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Phenolic Profiling of Portuguese Propolis by LC–MS Spectrometry: Uncommon Propolis Rich in Flavonoid Glycosides

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ABSTRACT:

Introduction – Propolis is a chemically complex resinous substance collected by honeybees (*Apis mellifera*) from tree buds, comprising plant exudates, secreted substances from bee metabolism, pollen and waxes. Its chemical composition depends strongly on the plant sources available around the beehive, which have a direct impact in the quality and bioactivity of the propolis. Being as Portugal is a country of botanical diversity, the phenolic characterisation of propolis from the different regions is a priority.

Objective – Extensive characterisation of the phenolic composition of Portuguese propolis from different continental regions and islands.

Method – Forty propolis ethanolic extracts were analysed extensively by liquid chromatography with diode-array detection coupled to electrospray ionisation tandem mass spectrometry (LC–DAD–ESI–MS^n).

Results – Seventy-six polyphenols were detected in the samples and two groups of propolis were established: the common temperate propolis, which contained the typical poplar phenolic compounds such as flavonoids and their methylated/esterified forms, phenylpropanoid acids and their esters, and an uncommon propolis type with an unusual composition in quercetin and kaempferol glycosides – some of them never described in propolis.

Conclusion – The method allowed the establishment of the phenolic profile of Portuguese propolis from different geographical locations, and the possibility to use some phenolic compounds, such as kaempferol-dimethylether, as geographical markers. Data suggest that other botanical species in addition to poplar trees can be important sources of resins for Portuguese propolis.

Keywords: Mass spectrometry; flavonoid glycosides; flavonoids; phenolic compounds; propolis

Introduction

Amongst all bee products, propolis is one of the most complex and, at the same time, very fascinating. Propolis is the name given to an extremely sticky, resinous substance, collected from various floral sources, transformed and used by the honeybees (*Apis mellifera* L.) to seal holes in their honeycombs, smooth out the internal walls and protect the entrance against intruders. The origin of the word propolis comes from the Greek: *pro-* standing for 'in defense', and *polis-* meaning 'the city', that is, defense of the city (or the hive) (Burdock, 1998).

Propolis acts as a strong biocide within the hive, being responsible for the low incidence of bacteria and fungi (Marcucci, 1995). This bee product has been widely used in folk medicine since ancient times and recently has gained popularity all over the world as an important ingredient of healthy foods and cosmetics. Propolis is thought to improve human health and to prevent diseases such as inflammation, heart disease, diabetes and even cancer (Banskota *et al*., 2001).

Frequently referred to as 'bee glue', it is mainly composed of resin (phenolic compounds) but also contains significant amounts of vegetable balsam, wax, essential oils and pollen. Other organic compounds such as amino acids, vitamins, mineral salts and insoluble debris are found as residual components (Marcucci, 1995; Burdock, 1998). The variability of propolis chemical composition is strongly dependent on the plant sources available around the hive and on the geographical and climatic conditions, although bees show a preference for specific resin sources (Bankova *et al*., 2000). In Europe, North America and other temperate zones the main resin sources are the exudates of apical buds of *Populus* species, in particular those from *Populus nigra* (Marcucci, 1995). Propolis from these regions shows similar phenolic composition, with the main compounds being flavonoids (pinocembrin,

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pinobanksin, chrysin and galangin), phenolic acids and their esters (Bankova *et al.*, 2000; Falcão *et al.*, 2010). Different phenolic profiles were found in propolis from Canary Islands, in which furofuran lignans were the main compounds (Bankova *et al.*, 2000). Moreover, terpenes were the major components in propolis from Mediterranean Sea areas, the origin of which was most probably the Cupressaseae family and the species *Ferula communis* (Popova *et al.*, 2009, 2011). In tropical areas, particularly in Brazil, propolis originating from *Baccharis dracunculifolia* (green propolis) presented a composition rich in prenylated phenylpropanoids and in caffeoyl quinic acids, whereas propolis from Cuba, Venezuela and some areas in Brazil, arising from flowers of *Clusia* spp., were shown to be rich in prenylated benzophenone derivatives (Bankova *et al.*, 2000). Chen *et al.* (2003) have also identified a new family of compounds in propolis samples from Taiwan, the C-prenylflavonoids (or propolins).

In recent years, different analytical approaches have been used for phenolic characterisation of propolis from all over the world (Bankova *et al.*, 2002; Popova *et al.*, 2004; Watson *et al.*, 2006) and of these, the most common technique of choice by far is liquid chromatography coupled with mass spectrometry (LC–MS) (Volpi and Bergonzini, 2006; Gardana *et al.*, 2007; Falcão *et al.*, 2010; Pellati *et al.*, 2011). LC–MS is a powerful tool for the analysis of natural products. The high sensitivity of the MS analytical approach provides the potential for discovery of new minor constituents, which are difficult to obtain by conventional means. More detailed structural information can be obtained by resorting to tandem mass spectrometry (MS/MS), which allows the characterisation of unknown compounds even without reference to standards (Cuyckens and Claeys, 2004).

In our previous work (Falcão *et al.*, 2010), the phenolic compounds of northeast Portuguese propolis were studied by the combined off-line analysis of HPLC and electrospray ionisation (ESI) MS in the negative ion mode. This approach allowed the characterisation of 37 phenolic compounds, which included the typical phenolic acids and flavonoids found in propolis from temperate zones, but also new methylated, esterified and/or hydroxylated derivatives of common poplar flavonoids and pinocembrin/pinobanksin derivatives containing a phenylpropanoic acid derivative moiety in their structure.

