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Phenolic quantification and botanical origin of Portuguese propolis

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ABSTRACT

The production of propolis by honeybees results from a selective collection of exudates from leaf buds and plants present in the hive neighborhood leading to a resin with many potentialities in the pharmaceutical industry. This study aims to quantify the phenolic content in propolis from different Portuguese regions and in the potential floral sources, *Populus x Canadensis* Moench buds and *Cistus ladanifer* L., in order to establish links with geographical and botanical origin.

The Portuguese propolis revealed a phenolic profile with marked differences in concentrations: the richness in flavonoids is common in all regions, but more evident in propolis from central interior, south and Madeira. The composition of poplar type propolis common in temperate zones was observed in the north, central coast and Azores, while the central interior and south samples, with a composition rich in kaempferol derivatives, resemble the *C. ladanifer* exudates, a spontaneous bush widespread in the Mediterranean. The compound kaempferol-3,7-dimethyl-ether, absent in the poplar type propolis, can be regard as a possible marker for the discrimination of these two types of propolis.

1. Introduction

The honeybee exploit nature as a store to fulfill its nutritional needs, collecting nectar and pollen, but also as a source for substances with other goals such as construction material or to maintain the antiseptic environment in the hive (Burdock, 1998; Bogdanov and Bankova, 2011). With the time evolution, bees were able to find in the surroundings of their nest the best source of materials for the desired proposes. Thus, it is not surprising the potentialities exhibit by propolis, a complex natural product gathered by the honeybees from resinous exudates of buds, leaves, branches and barks present in the vicinity of the beehive. Also named as the "bee glue" it plays an important role to guarantee the bee colony health.

Since ancient times, propolis is used in traditional medicine and now is gaining popularity in health foods as well in cosmetic products (Bogdanov and Bankova, 2011). A great number of research studies focused on the pharmacological and biological properties present by propolis, including antihepatotoxic, antitumor, antioxidative, antimicrobial, anti-inflammatory and immunomodulatory among others (Banskota et al., 2001; Bankova et al., 2000). These bioactivities are closely linked with the chemical composition, particularly with the richness in phenolic compounds, which accounts for approximately half of the resin content, while beeswax, volatiles and pollen represents the other 30%, 10% and 5%, respectively, of it. (Bogdanov and Bankova, 2011). The propolis chemical composition varies greatly with the plant origin of the resin and thus with the geographic and climatic characteristics of the site (Bankova, 2005). The specificity of local flora is very important, not all plants are resin providers and bees have a marked preference for one or a few sticky resin sources, which are, at the same time sources of biologically active phytochemicals (Salatino et al., 2011). Based on this knowledge, propolis was typified according to their plant origin and its main chemical constituents (Bankova, 2005). In temperate zones of the world, poplar buds (*Populus* spp.) are the main sources of the bee glue with flavones, flavanones, fenolic acids and their esters as major compounds. Exceptions can be found, for example, the birch propolis type found in Russia, which has its origin in species like *Betula verrucosa*, where the main compounds are flavones and flavonols different from those found in poplar propolis (Bankova, 2005). Also a Mediterranean propolis type was found in Sicily, Crete and Malta, whose main compounds are diterpenes most probably originated in coniferous plant of the genus *Cupressaceae* (Popova et al., 2009). Tropical propolis has a totally different compositional pattern: the green propolis type, found in Brazil, has its main plant source on the leaves of *Baccharis* spp. and mainly contains prenylated phenylpropanoids (Bankova et al., 2005). In Venezuela and Cuba, the main plant sources are the flower exudates of *Clusia*

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species, originating a propolis rich in prenylated benzophenones (Bankova et al., 2005). C-prenylflavonoids (or propolins) have been described in propolis from Pacific islands, where the resin sources are the fruit exudates of the tree *Macaranga tanarius* (Chen et al., 2003). The propolis typification on the basis of plant sources knowledge is a useful tool for its chemical standardization and thus for ensuring the quality and safety necessary for its commercialization (Bankova, 2005; Salatino et al., 2011).

