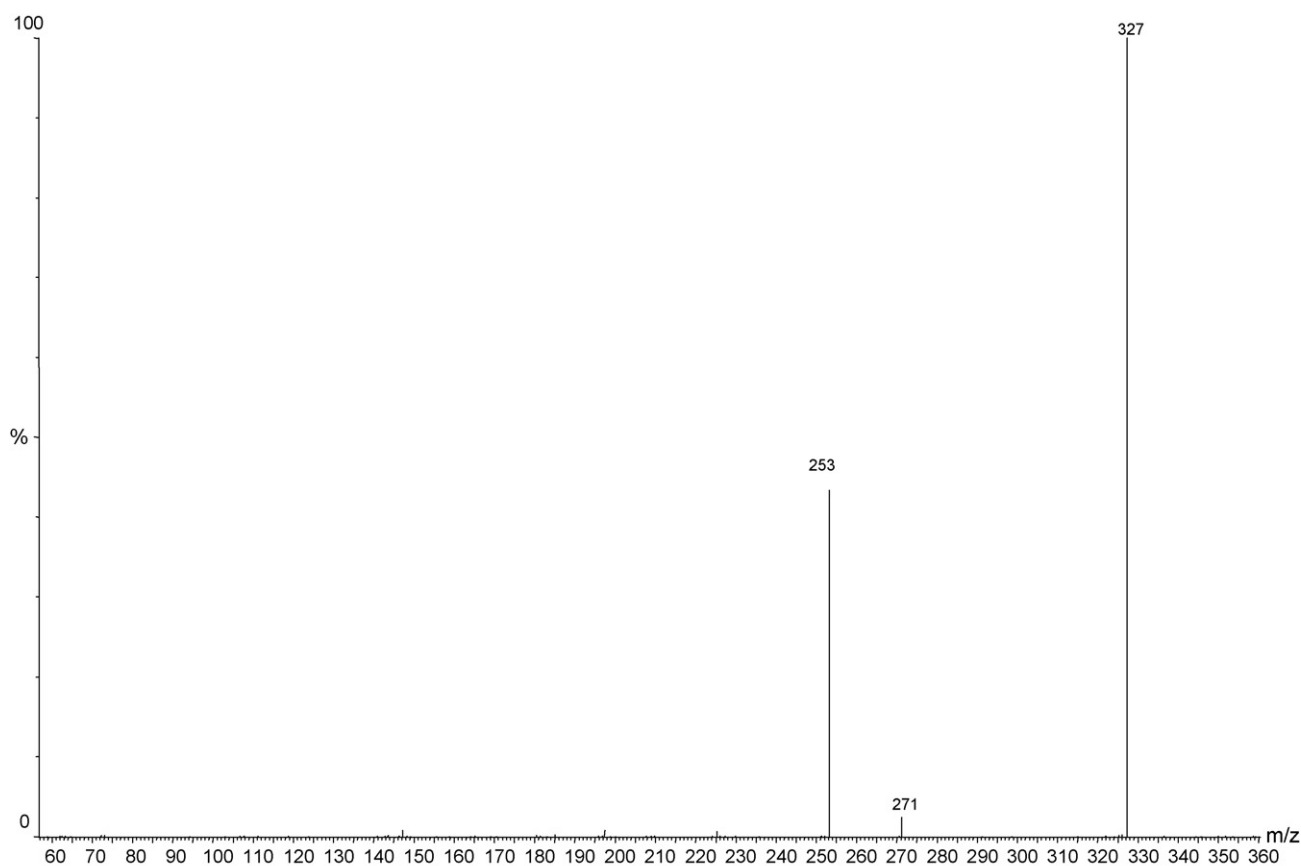
Fig. 4. Pinobanksin-3-propionate (M_W 328, peak 35) fragmentation pattern.

to a quercetin derivative and the molecular ion with $[m/z]^-$ 315 supports for a methoxyl quercetin. The methoxy group cannot be at $C_{3'}$ in the ring B; in fact, 3′-methoxy quercetin corresponds to isorhamnetin, which has a retention time different from that of peak 8. On the other hand, the methoxy group cannot be at the A ring, since MS/MS yielded the ion product with $[m/z]^-$ 151, thereby excluding methoxy substitution in this ring. Hence, peak 8 can be reasonably assigned as a 3-methoxy quercetin (substitution at C_3 of the C ring). Based on the UV spectrum, the molecular ion with $[m/z]^-$ 299 and the fragments with $[m/z]^-$ 255 [M–COOH]$^-$ and $[m/z]^+$ 245 [M–CH$_2$COOH]$^+$, peak 53 was identified as arthepillin C. Peak 50 was assigned as a p-coumaric acid with an isoprenyl group

bonded to the ring. This attribution is based on the UV behaviour (typical for p-coumaric acid derivatives) and on the fragments with $[m/z]^-$ 187 [M–COOH]$^-$ and $[m/z]^+$ 215 [M–H$_2$O]$^+$.

