

Phenolic characterization of Northeast Portuguese propolis: usual and unusual compounds

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Abstract In this study, an ethanolic extract from Portuguese propolis was prepared, fractionated by high-performance liquid chromatography, and the identification of the phenolic compounds was done by electrospray mass spectrometry in the negative mode. This technical approach allowed the identification of 37 phenolic compounds, which included not only the typical phenolic acids and flavonoids found in propolis from temperate zones but also several compounds in which its occurrence have never been referred to in the literature. Four of the novel phenolic compounds were methylated and/or esterified or hydroxylated derivatives of common poplar flavonoids, although six peculiar derivatives of pinocembrin/pinobanksin, containing a phenylpropanoic acid derivative moiety in their structure, were also identified. Furthermore, the Portuguese propolis sample was shown to contain a *p*-coumaric ester derivative dimer.

Keywords Phenolic compounds · Flavonoids · Phenolic acids · Mass spectrometry · Electrospray ionization

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Introduction

Propolis or bee glue is a sticky dark-colored substance produced from the collected buds or exudates of plants (resin) by bees (*Apis mellifera* L.). The resin is masticated, salivary enzymes are added, and the partially digested material is mixed with beeswax and used in the hive to seal the walls, strengthen the borders of combs, and embalm dead invaders. Propolis is a building material, but it is also the most important “chemical weapon” of bees against pathogen microorganisms [1].

Propolis has been used as remedy by humans since ancient times [2]. In the last years, this product has been the subject of intensive studies, highlighting its biological and pharmacological properties, such as the antimicrobial [3], antioxidative, antihepatotoxic [4], antitumoral, antiinflammatory [5], anti-HIV-1 [6], antineurodegenerative [7], and antituberculosis [8]. Propolis was also tested as food preserver due to its bactericidal and bacteriostatic properties [9]. Furthermore, most of its components are natural constituents of food and recognized as safe substances [9].

The successful medical applications of propolis led to an increased interest in its chemical composition [10]. In general, resin comprising flavonoids and related phenolic acids represent approximately half of the propolis constituents, while beeswax, volatiles, and pollen represent approximately 30%, 10%, and 5%, respectively [11]. Still, the chemical composition of the bee glue is extremely dependent on the plants found around the hive, as well on the geographic and climatic characteristics of the site. Buds from *Populus* species are the main source of resins in Europe and North America propolis (“poplar type” propolis) [2]. Alternatively, in regions where these plants are not native, other species such as *Clusia* in Cuba and *Baccharis* in Brazil are used as resin sources, increasing its diversity and complexity [12].

Less commonly, species such as *Betula*, *Ulmus*, *Pinus*, *Quercus*, *Salix*, and *Acacia* are also used [13].

More than 300 constituents have been identified in different types of propolis [11], with the phenolics being the most abundant compounds. In propolis from temperate zones, the most frequently reported phenolic components include the flavonoids pinocembrin, galangin, and chrysin and the phenolic acids caffeic acid, ferulic acid, and the cinnamic acid [2]. Instead, the propolis from tropical zones, in particular those from the Southeastern region of Brazil, were shown to be rich in prenylated phenylpropanoids [11], although non-typical flavonoids from “poplar type” propolis, such as kaempferide and isosakuranetin, have been found [14, 15]. Moreover, Cuban propolis has recently caught the attention of scientists because its peculiar enrichment in polyisoprenylated benzophenones turns it chemically distinct from both the European and the Brazilian bee glue [16].

A large number of analytical methods have been used for the analysis of phenolic compounds in propolis, including spectrophotometry [17], thin-layer chromatography [18], gas chromatography–mass spectrometry (GC–MS) [19], high-performance liquid chromatography (HPLC) [20], liquid chromatography–mass spectrometry (LC–MS) [21–23], electrospray ionization–mass spectrometry (ESI–MS) and electrospray ionization–tandem mass spectrometry (ESI–MSⁿ) [24], capillary electrophoresis [25], and nuclear magnetic resonance (NMR) [26]. Among these methods, MS with ESI is one of the most-used techniques in the research of natural products, as it is able to analyze complex mixtures because of its high selectivity. This method also provides adequate structural information, and thus, it facilitates the structural identification of unknown compounds.

The chemical composition of European propolis was determined by GC–MS [11, 19], LC–MS, and ESI–MS [21–24]. Also Moreira et al. [27] have recently described the phenolic content and the antioxidant potential of Portuguese propolis from two different regions, including the Northeast, but still, their phenolic components were not elucidated. In this context, this paper aims to characterize the phenolic composition of Northeast Portuguese propolis by ESI–MS. For that, the ethanolic extract was fractionated by reversed-phase HPLC, and the major phenolic compounds of each fraction were identified by detailed analysis of their fragmentation pathways. New compounds were found in propolis for the first time, and their structure will be assigned in the present study.

Materials and methods

Chemicals

The phenolic standards naringenin, quercetin, kaempferol, pinocembrin, chrysin, caffeic acid, ferulic acid,

cinnamic acid, and *p*-coumaric acid were purchased from Sigma Chemical Co (St Louis, MO, USA). Genkwanin and luteolin were obtained from Lancaster Synthesis (Morecambe, England). The analytical grade reagents formic acid and ethanol were obtained from Panreac (Barcelona, Spain), and methanol and acetonitrile with HPLC purity were purchased from Lab-Scan (Lisbon, Portugal). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Samples

Three distinct propolis samples were collected in the fall of 2007 from *Apis mellifera* hives located in different apiaries in the Northeast of Portugal. They were obtained after the honey extraction, by scratching the hive walls and frames, followed by the removal of debris of wood and bees. These propolis samples were then stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

Extraction of phenolic compounds

Prior to the extraction, the propolis was grounded and homogenized. The samples were extracted with 80% of ethanol/water (1/10, v/v) at $70\text{ }^{\circ}\text{C}$ for 1 h, the resulting mixtures were filtered, and the residues were re-extracted in the same conditions. After the second extraction, the filtrated solutions were combined, concentrated, frozen at $-20\text{ }^{\circ}\text{C}$, and freeze-dried.