Recently, the phenolic profile of Portuguese propolis was characterized by liquid chromatography with diode-array detection coupled to electrospray ionization tandem mass spectrometry (LC/DAD/ESI-MS^n) (Falcão et al., 2013). Forty samples from different continental regions and islands were analyzed allowing the detection of seventy six polyphenols and the establishment of two different propolis groups: 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. The data suggest a diversified botanical origin for the Portuguese propolis besides poplar buds (Falcão et al., 2013).

Following those finds, we now present the phenolic quantification of propolis from the different Portuguese continental regions and islands. The results assort the compounds with major contribution to the propolis composition and allow the establishment of links with the geographical origin of this beehive product, a key factor for propolis commercial valorization. The inclusion of two potential floral sources of Portuguese propolis in this study, the buds exudates and surface material present on the leaves and stems of *Populus x Canadensis*, male and female specimens and *Cistus ladanifer* L. enable the correlation between the phenolic profile and the plant source of the resin, and the proposal of kaempferol-3,7-dimethyl-ether as a marker compound for *C. ladanifer* propolis.

2. Materials and methods

2.1. Chemicals and reagents

Chrysin, quercetin, pinocembrin, caffeic acid, ferulic acid, caffeic acid phenylethyl ester (CAPE), salicylic acid were purchased from Sigma Chemical Co (St. Louis, MO, USA). Apigenin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, acacetin were from Extrasynthese (Genay, France). Analytical grade formic acid and HPLC grade ethanol were obtained from Panreac (Barcelona, Spain). HPLC grade methanol and acetonitrile were purchased from LabScan (Lisbon, Portugal). Water was treated in a Milli-Q water purification system (Topway Global Inc., Houston, TX, USA).

2.2. Samples origin

The study was performed on propolis and plant present in the hive neighborhood and reported (Falcão et al., 2013; Martos et al., 1997) as propolis floral sources. Forty propolis samples were collected from six different geographical regions (Fig. S1) in Portugal continental north (N1-6, Bragança; N7, Miranda do Douro; N8, Mirandela; N9-10, Chaves; N11, Montalegre; N12-13, Boticas; N14, Barcelos); central interior (CI1, (Falcão et al., 2013) Guarda; CI2, Penamacor; CI3, Fundão; CI4, Nisa); central coast (CC1, Figueira da Foz; CC2, Leiria; CC3, Coruche; CC4, Ramada); south (S1-3, Aljezur; S4, Moncarapacho); Azores Archipelago (A1, Terceira Island; A2-11, S. Miguel Island); and from Madeira island (M1-3, Funchal, Madeira Island). Propolis sampling sites are located on the map provide in supplementary material (Fig. S1). All the samples were obtained between 2007 and 2009 after the honey harvesting season (July/September), by conventional scraping or through plastic screens.

For the floral sources of the bee glue we collect, in Bragança region, northeast Portugal, in the spring of 2009, the leaf-buds of *Populus x Canadensis* Moenchen, male (PM) and female (PF) specimens and the leaves and stems of, *C. ladanifer* (C). The voucher specimens are deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança with the reference number BRESA 5174, BRESA 5355 and BRESA 5356 for C, PF and PM, respectively. All samples were stored at −20 °C until analysis.

2.3. Phenolic compounds extraction

Prior to the extraction, the resin available in the stems and leaves of *Cistus* specimens was scraped, grounded and homogenized. For *Populus*, the entire leaf-bud was grounded and homogenized. The phenolic extraction for propolis and for the floral sources was performed according with our previous work (Falcão et al., 2010). Briefly, 1 g of sample was mixed with 10 mL of 80% of ethanol/water 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.

2.4. LC/DAD/ESI-MS^n analysis of the plant sources

The LC/DAD/ESI-MS^n analyses were performed on a Finnigan Surveyor Plus HPLC instrument equipped with a diode-array detector and coupled to a mass detector. The chromatographic and MS conditions used were described before (Falcão et al., 2013). The mass spectrometer used was a Finnigan Surveyor LCQ XP MAX quadrupolo ion trap mass spectrometer equipped with an ESI source. Control and data acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