More recent works on Portuguese propolis from other regions of Portugal (Miguel *et al.*, 2010; unpublished results) revealed, however, the presence of propolis diversity, observed through differences in the physicochemical parameters and in total phenolic content. This is consistent with the botanical diversity of the country, and the need that bees have for search alternative resin sources in regions where poplar is not present. Our recent work in the study of propolis from distinct Portuguese continental regions and islands clearly identified the existence of different types of propolis, based on the distinct profile of physicochemical parameters identified (unpublished results). The phenolic characterisation of propolis from the different regions is therefore a priority.

In this article we present the results of an extensive study on the phenolic profile of distinct Portuguese continental regions and islands, performed by liquid chromatography with diode-array detection coupled to electrospray ionisation tandem mass spectrometry (LC–DAD–ESI–MS^n), in an attempt to establish the Portuguese propolis phenolic profile. A propolis type with an unusual composition of flavonoid glycosides is described herein.

Experimental

Chemicals and reagents

Chrysin, galangin, quercetin, pinocembrin, naringenin, hesperetin, ellagic acid, benzoic acid, cinnamic acid, caffeic acid, ferulic acid, isoferulic acid, *p*-coumaric acid, caffeic acid isoprenyl ester, caffeic acid phenylethyl ester (CAPE) and quercetin-3-*O*-rhamnoside were purchased from Sigma Chemical Inc. (St Louis, MO, USA). Apigenin, acacetin, kaempferol, chrysoeriol, chrysin-7-methylether, chrysin-5,7-dimethyl ether, pinocembrin-7-methyl ether, pinocembrin-5,7-dimethyl ether, 3,4-dimethyl-caffeic acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-neohesperidoside, isorhamnetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside were from Extrasynthese (Genay, France). Isorhamnetin and *p*-coumaric acid methyl ether were from Phytolab (Vestenbergsgreuth, Germany). Genkwanin and luteolin were obtained from Lancaster Synthesis (Morecambe, England). Analytical grade formic acid and HPLC grade ethanol were obtained from Panreac (Barcelona, Spain). HPLC-grade methanol and acetonitrile were purchased from Lab-Scan (Lisbon, Portugal). Water was treated in a Milli-Q water purification system (Topway Global Inc.,Houston, TX, USA).

Propolis samples

This work was performed with 40 Portuguese propolis samples. As shown in Fig. 1, the samples were collected from continental north (N), central interior (CI), central coast (CC), south (S), Azores archipelago (A) and from Madeira island (M). Table 1 shows the general status of propolis samples, the year of collection and geographical sites, as well as the corresponding collection method. All the samples were obtained after the honey harvesting season (July–September), by conventional scraping or through plastic screens. These propolis samples were then stored at -20°C until analysis.

Phenolic compounds extraction

The extraction procedure was made according to our previously work (Falcão *et al.*, 2010). The raw propolis samples (1 g) were first grounded,

Figure 1. Propolis sampling sites.

Table 1. Propolis samples description

Code	Geographical location	Year	Collection method[a]	Propolis type
N1	Bragança	2007	1	Common
N2	Bragança	2007	2	Common
N3	Bragança	2008	2	Common
N4	Bragança	2009	1	Common
N5	Bragança	2009	1	Common
N6	Bragança	2009	1	Common
N7	Miranda do Douro	2009	1	Common
N8	Mirandela	2009	2	Common
N9	Chaves	2009	2	Common
N10	Chaves	2009	2	Common
N11	Montalegre	2009	1	Uncommon
N12	Boticas	2009	1	Uncommon
N13	Boticas	2009	1	Uncommon
N14	Barcelos	2010	1	Common
CI1	Guarda	2009	1	Uncommon
CI2	Penamacor	2009	1	Common
CI3	Fundão	2009	1	Common
CI4	Nisa	2009	2	Common
CC1	Figueira da Foz	2009	2	Common
CC2	Leiria	2009	1	Common
CC3	Coruche	2009	2	Common
CC4	Ramada	2009	1	Common
S1	Aljezur	2009	1	Common
S2	Aljezur	2009	1	Common
S3	Aljezur	2009	1	Common
S4	Moncarapacho	2009	1	Uncommon
A1	Terceira Island, Azores Archipelago	2009	1	Common
A2	S. Miguel Island, Azores Archipelago	2009	1	Common
A3	S. Miguel Island, Azores Archipelago	2009	1	Common
A4	S. Miguel Island, Azores Archipelago	2009	1	Common
A5	S. Miguel Island, Azores Archipelago	2009	1	Common
A6	S. Miguel Island, Azores Archipelago	2009	1	Common
A7	S. Miguel Island, Azores Archipelago	2009	1	Common
A8	S. Miguel Island, Azores Archipelago	2009	1	Common
A9	S. Miguel Island, Azores Archipelago	2009	1	Common
A10	S. Miguel Island, Azores Archipelago	2009	1	Common
A11	S. Miguel Island, Azores Archipelago	2009	1	Common
M1	Funchal, Madeira Island	2009	1	Common
M2	Funchal, Madeira Island	2009	1	Uncommon
M3	Funchal, Madeira Island	2009	1	Common

[a]1, conventional scraping; 2, plastic screen.

homogenised and then mixed with 80% ethanol (10 mL) and kept at 70 °C for 1 h. The resulting mixture was filtered and the residue was re-extracted in the same conditions. After the second extraction, the filtered solution was combined, concentrated, frozen at −20°C and freeze-dried.

