

Selective Analysis of Phenolic Compounds in Propolis by HPLC-MS/MS

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Abstract: Phenolic compounds (flavonoids and phenolic acid derivatives) are major active constituents of the resinous fraction of propolis, and also represent its allergenic principles. We have developed a chromatography electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS/MS) method to characterise the polyphenolic fraction of propolis rapidly and qualitatively. With precursor ion scanning, selective detection of caffeic esters was easily achieved, confirming the identification of prenyl caffeate, benzyl caffeate and phenylethyl caffeate by comparison with synthetic standards. The ionisation and fragmentation behaviour of the major propolis flavonoids was rationalised and applied to selected real samples. Taken together, the results of this study show that the introduction of precursor ion analysis leads to a significant improvement in the characterisation of the phenolic fraction of propolis, paving the way to the establishment of a better quality control for this important natural remedy. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: Flavonoids; caffeates; precursor ion MS/MS.

INTRODUCTION

Phenolic compounds are major constituents of the resinous fraction of propolis, a material accounting for ca. 50% of this important honeybee product, which is still widely used as a home remedy for its antibacterial, antiviral and antiinflammatory properties (Castaldo and Capasso, 2002). Flavonoids and phenolic acid derivatives are the two major classes of propolis phenolics, and are responsible for its healing properties (Usia *et al.*, 2002). The phenolic fraction is the most hydrophilic fraction of propolis, but, on account of its low water solubility at or around physiological pH, ethanol is required for its solubilisation. For this reason, ethanolic or hydro-alcoholic solutions are the most common pharmaceutical preparations of propolis. The geographical origin strongly influences the composition of propolis, and it is therefore impossible to establish a universal profile for this material. Within the polyphenolics of propolis, a series of caffeate esters endowed with a specific spectrum of bioactivity were identified, with phenethyl caffeate (CAPE) and prenyl caffeate representing the major antiinflammatory/anticancer and the major allergenic constituents (Grunberger *et al.*, 1998; Walgrave *et al.*, 2005) in propolis from temperate climates.

The composition of propolis has been analysed using a variety of methods, mainly based on HPLC and GC-MS. Propolis from New Zealand was studied using these techniques (Stüwe *et al.*, 1989) and HPLC-MS/ESI-MS was also employed for fingerprint characterisation of propolis from different countries (Markham *et al.*, 1996; Sawaya *et al.*, 2004; Volpi and Bergonzini, 2006). Extraction and isolation of several fractions of Chinese propolis and their structural characterisation by ¹³C-NMR after suitable purification were later reported (Midorikawa *et al.*, 2001). Chemical composition of European propolis (deriving from Bulgaria, Italy and Switzerland) was determined by GC-MS (Usia *et al.*, 2002), while the chemical composition and antiviral and antimicrobial activities of Egyptian propolis have been studied (Bankova *et al.*, 2002). When different analytical techniques were compared, an HPLC-APCI-MS technique turned out to be the best choice for the reliable identification of a large number of propolis components (Rauha *et al.*, 2001). However, most of these studies relied on a relatively small number of reference compounds. On the other hand, many general studies in HPLC-MS on flavonoids have been reported in the literature (Ma *et al.*, 1997; Fabre *et al.*, 2001; Pietta *et al.*, 2002; De Rijke *et al.*, 2003; Cuyckens and Claeys, 2004). On this basis, in order to expand the range of propolis constituents amenable to analysis, we have now used HPLC tandem mass spectrometry with ion trap (IT) and with a triple quadrupole (TQ) mass analysers to evaluate a comprehensive series of flavonoids

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and caffeate esters and to optimise the conditions for their analysis. In particular, precursor ion mode analysis, a feature of triple quadrupole instruments, made it possible to clearly discriminate between flavonoids and caffeate esters, two classes of compounds that share many fragmentation peaks. This distinction is very important for the analysis of propolis, especially to assess its allergenic potential, related to its contents of prenyl caffeate. In order to validate our analysis, samples of propolis of different geographic origins were investigated.