2.5. HPLC quantification

The propolis and plant source extracts were analyzed by reversed-phase high-performance liquid chromatography (HPLC) with UV detection, according with our previous work (Falcão et al., 2010). Briefly, the chromatographic system consisted on a Knauer Smartline separation module equipped with a Knauer smartline autosampler 3800, a cooling system set to 4 °C and a Knauer UV detector 2500. Data acquisition and remote control of the HPLC system was done by ClarityChrom® software (Knauer, Berlin, Germany). The column was a 250 mm × 4 mm id, 5 μm particle diameter, end-capped Nucleosil C18 (Macherey-Nagel) and its temperature was maintained at 30 °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 analysis, the ethanolic extract (10 mg) was dissolved in 1 mL of 80% of ethanol. Salicylic acid, as the internal standard (IS), was added to all extracts. Each sample was filtered through a 0.2 μm Nylon membrane (Whatman) and then 10 μl of the solution was injected. Chromatographic data were acquired at 280 nm.

Quantification was achieved using calibration curves for caffeic acid, ferulic acid, quercetin, pinocembrin, chrysin, and caffeic acid phenylethyl ester, obtained with seven concentration levels. When the standard was not available, the compound quantification was expressed in equivalent of the structurally closest phenolic compound. The calibration parameters are shown in Table 1. The linearity was investigated by calculation of the regression plots by the least squares method and expressed by the correlation

Table 1
Calibration parameters for the phenolic acids and flavonoids used as standards (mg/mL). The compound class to be quantified by each standard is also represented.

Compound	Linearity range	Slope	Intercept	R^2	LOD (mg/mL)	LOQ (mg/mL)	Group to be quantified
Caffeic acid	0.05–0.6	22.3	−0.2	0.9990	0.01	0.05	Phenolic acids
Ferulic acid	0.04–0.6	18.8	−0.1	0.9993	0.01	0.04	Methylated phenolic acids
Quercetin	0.05–2.0	8.0	−0.8	0.9999	0.02	0.05	Flavonols
Pinocembrin	0.08–1.0	16.2	−0.2	0.9991	0.02	0.08	Flavanones; dihydroflavonols
Chrysin	0.03–1.0	23.0	−1.0	0.9999	0.01	0.03	Flavones
CAPE	0.06–1.0	13.8	−0.1	0.9996	0.02	0.06	Phenolic acids esters

LOD = limit of detection; LOQ = limit of quantification. CAPE = caffeic acid phenethyl ester.

coefficient (R^2). Concentrations of all compounds in propolis samples were calculated based on the peak area ratio. The limit of detection (LOD) and quantification (LOQ) were obtained from the y-intercept standard deviation (S_b) and the slope (m) of the calibration curve (Ribani et al., 2007), thus $LOD = 3 \times S_b/m$ and $LOQ = 10 \times S_b/m$.

2.6. Statistics

The statistic analysis was performed using SPSS version 18.0 program, and the hierarchical cluster was obtained with the Ward linkage method, using standardized variables.

3. Results and discussion

3.1. LC/DAD/ESI-MS^n analysis of the plant sources

The chemical composition of the plant source determines the chemical profile of propolis, therefore the profile comparison is the best indicator for the evaluation of propolis origin. In the present work we explore the phenolic composition of two potential plant sources of Portuguese propolis. They were chosen due to its great abundance in the hive neighborhoods and to the empirical knowledge of local beekeepers, which frequently associate these plants as the resin source.

Poplar buds are described as the main source of propolis in temperate zones (Bankova et al., 2000). The analysis of poplar bud exudates from *Populus nigra* and *Populus balsamifera* showed in their composition terpenoids, phenolic acids and their esters, flavonoid aglycons and their chalcones (Greenaway et al., 1989; Isidorov and Vinogorova, 2003) with a different degree of complexity depending on the specie. *Populus x Canadensis* is a hybrid poplar very common in Portugal and consequently a potential source of resin for honeybee, to our knowledge, not yet described.