HPLC analysis

The phenolic profile of the propolis extracts was analyzed by reversed-phase HPLC, based on the method of Gardana et al. [23] with some modifications, as described below. The HPLC analysis was performed on a Knauer Smartline separation module equipped with a Knauer smartline autosampler 3800, a cooling system set to $4\text{ }^{\circ}\text{C}$, and a Knauer UV detector 2500. Data acquisition and remote control of the HPLC system were done by ClarityChrom[®] software (Knauer, Berlin, Germany). The column was a $250\text{ mm} \times 4\text{ mm}$ id, $5\text{ }\mu\text{m}$ particle diameter, end-capped Nucleosil C18 (Macherey-Nagel), and its temperature was maintained at $30\text{ }^{\circ}\text{C}$. The mobile phase comprised (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, which were previously degassed and filtrated. The solvent gradient started with 80% A and 20% B, reaching 30% B at 10 min, 40% B at 40 min, 60% B at 60 min, 90% B at 80 min, followed by the return to the initial conditions. For the HPLC analysis, the ethanolic extract (10 mg) was dissolved in 1 mL of 80% of ethanol. All samples were filtered through a $0.2\text{ }\mu\text{m}$ Nylon membrane (Whatman) and $10\text{ }\mu\text{L}$ of each solution was injected. Chromatographic data were acquired at 280 nm,

and the eluted fractions were manually collected according to the visualization of the UV profile.

Mass spectrometry analysis by ESI-MS and ESI-MSⁿ

The freeze-dried HPLC fractions were dissolved in methanol and directly injected into the ESI source by means of a syringe pump, at flow rate of 8 $\mu\text{L min}^{-1}$. Studies were performed in the negative mode using a Linear Ion trap LXQ (ThermoFinnigan, San Jose, CA, USA). Typical ESI conditions were nitrogen sheath gas 30 psi, capillary voltage 4.7 kV, source temperature 350 °C, entrance voltage -7.0 V, and tube lens voltage -71.8 V. CID-MS/MS and MSⁿ experiments were performed on mass-selected precursor ions using standard isolation and excitation configuration. The collision energy used was between 15 and 40 (arbitrary units). Data acquisition was carried out with Xcalibur[®] data system (ThermoFinnigan, San Jose, CA, USA).

Results and discussion

HPLC fractionation

The chromatographic profiles at 280 nm were similar for all the ethanolic extracts studied, independent of the sample location, consistent with the relationship between the phenolic profile and the surrounding apiary flora [2]. The representative chromatogram is shown in Fig. 1.

As also represented in this figure, the HPLC analysis allowed the collection of 35 fractions, suggesting that the propolis samples contained a large variety of phenolic compounds. Due to this high diversity, the identification of the compounds in each HPLC fraction was made by

electrospray ionization–mass spectrometry (ESI-MS and MSⁿ), even when its retention time matched with that of an available reference compound.

ESI-MS and MSⁿ

These analyses were carried out in the negative ion mode because of its higher sensitivity in the detection of the distinct classes of phenolic compounds [28]. Table 1 summarizes the data obtained for each of the analyzed fractions. In general, the identification of the corresponding compound was based on the search of the $[\text{M}-\text{H}]^-$ deprotonated molecule together with the interpretation of its MSⁿ fragmentations. Still, when standards were available, the identification of phenolic compounds was determined by comparison of the ESI-MSⁿ data to that of the standards. The structure of other phenolic compounds was also confirmed by the MS/MS data published in the literature, as indicated in Table 1. With respect to these latter compounds, it should be noted that their fragmentation pathways will not be discussed in detail in the present work, as these were previously reported [21–24]. The chemical structures of these compounds are shown in Fig. 2.

Overall, the mass spectrometry analysis of the HPLC fractions allowed the identification of 37 phenolic compounds (Table 1), which included not only phenolic acids and flavonoids already reported in propolis but also new compounds. Due to the high number of phenolic components, their identification will be discussed according to the mentioned classes.

Phenolic acids

Simple phenolic acids eluted in the first 13 min of the HPLC program, corresponding to fractions 1 to 5 (Table 1

Fig. 1 Chromatographic profile at 280 nm of the ethanolic extract obtained from the Portuguese propolis samples. The numbers in the figure correspond to the fractions collected for further analysis by ESI-MSⁿ

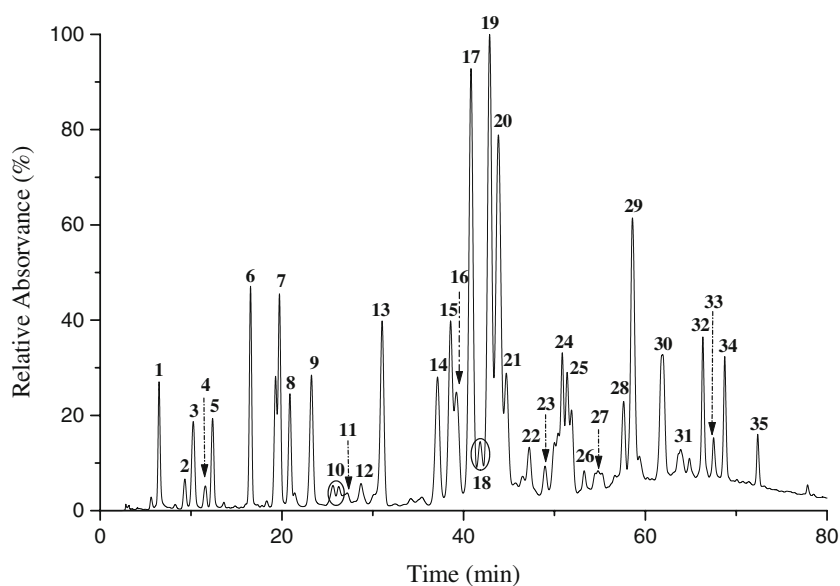
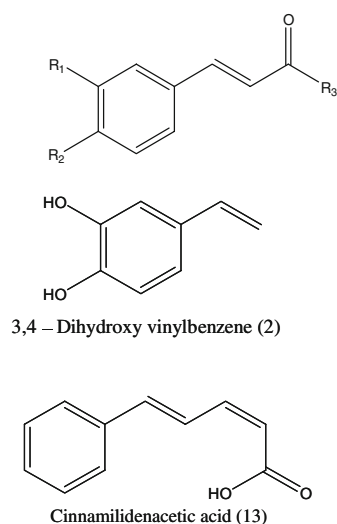


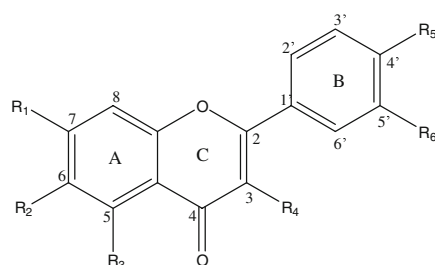
Table 1 Identification of HPLC-eluting fractions from propolis extract and correspondence with results obtained by mass spectrometry analysis