3.4. Flavonoid and phenolic acid content in propolis

The contents of total flavonoids and phenolic acids in different samples of propolis from different geographic areas are summarized in Table 3 . The amount of flavonoids and phenolic acids were determined using calibration curves obtained with standards, when available. Pinobanksin and its esters were assayed using pinobanksin calibration curves and their amounts were normalized by the molecular mass ratios. Similarly, caf-

Table 3
Total flavonoids and phenolic acids content (g/100 g) in raw samples of propolis from different geographic areas

Source	Flavonoids (%)	Phenolic acids (%)
France	10.6 ± 0.3	2.7 ± 0.2
France	13.0 ± 0.3	3.3 ± 0.1
France	9.3 ± 0.3	2.6 ± 0.2
France	12.4 ± 0.4	2.5 ± 0.2
France	12.8 ± 0.3	2.5 ± 0.2
France	1.5 ± 0.1	0.2 ± 0.0
Germany	10.0 ± 0.3	1.9 ± 0.1
Russia	1.1 ± 0.2	1.4 ± 0.1
Croatia	5.7 ± 0.2	2.7 ± 0.2
Croatia	3.2 ± 0.1	3.7 ± 0.0
Croatia	10.4 ± 0.1	4.9 ± 0.2
Croatia	10.7 ± 0.1	9.2 ± 0.3
Croatia	5.6 ± 0.2	6.7 ± 0.3
Croatia	8.5 ± 0.3	11.1 ± 0.4
Croatia	14.8 ± 0.2	9.4 ± 0.4
Croatia	12.4 ± 0.4	9.6 ± 0.2
Croatia	3.4 ± 0.2	1.4 ± 0.1
Croatia	22.6 ± 0.5	5.3 ± 0.3
Croatia	9.6 ± 0.5	8.1 ± 0.3
Croatia	9.7 ± 0.3	3.2 ± 0.2
Brazil	2.9 ± 0.2	14.6 ± 0.2
Brazil	0.7 ± 0.2	1.9 ± 0.4
Brazil	0.8 ± 0.2	1.6 ± 0.2
Brazil	2.4 ± 0.2	3.5 ± 0.2
Brazil	1.1 ± 0.1	3.7 ± 0.2
Brazil	1.5 ± 0.1	2.5 ± 0.1
Brazil	12.0 ± 0.5	3.4 ± 0.1
Brazil	2.3 ± 0.2	1.7 ± 0.2
Brazil	4.3 ± 0.3	2.3 ± 0.1
Argentina	3.1 ± 0.1	2.8 ± 0.1
Argentina	10.8 ± 0.3	1.5 ± 0.1
Argentina	12.9 ± 0.3	2.3 ± 0.0
Argentina	9.2 ± 0.2	0.3 ± 0.0
Argentina	15.2 ± 0.2	3.6 ± 0.1
Argentina	3.9 ± 0.1	0.6 ± 0.1
Argentina	13.7 ± 0.3	2.4 ± 0.3
Argentina	11.3 ± 0.5	1.7 ± 0.2
Argentina	19.0 ± 0.4	2.1 ± 0.1
Argentina	8.9 ± 0.2	2.5 ± 0.2
Argentina	5.1 ± 0.1	0.4 ± 0.1
Argentina	13.2 ± 0.2	1.3 ± 0.2
Argentina	9.7 ± 0.3	5.5 ± 0.3
Argentina	7.5 ± 0.2	2.1 ± 0.2
Argentina	14.4 ± 0.6	3.5 ± 0.2
Argentina	6.1 ± 0.1	0.5 ± 0.1
Argentina	11.3 ± 0.4	3.6 ± 0.2
Argentina	9.6 ± 0.3	2.1 ± 0.1
Argentina	10.9 ± 0.2	3.4 ± 0.1
Argentina	7.5 ± 0.1	2.6 ± 0.3
Argentina	9.4 ± 0.1	2.1 ± 0.1
Argentina	4.1 ± 0.3	1.3 ± 0.1
Argentina	9.4 ± 0.4	4.7 ± 0.2
Argentina	12.5 ± 0.3	2.9 ± 0.4
Argentina	15.2 ± 0.3	1.3 ± 0.1
Uruguay	6.9 ± 0.2	2.1 ± 0.2
Uruguay	6.6 ± 0.2	1.5 ± 0.1
Uruguay	12.1 ± 0.3	3.7 ± 0.2
Uruguay	3.8 ± 0.1	1.1 ± 0.2
Chile	16.9 ± 0.3	4.3 ± 0.2