For elucidation of its phenolic profile we collected the buds of male (PM) and female specimens (PF) in the neighborhood of the hives and analyzed by LC/DAD/ESI-MS^n in the negative ion mode as reported previously (Falcão et al., 2013), which allowed the elucidation of phenolic compounds by comparison of their chromatographic behavior, UV spectra and MS information, to those of 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. Both PM and PF ethanolic extracts presented a phenolic profile similar to the one observed in Portuguese common temperate propolis type earlier described (Falcão et al., 2013). The composition is rich in phenolic acids and their derivatives, mainly caffeic acid, 3,4-dimethyl-caffeic acid, caffeic acid isoprenyl ester and its isomer, caffeic acid benzyl ester and caffeic acid phenylethyl ester. Also flavonoids and its derivatives were found in the PM and PF extracts, with pinocembrin, chrysin, pinobanksin-3-*O*-acetate and galangin as major compounds. Comparing the two genders, some differences were found: the female poplar presented the compounds pinobanksin-5-methyl-ether-3-*O*-pentanoate, 3-hydroxy-5-methoxyflavanone, pinobanksin-3-*O*-butyrate, pinobanksin-3-*O*-pentenoate, pinobanksin-3-*O*-pentanoate, pinobanksin-3-*O*-hexanoate, which were previously described in Portuguese propolis (Greenaway et al., 1989; Isidorov and Vinogorova, 2003) and were absent from the male poplar phenolic profile.

C. ladanifer is a spontaneous shrubby plant widespread in the Mediterranean region (Chaves et al., 1998). Local beekeepers associate this material with propolis due to its abundance near the hives but also based on the typical odor of *Cistus* spp. that can be identified in some samples. The secretions on the surface of the leaves and stems of *C. ladanifer* were collected, extracted and analyzed by LC/DAD/ESI-MS^n. The representative chromatogram at 280 nm is shown in Fig. 1. This procedure allowed the detection of six flavonoids, mainly kaempferol derivatives (Table 2). These included kaempferol-3-*O*-glucoside (1), kaempferol-methyl-ether (4) and kaempferol-dimethyl-ether (6), of which the last two were recently described in propolis (Falcão et al., 2013). For a better assignment of the methyl positions on the flavonoid skeleton, a deeper look was made on the UV and MS data of the kaempferol-dimethyl-ether. The spectrum of this compound present maximum absorption bands II and I at 265 and 346 nm, respectively. Comparing with the spectral data of kaempferol (265, 364 nm), the introduction of methyl ethers on the free hydroxyls groups undergoes a hypsochromic shift of 18 nm in band I accompanied with a relative drop in the intensity, indicating a 3-*O*-methylation. This remarkable difference is generally used as a diagnostic tool in the identification of free hydroxyls at the C-3 position of the flavonoid molecule, since the methylation in other positions has little effect on the absorption spectrum (Santos-Buelga et al., 2003). The other methyl ether group may be in C-7, C-5 or C-4′ position. The introduction of more methyl ethers on the hydroxyls of the kaempferol increases lipophilicity and thus the retention time. Depending on the position of the methyl ether, the effect on the retention time is different, being the introduction on the C-7 and C-4′ position less polar than the C-5, since the internal hydrogen bond between the hydroxyl and the carbonyl at C-4 position is no longer possible and thus retention time decreases (Santos-Buelga et al., 2003). For the compound under discussion the retention time is higher than kaempferol, therefore the C-5 position for the second methyl group can be disregarded. The fragmentation pattern of the product ion *m/z* 313 produced the ion at *m/z* 298 arising from the loss of methyl radical from the deprotonated molecular ion, as the most prominent fragment. A minor fragment of *m/z* 165 was also identified which was resultant from the retro Diels–Alder mechanism, indicating the presence of the methyl group in C-7 position (Cuyckens and Claeys, 2004). So, the compound was tentatively identified as kaempferol-3,7-dimethyl-ether, nevertheless for an unequivocal determination of the group location, further structural studies with NMR are necessary. The flavonoid 5,3′-dihydroxy-3,7,4′,5′-tetramethoxyflavone observed in the composition of Tunisian propolis and assigned by Martos et al. (1997) to the leaf exudates of *Cistus* spp. was not here identified. We cannot judge, however, if this difference arises from the different extraction procedure used or from the geographical origin, or to the botanical specie, since no reference is given concerning the *Cistus* specie evaluated on that work. Besides these