LC–DAD–ESI–MS^n analysis

The LC–DAD–ESI–MS^n analyses were performed on a Finnigan Surveyor Plus HPLC instrument equipped with a DAD and coupled to a MS. The chromatographic system consisted of a quaternary pump, an autosampler, a degasser, a photodiode-array detector and an automatic thermostatic column compartment. The HPLC was run on a Macherey-Nagel Nucleosil C_{18}-column (250 mm × 4 mm i.d.; 5 μm particle diameter, end-capped) and its temperature was maintained at 25 °C. The mobile phase was composed of (A) 0.1% (v/v) formic acid in water, and (B) acetonitrile, which were previously degassed and filtered. 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 and return to the initial conditions. For the HPLC analysis, the freeze-dried extract (10 mg) was dissolved in 1 mL of 80% of ethanol. All samples were filtered through a 0.2 μm Nylon membrane (Whatman). The flow rate was 1 mL/min and split out 200 μL/min to MS. Spectral data for all peaks were acquired in the range 200–600 nm.

The MS used was a Finnigan Surveyor LCQ XP MAX quadrupole ion trap MS equipped with an ESI source. Control and data acquisition were carried out with the Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA). Nitrogen above 99% purity was used and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode, with ESI needle voltage set at 5.00 kV and the ESI capillary temperature at 325 °C. The full scan covered

the mass range from *m/z* 50 to 1000. The MS^n data were simultaneously acquired for the selected precursor ion. The collision induced decomposition (CID)–MS–MS and MS^n experiments were performed using helium as the collision gas, with a collision energy of 25–40 eV.

Results and discussion

The LC–DAD–ESI–MS^n study of the 40 Portuguese propolis samples allowed the elucidation of phenolic compounds by comparison of their chromatographic behaviour, UV spectra and MS information with reference compounds. When standards were not available, the structural information was confirmed with UV data combined with MS fragmentation patterns previously reported in the literature. This study was carried out using LC–MS in the negative ion mode because of its higher sensitivity in the analysis of the different polyphenol classes (Cuyckens and Claeys, 2004).

Overall, the Portuguese propolis samples could be arranged in two distinct groups (Table 1): (i) common temperate propolis type, which contained, for example, the typical poplar phenolic compounds such as the main phenolic components; and (ii) uncommon temperate propolis type, which in addition to the typical poplar flavonoids, also contained significant amounts of unusual flavonoid glycosides. Figure 2 shows the representative chromatographic profile at 280 nm for the two types of propolis found. The phenolic composition of the two groups is detailed below.

Common temperate propolis type

Overall, more than 85% of the Portuguese propolis samples under study were identified as common temperate propolis with a common phenolic matrix (Falcão *et al.*, 2010). This group included all the samples from the central coast and Azores archipelago, as well as the majority of samples from the north, and some from the central interior, south and Madeira. It is important, nevertheless, to note that some of the samples included in this group showed deviations to the typical phenolic profile of temperate regions. In particular, all samples from the central coast, samples N11–N13 from the north, samples S2–S3 from the south and sample M3 from Madeira contained an additional flavonol, the kaempferide. Moreover, samples CI2–CI4 from central interior and S2–S4 (south) were poor in pinobanskin derivatives but contained a kaempferol-dimethyl ether, which is not typically observed in the temperate propolis profile. Sample M1 from Madeira exhibits a small number of phenolic compounds.

In general, the analytical approach used in the present study allowed the identification of 62 compounds in common temperate propolis type samples (Table 2). These included the 37 phenolic compounds previously reported (Falcão *et al.*, 2010) plus (i) 19 new confirmed phenolic compounds (6, 9, 11, 12, 18, 19, 22–24, 26–28, 36–38, 44, 47, 54, 62) and (ii) six new compounds (46, 49, 55, 56, 59, 60) for which the structure will be partially elucidated herein.

In addition to our previous results, benzoic acid (molecular weight (MW) 122 Da) has now been detected as a minor peak in all samples (compound 6 at 23.5 min), and cinnamic acid (*m/z* 147, compound 11) was found in most of the samples. Note