Fraction number	Retention time (min)	[M-H] ⁻	MS ² (% base peak) ^c	Compound
1	6.48	179	135	Caffeic acid ^a
2	9.32	135	107 (100), 91 (25)	3,4-Dihydroxy vinylbenzene ^b
3	10.2	163	119	<i>p</i> -Coumaric acid ^a
4	11.6	193	177 (16), 149 (47), 133 (100)	Ferulic acid ^a
5	12.4	193	177 (16), 149 (47), 133 (100)	Isoferulic acid ^c [23]
6	16.5	207	163 (60), 102 (100)	3,4-Dimethyl-caffeic acid (DMCA) ^c [23]
7	19.5	285	267 (100), 252 (13), 239 (29), 165 (1)	Pinobanksin-5-methyl-ether ^c [23]
8	20.9	177	163 (100), 119 (15)	<i>p</i> -Coumaric acid methyl ester ^c [23]
9	23.2	271	253 (100), 225 (19), 151 (10)	Pinobanksin ^c [23]
10	25.9	269	254 (94), 227 (100), 165 (48)	Pinocembrin-5-methyl-ether ^c [23]
11	27.2	269	225(100), 151 (17)	Apigenin ^a [22]
12	28.7	267	252 (100), 223 (12)	Chrysin-5-methyl-ether ^c [23]
		299	284	Unknown ^d
13	31.0	173	129	Cinnamylidenacetic acid ^c [23]
		329	314 (100), 299 (30), 285 (3)	Unknown ^d
14	37.1	247	179 (100), 135 (13)	Caffeic acid isoprenyl ester ^c [22, 23]
15	38.6	247	179 (100), 135 (13)	Caffeic acid isoprenyl ester (isomer) ^c [22, 23]
16	39.2	269	179 (100), 135 (60)	Caffeic acid benzyl ester ^c [22, 23]
17	40.8	255	213 (100), 211 (55), 151 (35)	Pinocembrin ^a
18	41.8	285	179 (8), 145 (40), 139 (100)	Unknown
19	42.7	253	209 (100), 151 (5)	Chrysin ^a
20	43.8	313	271 (20), 253 (100)	Pinobanksin-3- <i>O</i> -acetate ^c [23]
21	44.7	283	179 (100), 135 (24)	Caffeic acid phenylethyl ester (CAPE) ^c [22, 23]
22	47.2	283	268	Chrysin-6-methyl-ether ^c [23, 29]
23	48.9	231	163 (100), 119(12)	<i>p</i> -Coumaric acid isoprenyl ester ^c [23]
24	50.4	295	179 (100), 135 (80)	Caffeic acid cinnamyl ester ^c [23]
25	51.6	327	271 (10), 253 (100)	Pinobanksin-3- <i>O</i> -propionate ^c [23]
		599	555 (40), 437 (100), 255 (<1)	Unknown
		403	385 (57), 293 (100), 281 (25), 267 (31), 255 (61)	Unknown ^d
		433	415, 401 (48), 323 (13), 309 (100), 255 (3)	Unknown ^d
		461	443 (74), 419 (16), 401 (65), 351 (100), 291 (43), 253 (2)	Unknown ^d
26	53.3	369	285 (92), 267 (70), 252 (<1), 239 (100), 165 (<1)	Unknown ^d
27	54.8	269	254 (100), 251 (57), 165 (30)	Unknown ^d
28	57.6	341	271 (4), 253 (100)	Pinobanksin-3- <i>O</i> -butyrate or isobutyrate ^c [23]
		417	402 (16), 385 (8), 281, (89), 267 (100)	Unknown ^d
29	58.6	377	359 (35), 335 (21), 333 (44), 317 (60)	Unknown
		475	433 (31), 415 (100), 400 (10), 253 (2)	Unknown ^d
30	61.9	355	271 (3), 253 (100)	Pinobanksin-3- <i>O</i> -pentanoate or 2-methylbutyrate ^c [23]
31	64.3	403	297 (15), 283 (19), 271 (17), 253 (100)	Unknown ^d
		565	455 (8), 417 (17), 283 (100), 269 (25)	Unknown ^d
32	66.3	293	275 (16), 249 (8), 209 (7), 197 (39), 185 (100)	Fatty acid ^c [31]
		297	295 (70), 279 (100), 237 (24), 183 (21), 171 (36)	Fatty acid ^c [31]
33	67.5	471	423 (100), 405 (30), 393 (53)	Unknown
34	68.7	471	423 (100), 405 (30), 393 (53)	Unknown
35	72.4	339	183	Unknown

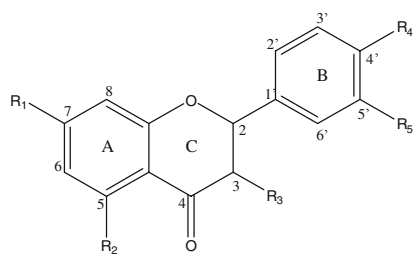
^a Confirmed with standard^b Confirmed with MSⁿ fragmentation^c Confirmed with references^d Compounds to be elucidated in the present study^e The numbers in parentheses denote the relative abundance of the respective fragment ion



Phenolic acids	R ₁	R ₂	R ₃
Caffeic acid (1)	OH	OH	OH
<i>p</i> -Coumaric acid (3)	H	OH	OH
Ferulic acid (4)	OH	OCH ₃	OH
Isoferulic acid (5)	OCH ₃	OH	OH
3,4-Dimethyl-caffeic acid (6)	OCH ₃	OCH ₃	OH
<i>p</i> -Coumaric acid methyl ester (8)	H	OH	OCH ₃
<i>p</i> -Coumaric acid isoprenyl ester (23)	H	OH	
Caffeic acid isoprenyl ester (15)	OH	OH	
Caffeic acid benzyl ester (16)	OH	OH	
Caffeic acid phenylethyl ester, CAPE (21)	OH	OH	
Caffeic acid cinnamyl ester (24)	OH	OH	



Flavones and Flavonols	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
Apigenin (11)	OH	H	OH	H	OH	H
Chrysin (19)	OH	H	OH	H	H	H
Chrysin-5-methyl-ether (12)	OH	H	OCH ₃	H	H	H
Kaempferol-5-methyl-ether (12)	OH	H	OCH ₃	OH	H	OH
Chrysin-6-methyl-ether (22)	OH	OCH ₃	OH	H	H	H



Flavanones and Dihydroflavonols	R1	R2	R3	R4	R5
Pinocembrin (17)	OH	OH	H	H	H
Pinocembrin-5-methyl-ether (10)	OH	OCH ₃	H	H	H
Hesperitin-5,7-dimethyl-ether (13)	OCH ₃	OCH ₃	H	OH	OCH ₃
Pinobanksin (9)	OH	OH	OH	H	H
Pinobanksin-5-methyl-ether (7)	OH	OCH ₃	OH	H	H
Pinobanksin-3- <i>O</i> -acetate (20)	OH	OH	OCOCH ₃	H	H
Pinobanksin-3- <i>O</i> -propionate (25)	OH	OH	OCOC ₂ H ₅	H	H
Pinobanksin-5-methyl-ether-3- <i>O</i> -pentanoate (26)	OH	OCH ₃	OCOC ₄ H ₉	H	H
Pinobanksin-3- <i>O</i> -butyrate or isobutyrate (28)	OH	OH	OCOC ₃ H ₇	H	H
Pinobanksin-3- <i>O</i> -pentanoate or 2-methylbutyrate (30)	OH	OH	OCOC ₄ H ₉	H	H
5-Methoxy-3-hydroxy-flavanone (27)	H	OCH ₃	OH	H	H