Table 3 (*Continued*)

Source	Flavonoids (%)	Phenolic acids (%)
Chile	2.7 ± 0.2	1.1 ± 0.1
Chile	14.4 ± 0.2	2.7 ± 0.2
Chile	19.4 ± 0.2	2.9 ± 0.3
Chile	11.1 ± 0.3	2.5 ± 0.2
Chile	14.4 ± 0.2	2.7 ± 0.3
Chile	19.4 ± 0.2	2.9 ± 0.3
Peru	<0.1%	<0.1%
Paraguay	<0.1%	0.3 ± 0.0
Canada	10.3 ± 0.2	3.4 ± 0.2
Canada	10.4 ± 0.3	3.2 ± 0.2
Macedonia	5.1 ± 0.1	1.6 ± 0.1
Macedonia	25.6 ± 0.3	2.6 ± 0.3
Macedonia	16.7 ± 0.3	9.3 ± 0.4
Macedonia	13.1 ± 0.3	4.1 ± 0.2
Korea	7.2 ± 0.2	1.9 ± 0.2
China	6.7 ± 0.2	1.5 ± 0.2
China	21.9 ± 0.4	2.9 ± 0.2
China	11.3 ± 0.3	2.2 ± 0.1
China	5.9 ± 0.3	2.8 ± 0.2
China	9.1 ± 0.3	2.5 ± 0.2
China	7.0 ± 0.2	2.1 ± 0.1
China	11.6 ± 0.3	2.6 ± 0.3
China	8.4 ± 0.2	1.9 ± 0.3
China	7.1 ± 0.2	1.1 ± 0.2
China	4.1 ± 0.1	1.7 ± 0.2
China	5.8 ± 0.3	1.6 ± 0.2
China	10.9 ± 0.3	2.7 ± 0.3
Poland	1.2 ± 0.1	0.5 ± 0.2
Poland	7.0 ± 0.2	1.8 ± 0.3
Poland	10.3 ± 0.3	1.6 ± 0.2
Poland	14.6 ± 0.3	1.9 ± 0.2
Poland	14.1 ± 0.3	2.4 ± 0.3
Poland	9.9 ± 0.3	3.7 ± 0.2
Poland	12.7 ± 0.4	4.4 ± 0.2
Poland	10.2 ± 0.3	2.9 ± 0.3
Poland	12.7 ± 0.4	6.6 ± 0.3
Poland	15.5 ± 0.5	4.8 ± 0.3
Poland	15.9 ± 0.4	3.1 ± 0.3
Poland	10.1 ± 0.3	4.8 ± 0.4
Bulgaria	7.6 ± 0.2	3.9 ± 0.3
Italy	13.5 ± 0.2	0.7 ± 0.2
Italy	11.2 ± 0.2	2.5 ± 0.3
Italy	9.3 ± 0.1	2.9 ± 0.2
Italy	3.7 ± 0.1	1.2 ± 0.2
Italy	12.3 ± 0.3	3.8 ± 0.3
Italy	15.4 ± 0.5	7.3 ± 0.2
Italy (organic)	11.9 ± 0.3	2.9 ± 0.3
Italy (organic)	13.3 ± 0.4	2.3 ± 0.2
Italy (organic)	16.8 ± 0.5	9.3 ± 0.2

feic acid and *p*-coumaric acid esters were evaluated using caffeic acid and *p*-coumaric acid calibration curves, respectively.

Except for some Brazilian propolis, whose flavonoid content is very low, the most abundant flavonoids were in the order chrysin (2–4%), pinocembrin (2–4%), pinobanksin-acetate (1.6–3%) and galangin (1–2%). When considering the total flavonoid content, propolis with a content less than 11% should be considered of low quality, whereas propolis with a

content of 11–14%, 14–17% or >17% should be classified as propolis of acceptable, good and high quality, respectively. On the other hand, cinnamic acid derivatives (e.g. *p*-coumaric esters and CAPE) can have various positive effects on human health [21]; therefore, also their quantitative and qualitative determination is desirable.

4. Conclusion

Propolis samples from different geographical areas contain several different compounds and LC–DAD–MS in the negative ion mode provides an effective fingerprinting method for their screening. Moreover, tandem mass spectrometry with collision-induced dissociation (CID–Ms^2) allows structural identification, especially when standard compounds are not available. The obtained results suggest that flavonoids could be used as "quality marker" since these polyphenols represent the main propolis components. When Brazilian propolis is considered, phenolics acids (e.g. artepillin C) could be used to describe propolis quality.

The proposed analytical method may be applied for the routinary screening of raw propolis and its commercial formulations.

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