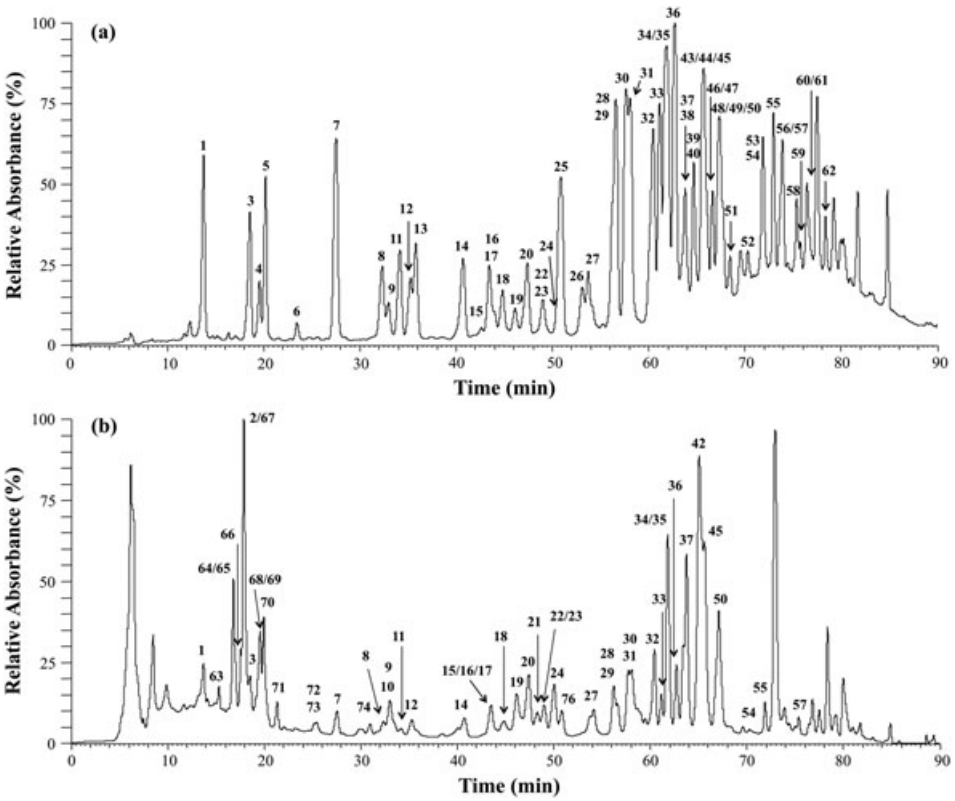


Figure 2. Representative chromatographic profile at 280 nm of ethanolic propolis extracts: (a) common temperate propolis type; (b) uncommon propolis type.

Table 2. Characterisation of the phenolic compounds by LC–DAD–ESI–MS^n from Portuguese propolis

Number	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	HPLC–ESI–MS^n *m/z* (% base peak)	Compound
1	13.7	292, 322	179, $[M+46]^-$: 225		Caffeic acid[a,b]
2	18.1	253, 367	301	MS^2[301]: 301 (100), 257 (77), 229 (96)	Ellagic acid[a,b]
3	18.5	310	163, $[M+46]^-$: 208		*p*-Coumaric acid[a,b]
4	19.5	295sh, 322	193, $[M+46]^-$: 238		Ferulic acid[a,b]
5	20.2	298, 319	193, $[M+46]^-$: 238		Isoferulic acid[a,b]
6	23.5	229	121, $[M+46]^-$: 166		Benzoic acid[a,b]
7	27.5	295sh, 322	207, $[M+46]^-$: 253		3,4-Dimethyl-caffeic acid[a,b]
8	32.3	286	285	MS^2[285]: 267 (100), 252 (13), 239 (27)	Pinobanksin-5-methyl ether[b,c]
9	32.9	256, 370	301	MS^2[301]: 179 (100), 151 (60)	Quercetin[a,b]
10	33.0	253, 268sh, 349	285	MS^2[285]: 285 (100), 267 (54), 241 (63), 175 (52)	Luteolin[a,b]
11	34.1	277	147, $[M+46]^-$: 193	MS^2[147]: 103	Cinnamic acid[a,b]
12	35.2	256, 355	315	MS^2[315]: 300; MS^3[300]: 271 (100), 255 (59), 151 (< 1)	Quercetin-3-methyl ether[b,d]
13	35.7	307	177, $[M+46]^-$: 223		*p*-Coumaric acid methyl ester[a,b]
14	40.6	292	271	MS^2[271]: 253 (100), 225 (26), 151 (10)	Pinobanksin[b,c]
15	42.5	286	269	MS^2[269]: 255 (48), 227 (100), 165 (30)	Pinocembrin-5-methyl ether[b,c]
16	43.3	268, 337	269	MS^2[269]: 225 (100), 151 (29)	Apigenin[a,b]
17	43.3	268, 313	267	MS^2[267]: 253 (100), 224 (25)	Chrysin-5-methyl ether[b,c]
18	44.6	265, 364	285	MS^2[267]: 285 (100), 257 (13), 151 (20)	Kaempferol[a,b]
19	45.9	253, 370	315	MS^2[315]: 300	Isorhamnetin[a,b]
20	47.2	265, 352	299	MS^2[299]: 284	Kaempferol-methyl ether[b,c]
21	48.4	265, 340	329	MS^2[329]: 314; MS^3[314]: 299 (100)	Kaempferol-methoxy-methyl ether[b]
22	48.9	253, 355	329	MS^2[329]: 314; MS^3[314]: 299 (48), 285 (100), 271 (78), 243 (28)	Quercetin-dimethyl ether[b,e]
23	48.9	289	327	MS^2[327]: 285 (100), 267 (18), 239 (31)	Pinobanksin-5-methyl ether-3-*O*-acetate[b,d]
24	49.9	256, 349	359	MS^2[359]: 344; MS^3[344]: 329 (100), 301 (35), 179 (< 1), 151 (< 1)	Quercetin-tetramethyl ether[b]
25	50.7	310	173, $[M+46]^-$: 219		Cinnamylidenacetic acid[b,c]
26	53.0	265, 300sh, 352	283	MS^2[283]: 268 (100), 239 (60), 211 (10)	Galangin-5-methyl ether (isomer)[b,e]
27	53.5	256, 367	315	MS^2[315]:300 (34), 193 (76), 165 (100)	Rhamnetin[b,f]
28	56.1	256, 355	329	MS^2[329]: 314; MS^3[314]: 299; MS^4[299]: 271 (100), 151 (< 1)	Quercetin-dimethyl ether[b,e]
29	56.5	298, 325	247	MS^2[247]:179 (100), 135 (15)	Caffeic acid isoprenyl ester[a,b]
30	57.5	298, 325	247	MS^2[247]:179 (100), 135 (15)	Caffeic acid isoprenyl ester (isomer)[b,c]
31	58.1	298, 325	269	MS^2[269]: 178 (100), 134 (32), 161 (12)	Caffeic acid benzyl ester[b,c]
32	60.3	289	255	MS^2[255]: 213 (100), 211 (32), 151 (48)	Pinocembrin[a,b]
33	61.1	295, 325	283	MS^2[283]: 179 (100), 135 (28)	Caffeic acid phenylethyl ester[a,b]
34	61.7	268, 313	253	MS^2[253]: 225 (17), 209 (100), 151 (5)	Chrysin[a,b]
35	61.7	292	313	MS^2[253]: 271 (18), 253 (100)	Pinobanksin-3-*O*-acetate[b,c]
36	62.5	265, 300sh, 358	269	MS^2[269]: 269 (100), 241 (61), 227 (20), 197 (22), 151 (20)	Galangin[a,b]
37	63.3	268, 331	283	MS^2[283]: 269	Acacetin[a,b]
38	63.9	265, 364	299	MS^2[299]: 284, 151 (< 1); MS^3[284]: 151	Kaempferide[b,d]
39	64.6	265, 300sh, 350sh	283	MS^2[283]: 269	6-Methoxychrysin[b,c]
40	64.8	294, 310	231	MS^2[231]: 163 (100), 119(12)	*p*-Coumaric acid isoprenyl ester[b,c]