Fig. 2 Structures of several phenolic acids and flavonoids isolated from the Portuguese propolis. The numbers in parentheses denote the number of the fraction according to Fig. 1. Compounds corresponding to the fractions 12, 13, 26, and 27 were elucidated for the first time in this study

and Fig. 2). The comparison of the ESI-MS/MS data with that of standards and/or that described in the literature allowed the identification of caffeic acid (m/z 179, fraction 1), 3,4-dihydroxy vinylbenzene (m/z 135, fraction 2), *p*-coumaric acid (m/z 163, fraction 3), and ferulic and isoferulic acids (m/z 193, fractions 4 and 5). These phenolic compounds demonstrated a common fragmentation pattern, with a loss of the carboxyl group (CO₂, -44 Da). Besides

the simple phenolic acids, some esterified and/or methylated derivatives of these compounds were also found in the Portuguese propolis sample. These included five esters of caffeic acid, which are commonly found in high amounts in the phenolic extracts of propolis from temperate zones [2, 23]. Namely, the caffeic acid isoprenyl ester and its isomer (m/z 247, fractions 14 and 15, respectively), the caffeic acid benzyl ester (m/z 269, fraction 16), the caffeic acid

phenylethyl ester (m/z 283, fraction 21), and the caffeic acid cinnamyl ester (m/z 295, fraction 24). All the caffeic acid esters had a major fragment at m/z 179, which corresponds to the phenolic acid, and another product ion at m/z 135, resulting from the loss of CO_2 from the phenolic acid. These data are in accordance with the fragmentation pathways reported for caffeic acid esters derivatives [22]. Besides the esters derivatives, a methylated derivative of this phenolic acid, the 3,4-dimethyl-caffeic acid (DMCA), was identified in fraction 6 (ion at m/z 207). The MS^2 spectrum of this ion showed a fragment at m/z 163 and a main product ion at m/z 102 (-105 Da), resulting from the loss of CO_2 and of the two methoxy substituents together with the CO_2 group, respectively.

p-Coumaric acid esters were identified by comparison of the MS/MS data to that described in the literature [23]. This approach allowed detecting *p*-coumaric acid methyl ester (m/z 177, fraction 8) and *p*-coumaric acid isoprenyl ester (m/z 231, fraction 23). As for the caffeic acid esters discussed above, the MS/MS spectra of these ions had a fragment corresponding to the phenolic acid (ion at m/z 163) and other resulting from the loss of a CO_2 group from the phenolic acid (ion at m/z 119).

Flavonoids

The flavonoids that have been previously described in propolis and were also detected in this Portuguese extract were identified by comparison of the MS/MS data of the compound to that of the standards and/or by comparison to literature data. It is worthy to note that as the distinct classes of flavonoids differ in their pattern of substitution, which strongly influences the fragment pathway, the interpretation of MS/MS data provides specific structural information about the type of molecules. In this context, the analysis of the phenolic extract allowed the detection of dihydroflavonols, flavones, flavanones, and flavonols, either as free form or their methylated/esterified forms (Fig. 2). In particular, it was possible to identify the aglycones forms of apigenin (m/z 269, fraction 11), pinobanksin (m/z 271, fraction 9), pinocembrin (m/z 255, fraction 17) and chrysin (m/z 253 fraction 19), the esterified derivatives pinobanksin-3-*O*-acetate (m/z 313, fraction 20), pinobanksin-3-*O*-propionate (m/z 327, fraction 25), pinobanksin-2-*O*-butyrate or isobutyrate (m/z 341, fraction 28) and pinobanksin-3-*O*-pentanoate or 2-methylbutyrate (m/z 355, fraction 30) and the methylated derivatives pinobanksin-5-methyl-ether (m/z 285, fraction 7), pinocembrin-5-methyl-ether (m/z 269, fraction 10), chrysin-5-methyl-ether (m/z 267, fraction 12), and chrysin-6-methyl-ether (m/z 283, fraction 22). Still, it should be noted that the two peaks collected as fraction 10 were distinct, and no differences were noted in their MS analysis.

It is possible that isomers can be present, although further NMR analysis must be performed.

The MS^2 spectrum of many of these flavonoids (Table 1) revealed the fragments at m/z 151 or at m/z 165, which are resultant from the retro Diels-Alder mechanism [28]. Also, neutral losses commonly described to occur in these compounds [28], such as the small molecules CO (-28 Da), CO_2 (-44 Da), $\text{C}_2\text{H}_2\text{O}$ (-42 Da), as well as the successive losses of these molecules, were also observed. In accordance with Cuyckens et al. [28], methylated flavonoids presented a significant $[\text{M}-\text{H}-\text{CH}_3]^+$ product ion.

Attending to the HPLC-UV profile showed in Fig. 1, it is possible to suggest that this Portuguese propolis samples are mostly rich in pinocembrin (fraction 17), chrysin (fraction 19), and pinobanksin-3-*O*-acetate (fraction 20), and thus, this fits well with the phenolic profile of propolis from temperate zones [2]. Still, it should be noted that the typical flavone galangin was not found in this sample, which may suggest a peculiar characteristic of the Northeast Portuguese propolis.

New compounds

The phenolic compounds that were identified for the first time in propolis could be considered in two main groups: (a) methylated and/or esterified or hydroxylated derivatives of flavonoids already described in propolis, and thus denoted as derivatives of common flavonoids, and (b) those containing a much peculiar structure, derived from phenolic compounds never found in propolis and thus were denoted as rare phenolic compounds. The elucidation of the structural characteristics of these phenolic components was based on the detailed analysis of their MS/MS and MS^n data, together with some information on the fragmentation of reference compounds.