EXPERIMENTAL

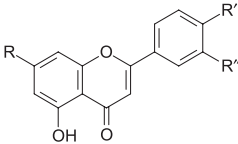
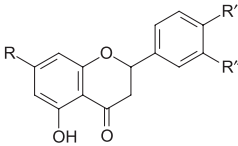
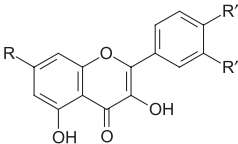
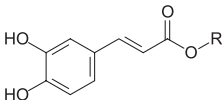
Standard and sample preparation. Flavonoids [chrysin, tectochrysin, apigenin and acacetin (flavones), galangin, kaempferide (flavonols), pinocembrin, pinostrobin and sakuranetin (flavanones)] were purchased from Extrasynthese (Genay, France). Prenyl-, benzyl- and phenylethyl-caffeates were synthesised as described elsewhere (Appendino *et al.*, 2002) and purity of compounds was verified to be >99% (melting point, NMR, HPLC). Standard compounds (Table 1) were dissolved at a concentration of 1.0 g/L in methanol and then

diluted with methanol to 100.0 mg/L and with methanol: water 50:50 v/v to 1.0 mg/L. HPLC-grade water was obtained from MilliQ System Academic (Millipore, Vimodrone, Italy). HPLC-grade methanol was filtered through a 0.45 µm filter before use. All solvents and reagents were from Aldrich (Milan, Italy).

Samples of propolis from different geographical origins were dissolved in methanol, diluted with methanol: water (50:50 v/v) and analysed by HPLC-MS after filtration (0.45 µm).

HPLC-UV-MS conditions. Analyses were performed using two HPLC-MS systems: (1) a Thermo-Finnigan Surveyor instrument, equipped with autosampler and a PAD-UV 6000 LP detector, coupled to an LCQ Deca XP Plus ion trap spectrometer (Thermo Electron, Rodano, Italy); (2) a Varian Pro-Star HPLC instrument equipped with a 410 autosampler and coupled to a 330 UV-visible diode array and a 1200L triple-stage quadrupole as detectors (Varian, Leini, Italy). Both mass spectrometers had ESI interfaces. With ion trap mass spectrometry, detailed studies (MSⁿ) on fragmentation pathways were performed by direct infusion. The triple-stage quadrupole spectrometer was used as the main detector for chromatographic analyses, thanks to its capability to be used

Table 1 Compounds studied, ordered by polyphenol class and molecular weight

	Name, MW	R	R'	R''
Flavones 	Chrysin, 254	OH	H	H
	Tectochrysin, 268	OMe	H	H
	Apigenin, 270	OH	OH	H
	Acacetin, 284	OH	OMe	H
Flavanones 	Pinocembrin, 256	OH	H	H
	Pinostrobin, 270	OMe	H	H
	Sakuranetin, 284	OMe	OH	H
Flavonols 	Galangin, 270	OH	H	H
	Kaempferide, 300	OH	OMe	H
	Quercetin, 302	OH	OH	OH
Caffeates 	Prenyl caffeate, 248		—	—
	Benzyl caffeate, 270	-CH ₂ Ph	—	—
	Phenylethyl caffeate (CAPE), 284	-CH ₂ CH ₂ Ph	—	—

in the precursor ion mode. All mass spectra were obtained in the negative ion mode but in the case of tectochrysin, as a consequence of its very low acidity, better sensitivity was obtained in the positive ion mode.

The chromatographic separations were performed on a Chrompack Inertsil 3 ODS 3 column (150 × 2 mm, 3 µm particle size; Varian, Leini, Italy). The injection volume was 10 µL and the flow rate 200 µL/min. Gradient elution was achieved using 0.05% formic acid in water and acetonitrile (organic modifier percentage varied from 24 to 100% in 20 min).