(Continues)

Table 2. *(Continued)*

Number	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	HPLC–ESI–MS^n *m/z* (% base peak)	Compound
41	64.6	292	461	MS^2[461]: 443 (68), 401 (75), 351 (100), 291 (55), 253 (2)	Pinobanksin-3-*O*-acetate-5-*O*-*p*-hydroxyphenylpropionate[b,c]
42	65.1	250, 268sh, 343	313	MS^2[313]: 298; MS^3[298]: 283 (100), 270 (20), 255 (7); MS^4[283]: 255 (100), 227 (4)	Chrysoeriol-methyl ether[b]
43	65.6	295, 324	295	MS^2[295]: 178 (100), 134 (24)	Caffeic acid cinnamyl ester[b,c]
44	65.6	294, 310	231	MS^2[231]: 163 (100), 119(12)	*p*-Coumaric acid isoprenyl ester (isomer)[b,e]
45	65.6	295	433	MS^2[433]: 415 (3), 401 (31), 323 (15), 309 (100)	Pinocembrin-5-*O*-3-hydroxy-4-methoxyphenylpropionate[b,c]
46	65.6	265, 346	313	MS^2[313]: 299 (10), 298 (100); MS^3[298]: 283 (100), 269 (8), 255 (41), 241 (2); MS^4[283]: 255 (100), 151 (< 1)	Kaempferol-dimethyl ether[b]
47	66.5	295, 324	295	MS^2[295]: 178 (100), 134 (24)	Caffeic acid cinnamyl ester (isomer)[b]
48	67.2	289	327	MS^2[327]: 271 (9), 253 (100)	Pinobanksin-3-*O*-propionate[b,c]
49	67.2	298, 322	419	MS^2[419]: 313 (33), 299 (100), 193 (66); MS^3[299]: 193	Ferulic acid derivative[b]
50	67.2	289	369	MS^2[369]: 285 (53), 267 (65), 239 (100)	Pinobanksin-5-methyl ether-3-*O*-pentanoate[b,c]
51	68.4	292	475	MS^2[475]: 433 (9), 415 (100), 400 (8), 253 (< 1)	Pinobanksin-7-methyl ether-5-*O*-*p*-hydroxyphenylpropionate[b,c]
52	70.2	289	269	MS^2[269]: 254 (100), 251 (54), 165 (22)	3-Hydroxy-5-methoxyflavanone [b,c]
53	71.9	292	341	MS^2[341]: 271 (5), 253 (100)	Pinobanksin-3-*O*-butyrate or isobutyrate[b,c]
54	71.9	292	353	MS^2[353]: 271 (7), 253 (100)	Pinobanksin-3-*O*-pentenoate[b,c]
55	72.9	292, 322	399	MS^2[399]: 355 (39), 179 (100), 135 (50)	Caffeic acid derivative[b]
56	73.9	292, 322	399	MS^2[399]: 355 (39), 179 (100), 135 (50)	Caffeic acid derivative (isomer)[b]
57	73.9	289, 345	565	MS^2[565]: 455 (10), 417 (36), 283 (100), 269 (43)	*p*-Coumaric acid-4-hydroxyphenylethyl ester dimer[b,c]
58	75.3	292	355	MS^2[355]: 271 (5), 253 (100)	Pinobanksin-3-*O*-pentanoate or 2-methylbutyrate[b,c]
59	76.2	292	367	MS^2[367]: 271 (100), 253 (45); MS^3[271]: 253 (100), 225 (27), 151 (12)	Pinobanksin-*O*-hexenoate[b]
60	76.4	292, 322	315	MS^2[315]: 179 (100), 135 (8)	Caffeic acid derivative[b]
61	76.4	292	403	MS^2[403]: 271 (16), 253 (100)	Pinobanksin-3-*O*-phenylpropionate[b,c]
62	78.3	292	369	MS^2[369]: 271 (14), 253 (100)	Pinobanksin-3-*O*-hexanoate[b,d]

[a]Confirmed with standard;
[b]confirmed with MS^n fragmentation;
[c]confirmed with references: Falcão *et al.* (2010);
[d]Gardana *et al.* (2007);
[e]Pellati *et al.* (2011);
[f]Justesen (2001).