Derivatives of common flavonoids The phenolic compounds related to previously reported flavonoids corresponded to the $[\text{M}-\text{H}]^-$ at m/z 299 (fraction 12), at m/z 329 (fraction 13), at m/z 369 (fraction 26), and at m/z 269 (fraction 27) (Table 1). The MS^2 spectrum of the $[\text{M}-\text{H}]^-$ at m/z 299 showed an ion at m/z 284 (-15 Da), which can be attributed to the loss of a methyl group. This latter fragment was ascribed to kaempferol, since its MS^3 spectrum demonstrated a similar fragmentation pathway to that of the reference compound (ions at m/z 257 and m/z 151). Thus, the results suggested that the ion at m/z 299 is a methylated derivative of kaempferol. Most commonly, these substituents are linked to C-5 or C-7 of A-ring [29], but its exact position cannot be discriminated by MS analysis. Still, in accordance with Gardana et al. [23], the five-derivative flavonoids tend to elute before the aglycones in HPLC reversed-phase conditions, and that was the reason why this position is considered in the present study (Fig. 2).

The ESI-MS spectrum of fraction 13 showed a $[M-H]^-$ ion at m/z 329 (Table 1), and its MS^2 fragmentation indicated the loss of three methyl groups (product ions at m/z 314, 299, and 285). As the MS^3 spectrum of the ion at m/z 299 showed product ions at m/z 285 ($-CH_3$) and m/z 242 ($CH_3-CO_2^-$) that are consistent with the fragmentation pathway of the reference compound hesperitin, the results suggested that the phenolic compound in fraction 13 is a dimethylated derivative of hesperitin, probably linked to C-5 and C-7, as proposed in Fig. 2.

The major $[M-H]^-$ ion in fraction 26 appeared at m/z 369, and its MS^2 spectrum demonstrated a main peak at m/z 285 (Table 1), which corresponded to the loss of a pentanoate group (-84 Da) and an ion at m/z 267, that is in accordance with the structure of a methylated chrysin. As described by Gardana et al. [23] and also observable in Table 1 for fractions 20, 25, 28, and 30, chrysin is the main fragment ion of the esterified pinobanksins. Thus, these data suggested that the phenolic compound eluted in fraction 26 is pinobanksin-5-methyl-ether-3-pentanoate. This structure was reinforced by the MS^3 spectrum analysis of the ion at m/z 285, since this was similar to that of pinobanksin-5-methyl-ether (fraction 7).

Fraction 27 showed an ESI-MS spectrum with a main $[M-H]^-$ ion at m/z 269. The MS^2 spectrum of this ion had some similarities to that of pinocembrin-5-methyl-ether (m/z 269, fraction 10), but still, the ion eluted in peak 27 showed a fragment resultant from the loss of water (-18 Da, fragment at m/z 251), indicating that this compound must have an OH group that can be lost very

easily. Considering the high intensity of the ion at m/z 254 (-15 Da, generated from the methoxy group fragmentation), as also observed for the pinocembrin-5-methyl-ether, the methoxy group is proposed to be linked to position C-5. Moreover, the high abundance of the ion at m/z 251 (-18 Da) may be explained considering OH in position 3. In fact, the loss of H_2O leads to the formation of a double bond that is conjugated with the aromatic ring, promoting this fragmentation pathway and generating an abundant fragment.

Rare phenolic compounds These comprised three of the co-eluted compounds in fraction 25 ($[M-H]^-$ ions at m/z 403, m/z 433, and m/z 461), one co-eluted compound in fractions 28 and 29 ($[M-H]^-$ ions at m/z 461 and m/z 475, respectively), and two co-eluted compounds in fraction 31 ($[M-H]^-$ ions at m/z 403 and m/z 565). It should be noted that, in general, the molecular weight of these compounds are considerably higher than the phenolic compounds commonly found in temperate propolis [23].

The analysis of the fragmentation pattern of the mentioned $[M-H]^-$ ions revealed that, with the exception of the ion at m/z 565 in fraction 31, all the remaining compounds belong to the flavonoid family. These latter compounds embraced pinocembrin derivatives, since their MS/MS spectra showed an ion at m/z 255, correspondent to the $[M-H]^-$ ion of pinocembrin and pinobanksin derivatives, as these yielded a product ion at m/z 253, which is the $[M-H]^-$ ion of chrysin. As already described in the present study, chrysin is the main fragment ion of the esterified pinobanksins. Also, as discussed in detail in the following

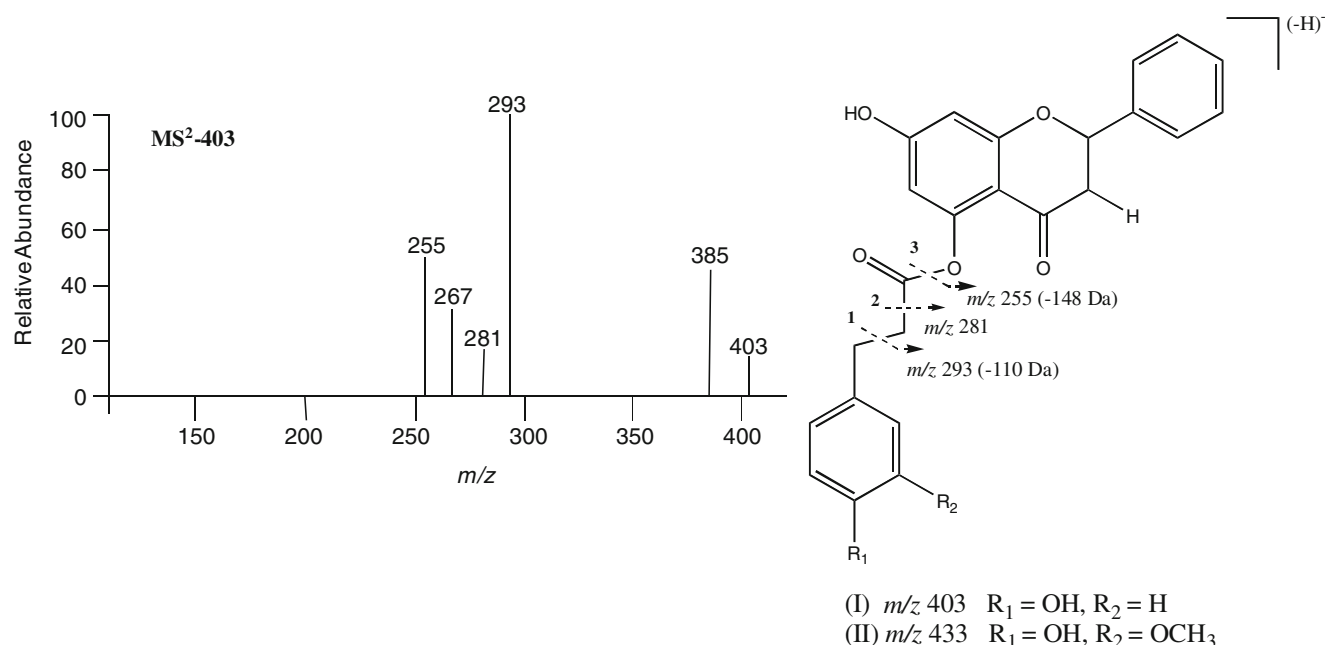


Fig. 3 ESI- MS^2 spectrum of the $[M-H]^-$ at m/z 403 detected in fraction 25. The tentative structure for this ion (I) and for ion at m/z 433 (II) is also shown. Some relevant fragments for the ion at m/z 403 are also represented in the figure

sections, the high molecular weight of these compounds, together with their fragmentation pathways under ESI-MS/MS, supported the hypothesis that these flavonoids are linked with the basic structures of phenolic acids, such as the phenylpropanoic acid moiety. To our knowledge, these structural characteristics were not previously described to occur in phenolic extracts from propolis or related products, although the flavanone derivative pinobanksin 3-cinnamate has been reported to occur in Cheilantheid ferns [30].