The effluent was delivered to the ion source using nitrogen as sheath and auxiliary gas. The ESI/TQ mass analyser parameters were as follows: in the positive ion mode, the needle potential was 4.1 kV, capillary voltage -40.0 V, Q0 offset 2.0 V; in the negative ion mode the needle potential was 5.0 kV, capillary voltage +40.0 V, Q0 offset -1.9 V; the drying gas temperature was 300°C. For the ESI/IT mass analyser in the negative ion mode, the needle potential was 3.5 kV, tube lens offset 80 V, in the positive ion mode, needle potential was 4.5 kV, tube lens offset -80 V; the heated capillary temperature was 270°C.

The acquisition method used was first optimised in the tuning sections for each compound in the form of the protonated or de-protonated ion (capillary, magnetic lenses and collimating multipole voltages) so as to maximise sensitivity. In the case of IT, collision energy (CE) was generally chosen to maintain about 10% of the precursor ion. IT mass spectra were collected in the full-scan positive and negative modes in the range 50–700 *m/z* and in the tandem MS mode in various ranges depending on the precursor ion *m/z* value in the range 75–310 *m/z* with 45–50% CE for flavonoids and 25–35% CE for caffeates. TQ was used as analyser in three different acquisition modes: (i) full mass scan (200–800 *m/z* range); (ii) SRM monitoring only retro Diels–Alder [product ions (-)151, (+)153, (-)165, (+)167, with 10–40 V CE] and caffeic, [(-)134 and (-)135, with 18–26 V CE] ions; and (iii) precursor ion mode [range 200–800 *m/z*, product ions (+)153, 167 *m/z* with 5–25 V CE, (-)151, 165 *m/z* with 5–25 V CE for flavonoids and (-)134, 135 *m/z* with 20 V CE for caffeates]. Signals were collected to measure different sensitivity (Table 3); mass width accuracy was in all cases ±1.5 *m/z*.

Linearity, limit of quantitation, precision and accuracy.

Statistical analyses were performed for the quantitative acquisition method on the TQ analyser. The full mass, SRM and precursor ion HPLC-MS methods were linear over the range 0.1–1000 ng/mL; the *r*² values of the calibration curves were in all cases >0.998 (*n* = 5). In order to evaluate the sensitivities of the different methods, the limit of quantitation (LOQ) was evaluated for the three different acquisition modes. LOQ, precision and accuracy values are reported in Table 3.

RESULTS AND DISCUSSION

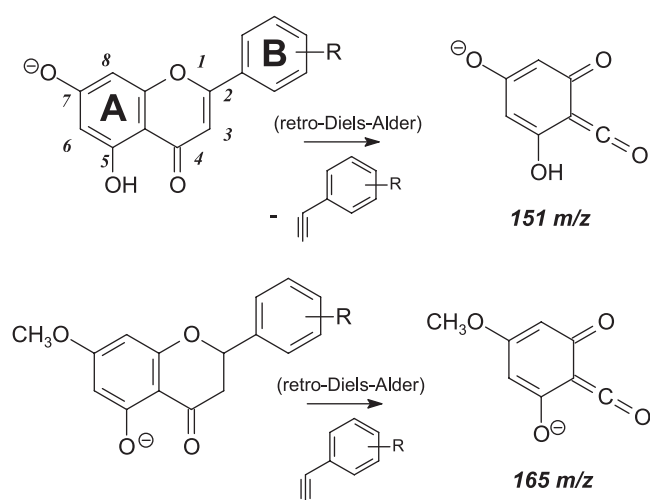
A detailed study of the fragmentation pattern of the flavonoids and the caffeates from propolis was made employing both ion trap and triple quadrupole mass analysers. In this way, the mass fragmentation behaviour of the analytes could be analysed both in terms of the collision-inducing devices and in terms of the potential applied. In the ion trap system, the collision energy is tailored on each precursor ion by applying a resonant radio frequency potential allowing specific resonant excitation. The number of product ions may be limited by the low mass cut-off typical of the region of stability inside the trap and their intensity will depend on the available excitation energy, an important limitation on the stability inside the trap of the precursor ion. The ion excitation mechanism in an ion trap is the same as that which operates when the instability necessary to detect ions is induced. Precursor ion instability conditions are often established in an ion trap before reaching high enough excitation energies to activate certain fragmentation pathways. Conversely, in the triple quadrupole, the accelerating potential between the ends of the collision cell is optimised so as to obtain product ions at the best sensitivity, activating all possible fragmentation pathways. As a result, no correlation is often found between the most intense fragment ions obtained with IT and those obtained with TQ (Table 2).