that in this study, these acids (as well as the remaining simple acids in the propolis extracts) were mainly detected in the LC–MS both with formation of the $[M+HCOOH]^-$ adduct ($[M+46]^-$). Formation of an adduct with formic acid was observed previously in the characterisation of this type of phenolic compound (de Rijke *et al*., 2003). In addition to those two acids, one ferulic acid derivative and three other caffeic acid derivatives were detected in this study. Indeed, although the structure of these compounds was not totally elucidated, it was possible to observe a UV spectrum equivalent to that of caffeic acid (292, 322 nm) in compounds 55, 56 and 60. Moreover, the MS^2 spectrum of the $[M-H]^-$ at *m/z* 399 (isomeric compounds 55 and 56) showed a base peak ion at *m/z* 179, which corresponds to caffeic acid and an ion at *m/z* 135 resulting from the loss of CO_2 from the phenolic acid. In a similar manner, the $[M-H]^-$ at *m/z* 419 in compound 49 was assigned to a ferulic acid derivative, as it presented UV_{max} at 298 and 322 nm and its MS^3 spectrum showed an ion at *m/z* 193, both consistent with ferulic acid.

It is worth noting the large number of methoxylated flavonols in these Portuguese propolis samples, namely quercetin and kaempferol derivatives. These polyphenols were not detected in our previous work due to the methodological approach used, where the major eluted peaks were collected as fractions and directly injected into the ESI source and subsequently analysed (Falcão *et al*., 2010). These compounds include several monomethyl ether and polymethyl ether quercetin derivatives, namely quercetin-3-methyl ether (12), isorhamnetin (19), quercetin-dimethyl ether (22), quercetin-tetramethyl ether (24), rhamnetin (27) and quercetin-dimethyl ether (28), which previously have been detected as major components of plant exudates, resins and waxes (Stevens *et al*., 1995). Within the kaempferol derivatives, it was possible to detect kaempferol-methyl ether (20), kaempferide (38) and kaempferol-dimethyl ether (46). Also, galangin-5-methyl ether (26), with a precursor ion at *m/z* 283, was identified in the majority of the propolis samples. A flavone derivative identified as acacetin (37) was also found in samples of Portuguese common temperate propolis type.

Besides the dihydroflavonols already described in Portuguese propolis, pinobanksin-3-*O*-pentenoate (54) and pinobanksin-3-*O*-hexanoate (62) were herein assigned by comparison of their UV spectrum and fragmentation pattern with the reported literature and also with the pinobanksin derivative (59) eluted at 76.2 min. The latter presented an absorption maximum peak at 292 nm, which is consistent with pinobanksin (Gardana *et al*., 2007). Moreover, its ESI–MS showed a $[M-H]^-$ ion at *m/z* 367 (Table 2), and the main product ion (*m/z* 271) in its MS^2 spectrum resulted from the loss of a hexenoate group (−96 Da) (Sharkey *et al*., 1959), suggesting an isomer of pinobanksin-*O*-hexenoate.

Uncommon propolis type

Six of the 40 Portuguese propolis samples had a particular phenolic profile different from that of the common temperate propolis samples (Table 1). As shown in Fig. 2, their chromatograms had a group of peaks at early retention times, associated with flavonoid glycosides. Due to their rarity in propolis matrices, we will describe these compounds in detail in the following section. Moreover, these six propolis samples contained four other phenolic compounds that were not detected in the common propolis samples (Table 2): ellagic acid (2), luteolin (10), a dimethoxylated flavonol (21) and a dihydroxy-dimethoxyflavone (42).

The UV spectrum of the dimethoxylated flavonol peak was equivalent to that of kaempferol and its full MS spectrum had a $[M-H]^-$ at *m/z* 329. Moreover, MS^2 and MS^3 experiments indicated the successive loss of methyl groups (−15 Da), with the formation of the product ions at *m/z* 314 and *m/z* 299, respectively. Overall, the data pointed to the presence of kaempferol-methoxymethyl ether with the methoxy group linked to the C-6 position, as previously described (Kumazawa *et al*., 2003).

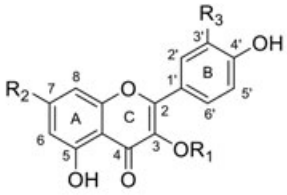
Experimental data of the dihydroxy-dimethoxyflavone (42) suggested the presence of chrysoeriol-methyl ether. In fact, its UV spectrum was equivalent to that of chrysoeriol (250, 268sh and 343 nm) and the MS^2 spectrum of the molecular ion (*m/z* 313) showed a loss of a methyl group, which resulted in the formation of a base peak product ion (*m/z* 298), the latter with a similar fragmentation pattern to that of chrysoeriol.

Flavonoid glycosides

As previously mentioned, uncommon propolis type samples typically contained flavonoid glycosides in their composition. We must highlight that phenolic glycosides are rare in propolis because of the hydrophobic nature of plant sources of the resin and due to the presence of β-glucosidase enzymes during propolis collection and processing. This last point has been under debate, as recent studies (Zhang *et al*., 2011) demonstrated the inefficiency of the enzyme to hydrolyse β-diglycosides. Also, Bankova *et al*. (2000) suggested the possibility of no chemical changes in plant material during propolis collection. Anyway, to our knowledge, the only flavonoids glycosides previously reported in propolis matrices were the isorhamnetin-3-*O*-rutinoside, isolated from Cretan propolis (Popova *et al*., 2009), and rutin (quercetin 3-*O*-rutinoside), which has been identified in European, Asian and South American propolis (Bonvehí and Call, 1994).