The MS analysis of the $[M-H]^-$ signals at m/z 403 and m/z 433 of fraction 25 demonstrated a similar fragmentation pattern, suggesting that they have related structures. The MS² spectrum of the ion at m/z 403 showed an ion at m/z 255 (Table 1 and Fig. 3), the latter attributed to pinocembrin,

as confirmed by its MS³ fragmentation (ions at m/z 213 and m/z 151). These results indicated that the phenolic compound of the $[M-H]^-$ ion at m/z 403 is a pinocembrin derivative. As the ion corresponding to pinocembrin (at m/z 255) was formed by the loss of 148 Da ($-C_9H_8O_2$) from the $[M-H]^-$ (cleavage at position 3 denoted on the structure I of Fig. 3), this result suggested that a 4-hydroxyphenylpropanoid group is present in the molecule. This hypothesis was also supported by the presence in the MS² spectrum of a main product ion at m/z 293, formed by the loss of 110 Da. This latter ion can result from the cleavage of the hydroxyphenylpropanoid in the β -position relative to the carboxylic group, as represented in the structure I of Fig. 3 (position 1). It should be noted that, although the substituent is

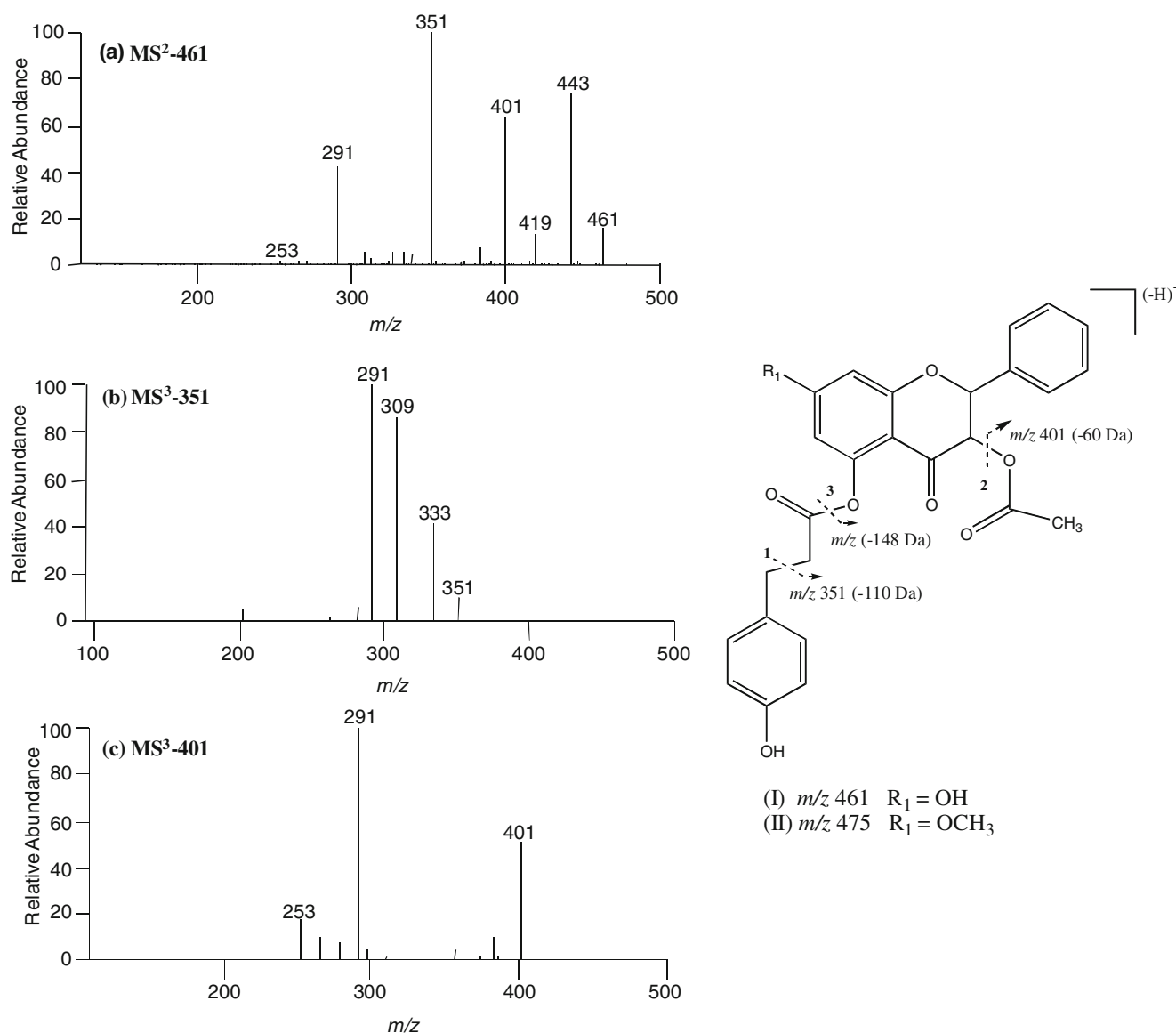


Fig. 4 ESI-MSⁿ spectra of the $[M-H]^-$ at m/z 461 detected in fraction 25. **a** ESI-MS² spectrum, **b** ESI-MS³ spectrum of the ion at m/z 351, **c** ESI-MS³ spectrum of the ion at m/z 401. The tentative structure for

this ion (*I*) and for ion at m/z 475 (*II*) is also shown. Some relevant fragments for the ion at m/z 461 are also marked in the figure

represented in the position C-5 of the flavanone structure, this can be arbitrarily located in C-5 or C-7. The elucidation of its precise position was not achieved through the MS technique and would require the NMR analysis of the compound.