In order to identify the best mass detection mode for the analysis of real samples of propolis, the main product ions obtained with the two mass analysers were compared (Table 2). The ions reported are not only the most intense ones (useful to SRM analysis), but also those derived from common fragmentation pathways (useful for precursor ion analysis). The most common fragmentation pathway for flavonoids was a retro Diels–Alder reaction leading to 153 and 167 *m/z* positive ions or 151 and 165 *m/z* negative ions. For the caffeates, homo- and heterolytic ester breakdown leading to 134 and 135 *m/z* negative ions were commonly observed (Figures 2 and 3).

The differences in intensities in Table 2 are in good accordance with the chemical structure of the two classes of compounds (flavonoids and caffeates). Flavonoids are generally stable compounds, and their fragmentation pathways depend on the substitution pattern of the aromatic rings, producing product ions at low level of intensity, and maintaining high level of unfragmented precursor ions. The heterocyclic linkage between rings A and B of flavonoids (Fig. 1) is their molecular moiety more prone to fragmentation: this site is less easily fragmented when a C-2–C-3 double bond is present, as in flavones, or when a further hydroxyl is present at C-3 (flavonols). Within each class of compounds, the relative energy of fragmentation is modulated by the presence of oxygenated substituents

Table 2 Main product ions obtained with IT and TQ analysers, in order of intensity. Percent relative intensities (in brackets) are referred to base peaks in IT and to maximum signal of precursor ions in TQ

Analyte	IT analyser		TQ analyser	
	Positive ions (<i>m/z</i>)	Negative ions (<i>m/z</i>)	Positive ions (<i>m/z</i>)	Negative ions (<i>m/z</i>)
Chrysin	153 (30)	209 (100), 151 (6)	153 (14)	107 (2), 151 (1)
Apigenin	153 (100), 119 (4)	225 (100), 151 (11)	153 (7), 119 (4)	117 (6), 151 (3)
Acacetin	270 (100), 153 (3)	268 (100), 151 (3)	241 (100), 153 (10), 270 (6)	268 (97), 151 (8)
Tectochrysin	254 (100), 167 (10)	—	226 (18), 254 (7), 167 (0.3)	252 (10), 224 (8), 165 (2)
Pinocembrin	131 (100), 153 (90)	213 (100), 151 (23)	153 (27)	151 (2), 213 (2)
Pinostrobin	167 (100), 131 (90)	254 (100), 251 (62), 191 (50), 165 (45)	167 (29), 131 (20)	165 (14), 254 (2)
Sakuranetin	147 (100), 167 (94)	165 (100), 191 (14), 119 (9)	167 (86), 147 (60)	119 (56), 165 (13)
Galangin	165 (95), 153 (55)	213 (100), 197 (88), 151 (0.1)	153 (9)	197 (6), 169 (6), 227 (3), 151 (1)
Kaempferide	286 (100), 153 (15)	284 (100), 151 (0.5)	153 (9), 286 (6)	284 (89), 151 (20)
Quercetin	257 (100), 153 (0.2)	179 (100), 151 (53)	153 (10), 165 (6)	151 (7), 179 (3)
Prenyl caffeate	—	179 (100), 178 (58), 134 (19), 135 (20)	—	134 (28), 135 (28), 178 (9), 179 (8)
Benzyl caffeate	—	178 (100), 134 (45)	—	134 (100), 178 (77)
CAPE	—	179 (100), 135 (35)	—	135 (73), 179 (61)

**Figure 1** Common RDA fragmentation patterns of flavonoids.

(especially at C-7) that, in some cases, accentuate the difference between the results obtained by IT and TQ (see Table 4).