The LC–DAD–ESI–MS^n data of the 14 flavonoid glycosides of the uncommon propolis type samples showed that these were quercetin and kaempferol derivatives. In this study, five of those compounds were identified by comparison of UV and LC–MS data with those of the corresponding reference product: quercetin-3-*O*-rutinoside (63), quercetin-3-*O*-glucoside (65), kaempferol-3-*O*-rutinoside (66), isorhamnetin-3-*O*-rutinoside (67) and quercetin-3-*O*-rhamnoside (69). The structure of the nine remaining compounds was assigned based on their UV spectra and interpretation of their fragmentation pathways observed in MS^n spectra, as will be discussed in detail. Sugar moieties in flavonoids were assigned to glucosides, rutinosides and glucuronides, because these are most common and frequent in nature and are now confirmed in propolis, although the presence of galactoside moieties seems also consistent, particularly due to its resistance to bee enzymes hydrolysis. Note that the glycosylation position of these polyphenols will not be ascribed in the present work, but these are commonly C-3 or C-7 (Santos-Buelga *et al*., 2003). Representative structures of the flavonoids glycosides found in the uncommon temperate propolis type samples are shown in Fig. 3, where the most probable linkage positions are considered. Thus, the structures proposed in Fig. 3 should be regarded only as an example.

Seven of those compounds, 64, 68, 70 and 72–75, corresponded to quercetin glycosides. The UV spectrum of compound 64 (similar to that of quercetin) and the identification of the $[M-H]^-$ ion at *m/z* 477 in the corresponding MS spectrum suggest that the



Compound	Name	R_1	R_2	R_3
63	Quercetin-3-*O*-rutinoside	Rut	OH	OH
64	Quercetin-3-*O*-glucuronide	GlcUA	OH	OH
65	Quercetin-3-*O*-glucoside	Glc	OH	OH
66	Kaempferol-3-*O*-rutinoside	Rut	OH	H
67	Isorhamnetin-3-*O*-rutinoside	Rut	OH	OCH_3
68	Isorhamnetin-*O*-pentoside	Pent	OH	OCH_3
69	Quercetin-3-*O*-rhamnoside	Rham	OH	OH
70	Isorhamnetin-*O*-glucuronide	GlcUA	OH	OCH_3
71	Kaempferol-methyl-ether-*O*-glucoside	Glc	OCH_3	H
72	Isorhamnetin-*O*-acetylrutinoside	Rut-Ac	OH	OCH_3
73	Rhamnetin-*O*-glucuronide	GlcUA	OCH_3	OH
74	Quercetin-dimethyl-ether-*O*-rutinoside	Rut	OCH_3	OCH_3
75	Quercetin-dimethyl-ether-*O*-glucuronide	GlcUA	OCH_3	OCH_3
76	Kaempferol-*O*-*p*-coumaroylrhamnoside	Rham-Coum	OH	H

Rut – Rutinose; GlcUA – Glucuronic acid; Glc – Glucose; Pent – Pentose; Rham – Rhamnose; Ac – Acetyl; Coum – *p*-Coumaroyl

Figure 3. Proposed structures for the flavonoid glycosides identified in Portuguese propolis.

compound with a MW of 478 Da is a glucuronic derivative of quercetin. In fact, the product ion at *m/z* 301 (quercetin) was formed by the loss of 176 Da, which is indicative of a glucuronyl unit (Cuyckens and Claeys, 2004). In addition to the aglycone fragment, the MS^2 spectrum of this quercetin-*O*-glucuronide also showed a major product ion at *m/z* 300, formed by the homolytic cleavage of the *O*-glycosidic bond, and has been proposed as indicative of quercetin glycosides (Constant *et al.*, 1997). The ion at *m/z* 300 was also observed in the MS^2 spectrum of the remaining quercetin glycosides. Compounds 68, 70 and 72–75 were identified as methylated derivatives of quercetin glycosides, and all presented a UV spectrum equivalent to that of isorhamnetin-3-*O*-rutinoside (Table 3). Compound 68 showed an ESI–MS^2 spectrum with a base peak ion at *m/z* 315 (−132 Da) and the fragmentation pattern of the latter ion corresponded to that of isorhamnetin. Thus, overall, the data indicated that the phenolic compound eluting at 19.6 min corresponded to an isorhamnetin pentoside derivative. Most probably, the pentoside residue moiety could be assigned to xylose or arabinose, because these two are the most commonly found in polyphenols (Cuyckens and Claeys, 2004). On the other hand, both compounds 70 and 73 presented a $[M-H]^-$ ion at *m/z* 491 and the base peak in the MS^2 spectrum (*m/z* 315) was formed due to the loss of a glucuronide residue (−176 Da). Moreover, the fragmentation pathway (observed in the MS^3 spectrum) of the ion at *m/z* 315 in compound 70 was equivalent to that of isorhamnetin (methyl group in C-3′ position), whereas that of compound 73 was equivalent to that of rhamnetin (methyl group in C-7 position). In accordance with these results, compounds 70 and 73 (MW 492 Da) were respectively assigned to isorhamnetin-*O*-glucuronide and rhamnetin-*O*-glucuronide. The MS analysis of compound 72 showed the $[M-H]^-$ at *m/z* 665, and the MS^2 spectrum showed a main product ion at *m/z* 315 (−350 Da, a combined loss of rutinose and one acetyl residue), which corresponded to isorhamnetin, and an ion at *m/z* 623 (−42 Da, loss of acetyl group). Both these product ions are coherent with the presence of an acetyl disaccharide moiety in the molecule. Note that flavonoid glycosides with an acylated glycosyl moiety part can be identified by the presence, in their MS^2 spectra, of the product ions $[acylsugar-H]^-$ and $[M-H-acyl]^-$ (Parejo *et al.*, 2004), which are reported here. Thus, the data discussed led us to propose compound 72 as isorhamnetin-*O*-acetylrutinoside. The linkage positions of the acyl group on the glycosidic part of the molecule and that of the acylsugar group on the aglycone could not be established on the basis of the UV spectra or on the MS data, as also found in the literature (Parejo *et al.*, 2004). Compounds 74 and 75 showed a similar base peak ion in their ESI–MS^2 spectrum (*m/z* 329) (Table 3) that was obtained by the loss of a rutinoside (−308 Da) and a glucuronide unit (−176), respectively. Furthermore, as the fragmentation pattern of that ion (*m/z* 329) was consistent with that described earlier for quercetin-dimethyl ether, we propose to assign compounds 74 and 75 to quercetin-dimethyl ether-*O*-rutinoside and quercetin-dimethyl ether-*O*-glucuronide, respectively.