The MS² spectrum of the ion at m/z 433 also showed a fragment at m/z 255 and a major product ion at m/z 309 (Table 1). As described above, the former fragment corresponded to pinocembrin, and the latter, which resulted from the loss of 124 Da, can be justified by the cleavage of a 3-methoxy-4-hydroxyphenylpropanoid substituent in the β -position relative to the carboxylic group, like in the ion at m/z 403. The presence of a methoxy group in the molecule was also confirmed by the ion peak at m/z 401 (-32 Da). The tentative structure of this compound is also represented in Fig. 3.

Fraction 25 showed other unknown phenolic compound, with a molecular ion at m/z 461 (Table 1). As for the $[M-H]^-$ ion at m/z 403 discussed above, the MS² spectrum of this ion (Fig. 4a) showed a fragment corresponding to the loss of 110 Da (ion at m/z 351), which can be justified by the cleavage at position 1 (structure I in Fig. 4) and thus suggesting that a phenylpropanoid group is linked to C-5 or C-7. The fragmentation pattern of the signal at m/z 461 also

demonstrated a strong ion at m/z 401 (-60 Da), which is consistent with the loss of an acetate group. Considering that the loss of a C-3 substituent is usually the base peak in a spectrum (as discussed above), it is probable that the acetate group of this molecule is linked at that position. The presence of an acetate group in the C-3 was also supported by the analysis of the MS³ spectrum of the ion at m/z 351 (Fig. 4b) that showed a main loss of the acetate group (-60 Da, ion at m/z 291). The general structure of this compound was also supported by the MS³ spectrum of the ion at m/z 401 (Fig. 4c), which demonstrated fragments resulting from the losses of 110 (ion at m/z 401) and 148 Da (ion at m/z 253), consistent with cleavages at positions 1 and 3, as denoted in Fig. 4. Moreover, the MS³ data of the ion at m/z 253 was in agreement with the structure of chrysin (fragments at m/z 209 and m/z 151), and thus, this compound can be considered a derivative of pinobanksin.

The MS² data of the ion $[M-H]^-$ at m/z 475 eluted in fraction 29 (Table 1) was consistent with a methylated derivative of the ion at m/z 461 (Fig. 4). The ion showed fragments at m/z 433 (-42 Da), a main fragment ion at m/z 415 (-60 Da) corresponding to the loss of the acetate group, and a fragment at m/z 400 (-75 Da), which can be

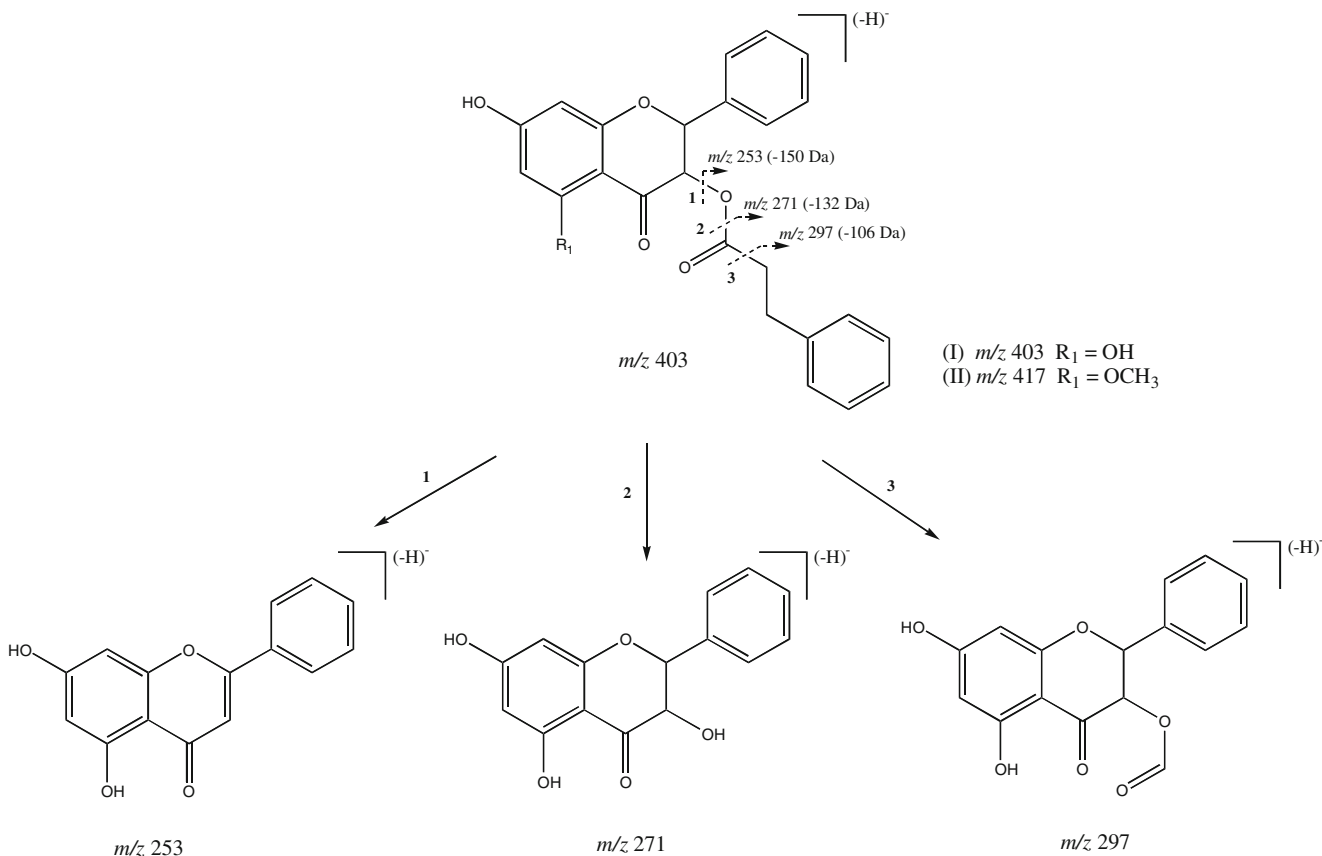


Fig. 5 Tentative structure for the ions $[M-H]^-$ at m/z 403 (I) and 417 (II), detected in fractions 28 and 31, respectively. Proposed scheme for fragmentation of the $[M-H]^-$ at m/z 403 (I)

justified by the loss of the acetate and the methyl groups. In opposition to the methylated compound discussed above (ion at m/z 433), the methyl group of this molecule was considered to be linked to C-7, because there was no evidence of a methoxylated hydroxyphenylpropanoid substituent in the molecule (fragment resulting from the loss of 124 Da). This was also supported by the MS³ data of the ion at m/z 415, which showed a major fragment at m/z 400 (–15 Da).