All the flavonoids showed $[M + H]^+$ ions of good intensity in the positive mode and, with the exception of tectochrysin, intense $[M - H]^-$ negative ions. The high stability of flavonoid ions, especially of flavonols, makes it difficult to obtain good sensitivity in MS/MS using an ion trap because the supply of collision energy to precursor ion is limited by its stability inside the detector. It is then more convenient to use TQ by virtue of its ability to provide as much collision energy as is required to obtain product ions at best sensitivity. Therefore TQ was used for this class of compounds in the SRM mode.

In sharp contrast to flavonoids, caffeic acid esters provided intense negative ions and were more prone to fragmentation, with a rather uniform spectrum, depending on the ester moiety. Thus, the most convenient detection mode for these compounds was precursor ion analysis. Owing to a commercial availability limited to CAPE, the other caffeates in Table 1 were obtained by synthesis, capitalising on a recent esterification protocol for phenolic acids (Appendino *et al.*, 2002).

Despite their pharmacological and toxicological relevance, caffeates are not routinely used as significant biomarkers for the quality of propolis. This is very surprising, since important and specific bioactivities have been demonstrated for them. Thus, benzyl-caffeate shows potent antibacterial properties, prenylcaffeate is a potent allergen and CAPE is an important inhibitor of NF- κ B (Da Cunha *et al.*, 2004). During the analysis of propolis, these compounds co-elute with flavonoids, but their fragmentation pattern is quite different, and can be exploited in terms of an unambiguous differentiation based on the detection of common product ions

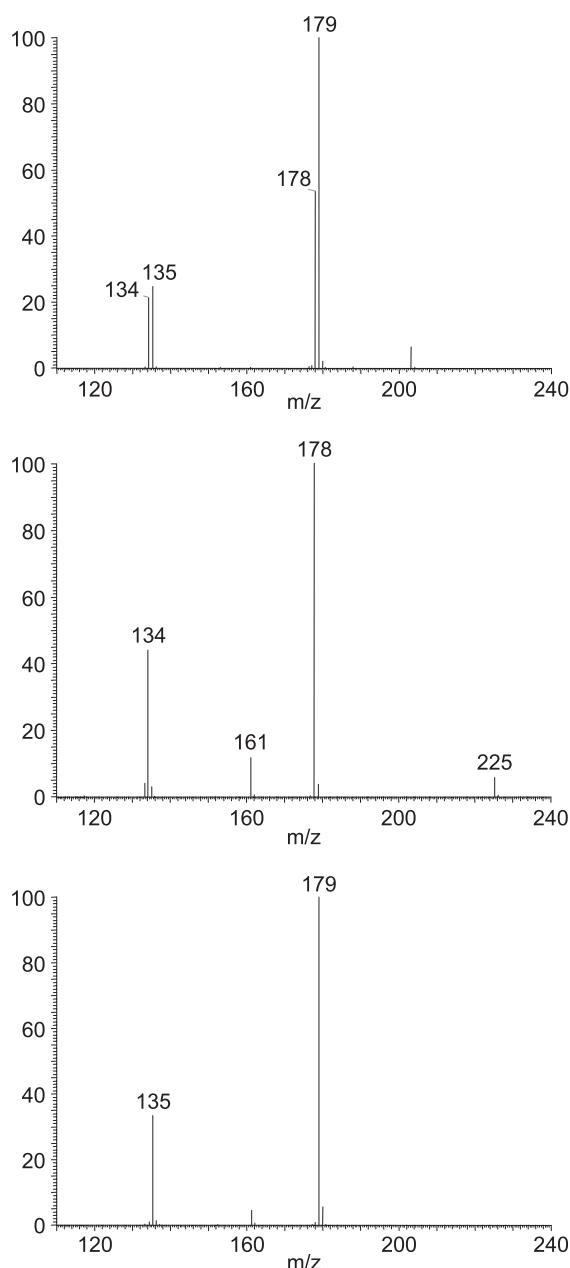


Figure 2 Negative ion MS² spectra of different caffeic acid esters: prenyl (top), benzyl- (middle) and CAPE (bottom).

resulting from both homolytic cleavage (radical product ion formation, m/z 134) and heterolytic cleavage (even electron product ion formation, m/z 135) depending on the alcohol moiety present.