Kaempferol glycosides were present in compounds 71 (*m/z* 461) and 76 (*m/z* 577). In the first case, the MS^2 data of the ion $[M-H]^-$ at *m/z* 461 was consistent with kaempferol-methyl ether-*O*-hexoside: it showed a product ion at *m/z* 446 (−15 Da) and a base peak product ion at *m/z* 299 (−162 Da, loss of a hexoside, most probably glucose). For compound 76 the ESI–MS^2 data obtained for its $[M-H]^-$ ion at *m/z* 577 presented a base peak product ion at *m/z* 285 with a fragmentation pattern similar to that of kaempferol, and a mass loss of 146 and 292 Da. The elution time for this compound is significantly greater than all the other flavonoid glycosides and the UV spectrum shape shows deviations from the flavonoid derivatives, with maxima at 265 and 319 nm (data not shown), where the band I is shifted to lower wavelengths with an increase in intensity. This UV behaviour was observed when an acylation by an aromatic acid occurs (Santos-Buelga *et al.*, 2003). The results for this compound are consistent with a kaempferol linked with an acylated glycoside, most probably a *p*-coumaroylrhamnose (Santos-Buelga *et al.*, 2003; Cuyckens and Claeys, 2004), however, further structural studies by NMR are necessary to determine the precise location of the groups in the flavonoid.

Table 3. List of flavonoid glycosides identified by LC–DAD–ESI–MS^n in the uncommon propolis type

Number	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	HPLC/ESI–MS^n *m/z* (% base peak)	Compound
63	15.3	256, 352	609	MS^2[609]: 301(100), 300 (87)	Quercetin-3-*O*-rutinoside[a,b]
64	16.8	256, 355	477	MS^2[477]: 301; MS^3[301]: 179 (100), 151 (39)	Quercetin-3-*O*-glucuronide[b]
65		256, 355	463	MS^2[463]: 301(100), 300 (64)	Quercetin-3-*O*-glucoside[a,b]
66	17.6	265, 349	593	MS^2[593]: 285	Kaempferol-3-*O*-rutinoside[a,b]
67	17.9	253, 355	623	MS^2[623]: 315 (100), 300 (22)	Isorhamnetin-3-*O*-rutinoside[a,b]
68	19.6	253, 346	447	MS^2[447]: 315 (100), 300 (8); MS^3[315]: 300; MS^4[300]: 300 (100), 271 (47), 243 (47), 151 (<1)	Isorhamnetin-*O*-pentoside[b]
69	19.9	256, 349	447	MS^2[447]: 301(100), 300 (47)	Quercetin-3-*O*-rhamnoside[a,b]
70	19.9	253, 346	491	MS^2[491]: 315; MS^3[315]:300; MS^4[300]: 271 (100), 255 (68), 151 (< 1)	Isorhamnetin-*O*-glucuronide[b]
71	20.1	265, 343	461	MS^2[461]: 446 (91), 299 (100), 284 (11); MS^3[299]: 284; MS^4[284]: 284 (100), 256 (60), 255 (52)	Kaempferol-methyl ether-*O*-glucoside[b]
72	21.3	253, 352	665	MS^2[665]: 623 (18), 315 (100), 300 (14); MS^3[315]: 300; MS^4[315]:271(100), 255(57), 151 (< 1)	Isorhamnetin-*O*-acetylrutinoside[b]
73	25.2	256, 349	491	MS^2[491]: 315; MS^3[315]: 300 (40), 193 (69), 165 (100)	Rhamnetin-*O*-glucuronide[b]
74	25.4	253, 349	637	MS^2[637]: 329 (100), 314 (18); MS^3[329]: 314; MS^4[314]: 299 (100), 285 (25), 271 (26), 243 (10)	Quercetin-dimethyl ether-*O*-rutinoside[b]
75	30.9	253, 349	505	MS^2[505]: 329 (100), 314 (18); MS^3[329]: 314; MS^4[314]: 299 (100), 285 (24), 271 (24), 243 (3)	Quercetin-dimethyl ether-*O*-glucuronide[b]
76	50.1	265, 322	577	MS^2[577]: 431 (6), 285 (100); MS^3[285]: 257 (100), 151 (41)	Kaempferol-*O*-*p*-coumaroylrhamnoside[b]

[a]Confirmed with standard;
[b]confirmed with MS^n fragmentation.

Summary

Overall, LC–MS data analysis of glycoside propolis type samples suggest the existence of one or more plant sources of propolis resin around the respective apiary, in addition to *Populus* species. The diversity of resin sources available for honeybees makes the identification of the exact botanical origin of the flavonoid glycosides found in those propolis samples difficult. As these collection regions are rich in conifer plants, however, it is possible that these can be the main source of the glycoside flavonoids (Popova *et al*., 2009) described herein.

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