The fragmentation pattern of the ion at m/z 403 eluted in fraction 31 was different from that of the ion at m/z 403 in fraction 25. In particular, the MS/MS spectrum of the former phenolic compound showed a major fragment ion at m/z 253 (Table 1), ascribed to chrysin according to its MS³ data. Additionally, the fragment ion at m/z 253 (–150 Da) may result from the loss of a phenylpropanoic acid, as shown in Fig. 5. The presence of phenylpropanoic acid in the molecule was also supported by the fragment ions at m/z 271 (–132 Da) and at m/z 297 (–106 Da), since they can correspond to a cleavage at positions 2 and 3, respectively. In this case, the substitution is proposed to

be at C-3 due to the high intensity of the fragment at m/z 253. This product ion was also the base peak in the MS³ spectrum of the ion at m/z 271 (data not shown).

As observed in Table 1, the major fragment of the ion at m/z 417 eluted in fraction 28 also resulted from the loss of 150 Da (ion at m/z 267), suggesting the presence of a phenylpropanoic acid moiety on the C-3 position of the flavonoid. Moreover, the molecule should contain a methoxy group at C-5 or C-7, as inferred by the fragment ion at m/z 385 (–32 Da). In conclusion, these results indicated that the phenolic compound correspondent to the molecular ion at m/z 417 is a methylated derivative at C-5 or C-7 position of the ion at m/z 403 (fraction 31), as represented in Fig. 5.

The MS/MS analysis of the other compound eluted in fraction 31 (ion at m/z 565) showed a main fragment at m/z 283 (–282 Da), suggesting that this compound is a dimeric species (Fig. 6a). Also, the MS/MS product ions at m/z 455 (–110 Da), at m/z 443 (–122 Da), and m/z 417 (–148 Da) indicated the presence of 4-hydroxyphenyl propanoic acid in the structure, as already described in this study

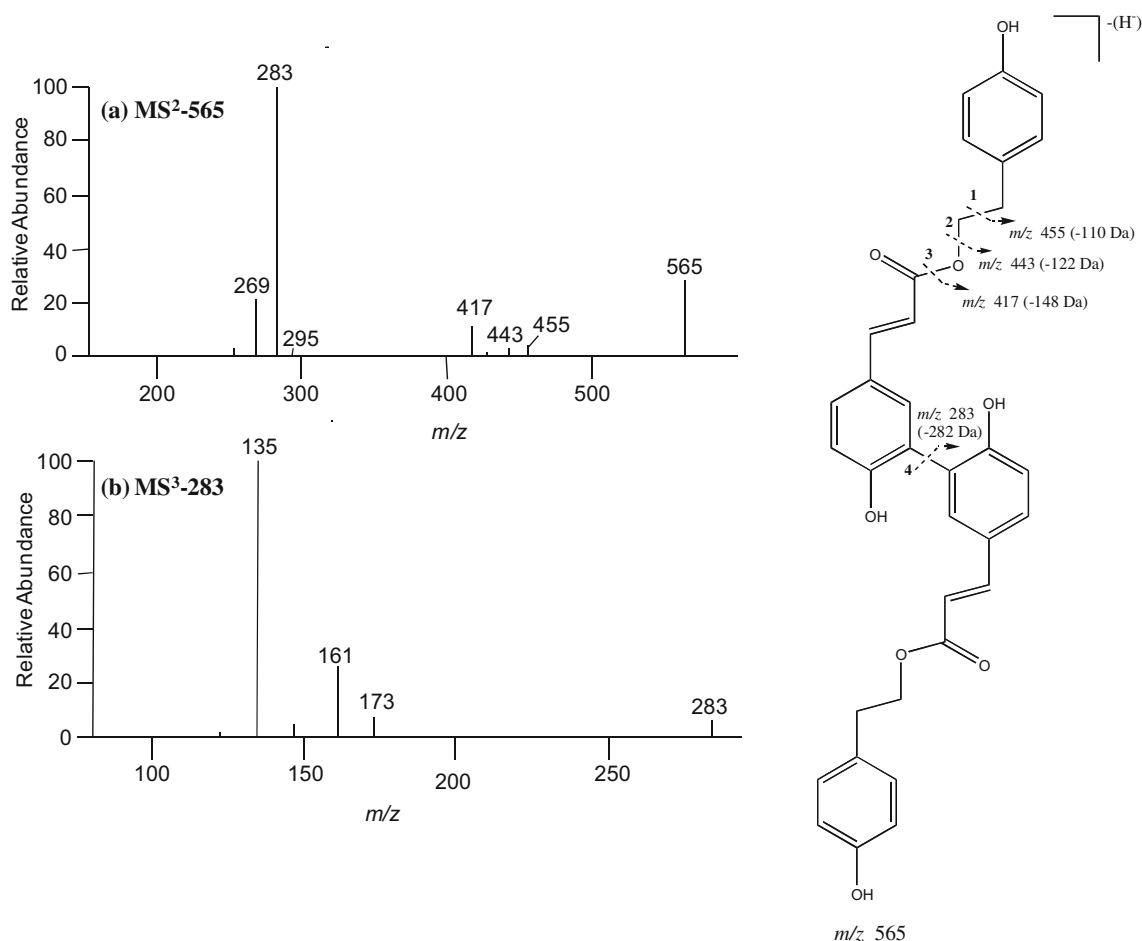


Fig. 6 ESI–MSⁿ spectra of molecular ion at m/z 565 detected in fraction 31. **a** ESI–MS² spectrum, **b** ESI–MS³ spectrum of the ion at m/z 283. The tentative structure of the compound is also shown

(cleavages at positions 1, 2, and 3 denoted in Fig. 6). The presence of this acid in the dimeric structure was also supported by the fragment at m/z 269 (correspondent to the loss of two fragments of 148 Da) and by the analysis of MS³ spectrum of the ion at m/z 283 (Fig. 6b). This latter showed, once again, fragments resultant from the losses of 110 (ion at m/z 173), 122 (ion at m/z 161), and 148 Da (ion at m/z 135). Thus, the analysis of the fragmentation data of the $[M-H]^-$ at m/z 565 suggested that the compound is a *p*-coumaric ester derivative dimer.

Conclusions

The present work allowed to characterize the phenolic compounds of Portuguese propolis for the first time. The most abundant compounds in this matrix were those commonly found in propolis of the temperate zones. Still, the Portuguese propolis also contained several new phenolic components, namely, some methylated and/or sterified or hydroxylated derivatives of flavonoids already described in propolis and also rare pinocembrin or pinobanksin derivatives that contain basic structures of phenolic acids. It is probable that these groups are linked to the positions C-5, C-7, or C-3 of the flavonoid skeleton, although their precise position would only be elucidated by NMR analysis. Moreover, it was possible to detect the presence of a *p*-coumaric ester derivative dimer (MW 566 Da) in this sample.

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