The MS/MS spectra and the relative hypothesised pathways of fragmentation are reported in Figs 2 and 3.

The predominant fragmentation mechanism for benzyl caffeate is the homolytic cleavage of the benzylic bond. This behaviour, relatively rare with an electrospray ionisation source, is presumably related to the great

stability of benzyl radical (rearranged to tropylium ion). The resulting odd electron ion at m/z 178 loses a CO₂ molecule, giving the radical species at m/z 134. The odd electron cleavage is not noticeable for phenethyl caffeate (phenylethyl radical is not so stabilised as benzyl radical); it only loses the neutral styrene molecule, giving as final species, after CO₂ loss, the anion of m/z 135.

Owing to the relative stability of prenyl radical, prenyl-caffeate showed an intermediate behaviour, where both the fragmentation mechanisms were operating, affording both radical and neutral product ions. These observations made it possible to discriminate flavonoids and caffeates, taking advantage of the precursor ion analysis typical of triple quadrupole, obtaining a chromatogram selectively containing only the signals corresponding to these compounds. To the best of our knowledge applications of precursor ion or neutral loss analysis have been used only to discriminate isobaric couples of flavonoids (Ye *et al.*, 2005) or selectively analyse glycosides (Qu *et al.*, 2004; Es-Safi *et al.*, 2005). The chromatograms shown in Fig. 4(A, B) highlight the difference between a full mass acquisition and a precursor ion acquisition mode for a sample of propolis. Using this technique, a new caffeate, phenylpropyl-caffeate or homo-CAPE, could be identified by its molecular weight and fragmentation. This compound is reported in the literature (Nagaoka *et al.*, 2003) as a synthetic analogue of CAPE, but not as a propolis component.

A validation study concerning the quantitative application of TQ different detection modes was performed (Table 3). Linearity, accuracy and precision good results were obtained. The limits of quantitation (LOQ) are an interesting tool with which to illustrate different sensitivity of each detection mode. Generally speaking, SRM acquisition mode gives a better sensitivity while the precursor ion mode offers selectivity and allows discrimination between flavonoids and caffeates classes. For caffeates only the precursor ion mode gives also significant sensitivity, providing clean chromatograms for this class of compounds in a real sample without further purification steps (Fig. 4).

The possibility to unambiguously discriminate between the two main classes of propolis polyphenols is critical in order to differentiate samples of different geographical origin. Samples from Ukraine, Argentina, China, Macedonia and Italy were used to test the analysis. Table 4 reports data about the total flavonoids and caffeates contents of these samples, measured by HPLC-MS, highlighting the power of the technique to differentiate different samples of propolis.

While only galangin is currently used in the commercial analysis of propolis, the use of benzyl-caffeate as a complementary standard has the potential to significantly improve the quality controls of this important natural remedy.

Table 3 Statistical analysis parameters

Analyte	(-) Full mass LOQ (ng/mL)	(-) retro Diels–Alder SRM LOQ (ng/mL)	(-) Precursor ion LOQ (ng/mL)	Precision (RSD% on 100 ng/mL standard analysis, <i>n</i> = 5)	Accuracy (% on 100 ng/mL standard analysis, <i>n</i> = 5)
Chrysin	66	3.4	5.9	2.3	7.8
Apigenin	65	0.58	1.6	2.0	5.1
Acacetin	51	1.7	1.8	2.5	4.0
Pinocembrin	40	1.5	0.77	2.7	3.0
Pinostrobin	55	4.9	16.7	4.7	9.1
Sakuranetin	46	1.3	2.8	2.3	8.7
Galangin	51	19.1	57.2	8.3	10.0
Kaempferide	54	1.1	1.1	2.2	4.6
Quercetin	51	5.9	0.8	3.1	5.1
Prenyl caffeate	38	0.35	0.24	2.0	6.5
Benzyl caffeate	35	0.084	0.28	2.4	8.8
CAPE	36	0.076	0.26	3.5	4.9

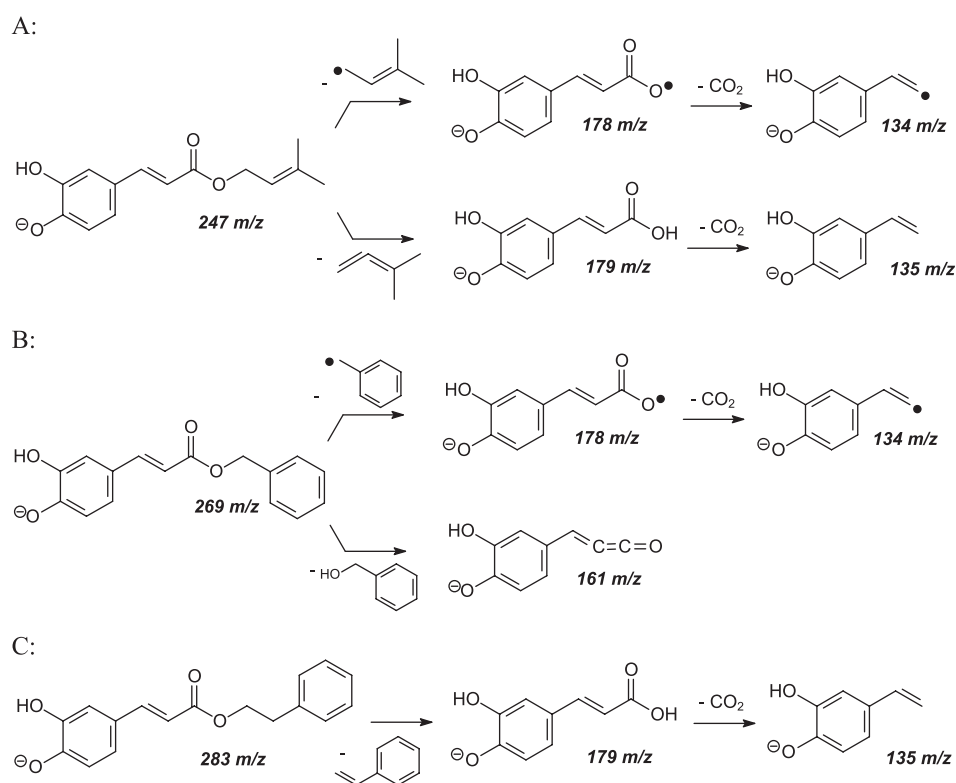
**Figure 3** Fragmentation pathways of different caffeic acid esters: prenyl (A), benzyl- (B) and CAPE (C).

Table 4 Distribution of analysed compounds in propolis of different geographical origin

Analyte	$\mu\text{g/g} \pm \text{standard deviation (SD), } n = 3$				
	Italy	China	Argentina	Ukraine	Macedonia
Chrysin	781.5 ± 80.1	2333.0 ± 350.0	2347.6 ± 215.7	922.0 ± 111.2	1649.8 ± 177.5
Apigenin	132.1 ± 21.6	178.6 ± 34.8	336.9 ± 41.7	177.4 ± 15.2	236.4 ± 34.1
Acacetin	1133.3 ± 256.2	1578.0 ± 141.9	831.5 ± 16.0	658.9 ± 67.0	1343.0 ± 200.2
Tectochrysin	130.9 ± 21.6	238.3 ± 24.6	224.7 ± 29.0	153.6 ± 18.7	987.1 ± 104.0
Pinocembrin	769.4 ± 77.9	2087.5 ± 347.0	3362.5 ± 418.8	1196.5 ± 91.9	2112.0 ± 184.5
Pinostrobin	575.6 ± 48.6	n.d.	n.d.	1479.3 ± 303.5	3816.0 ± 397.1
Sakuranetin	152.9 ± 20.6	146.0 ± 18.4	27.5 ± 4.1	2184.0 ± 196.1	2203.4 ± 269.0
Galangin	70.4 ± 11.8	1400.3 ± 126.6	2253.7 ± 294.4	952.9 ± 106.8	903.9 ± 88.2
Kaempferide	39.8 ± 3.6	38.8 ± 5.0	38.1 ± 4.6	91.9 ± 13.7	43.2 ± 7.0
Quercetin	153.5 ± 18.6	70.9 ± 9.7	n.d.	28.9 ± 4.1	118.3 ± 13.3
Prenyl caffeate	33.3 ± 2.6	56.2 ± 6.0	29.7 ± 4.2	53.9 ± 6.1	94.8 ± 10.3
Benzyl caffeate	527.1 ± 61.8	1477.0 ± 116.6	1180.1 ± 200.8	465.1 ± 31.9	400.6 ± 62.6
CAPE	1745.2 ± 245.2	2525.0 ± 199.3	1111.6 ± 125.6	1145.9 ± 98.6	1263.1 ± 212.6

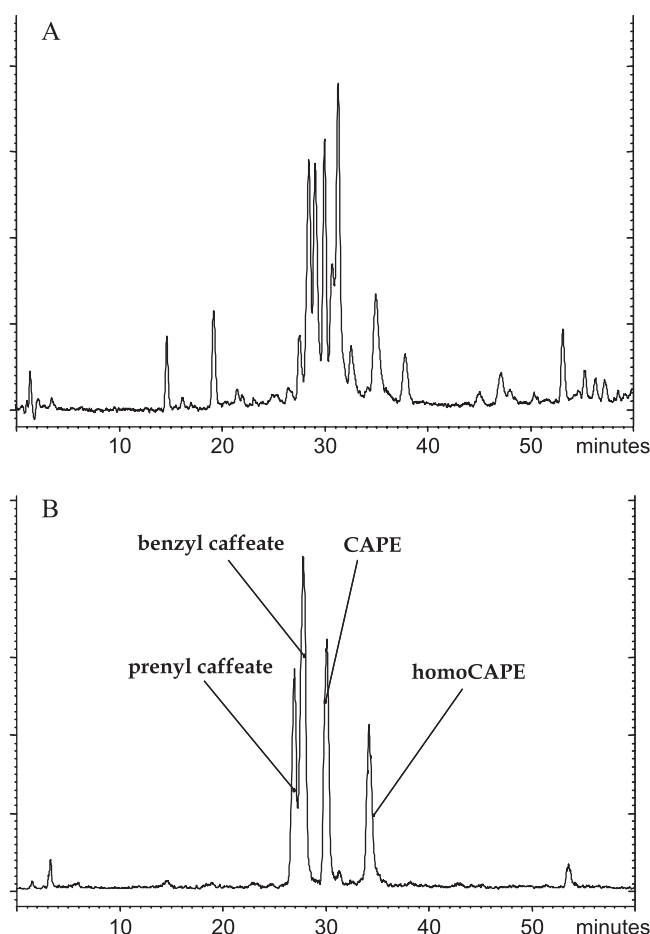


Figure 4 Full MS (A) and caffeates precursor ion MS (B) chromatograms of a real sample of propolis obtained with TQ analyser: (A) shows an unselective LC-MS analysis where co-elution occurs because of the great number of phenolic compounds in a propolis sample. In this case compound identification could be difficult. (B) shows the chromatogram of the same sample with acquisition of the only compounds whose precursor ions generated 134 and 135 m/z ions (caffeic acid moiety).

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