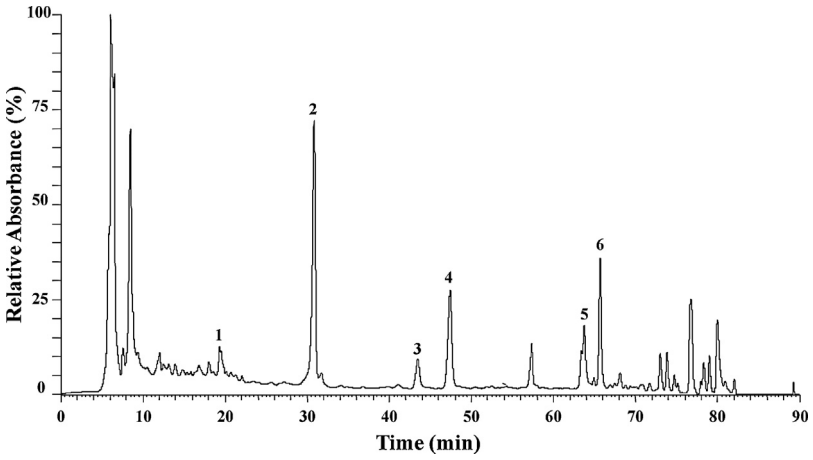

Fig. 1. Chromatographic profile at 280 nm for *Cistus ladanifer* exudates ethanolic extract: 1 – kaempferol-3-*O*-glucoside; 2 – kaempferol derivative; 3 – apigenin; 4 – kaempferol-methyl-ether; 5 – acacetin; and 6 – kaempferol-3,7-dimethyl-ether.

compounds, a kaempferol derivative (2) was identified without the total elucidation of its structure. The ESI-MS^2 data obtained for its $[M-H]^-$ ion at *m/z* 593 presented a base peak product ion at *m/z* 285 with a fragmentation pattern similar to that of kaempferol, and a mass loss of 308 Da. Although this loss could correspond to a rutinoside, the elution time is greater than for the commercial standard and the UV spectrum show deviations from that of kaempferol aglycone, with the band I enhanced in intensity and shifted back to 313 (Table 2). These results could indicate an acylation by an aromatic acid in the molecule (Santos-Buelga et al., 2003). For the elucidation of the structure, further structural studies by NMR are required. Additionally, two flavones were identified, namely apigenin (3) and acacetin (5), which were present in the propolis samples (Falcão et al., 2013). This flavonoid pattern in the *C. ladanifer* exudates is consistent to the one described by Chaves et al. (1998) with a composition rich in apigenin and kaempferol derivatives.

3.2. Phenolic quantification in propolis – geographical origin

The phenolic complexity of propolis is linked with the phenolic diversity within a plant resin but also due to the combination of many different plants visited by the honeybees, particularly in sites with phyto-geographic diversity. Not all the resin sources or phenolic compounds within a resin are, however, in the propolis composition at significant amounts. In fact, some compounds are sometimes detected only as trace elements, which mean that the plant is scarce around the hive or it is not the honeybee preferred plant. For a better understanding of the phyto-diversity impact in the Portuguese propolis, we quantified the phenolic composition of forty samples, from different Portuguese geographical locations, using HPLC. The former evaluation of the phenolic profile of the samples (Falcão et al., 2013, 2010) allowed the detection of seventy-six phenolic compounds, including an uncommon group of flavonoid glycosides rarely described in this type of bee product (Falcão et al., 2013). From the overall list, only forty-one compounds were herein detected above the limit of quantification. Fig. 2a and b shows the experimental chromatograms for the commercial standards used in the quantification and for a typical propolis sample, respectively. Due to the number of compounds in the propolis chromatographic profile we decided to use in the quantification procedure a reduced number of phenolic compounds, representative of each class: caffeic acid, ferulic acid, caffeic acid phenylethyl ester, quercetin, pinocembrin and chrysin. For those compounds that do not fit under the chosen standards, the quantification was made in equivalents terms using the phenolic compound of the same chemical class (Table 1). Salicylic acid was chosen as internal standard, considering the detector response and the retention time, which did not interfere with the compounds under investigation. The analytical method exhibits a good linear response for all compounds, with correlation coefficients (R^2) above 0.999, and good sensitivity with the LOD varying between 0.01 and 0.02 mg/mL and LOQ varying from 0.03 and 0.08 mg/mL (Table 1).

The majority of the propolis samples presented a similar phenolic profile, but with marked differences in their concentrations. For an easier interpretation of the results, the individual compounds were aggregated in phenolic classes, as shown in Table 3, however the individual phenolic concentration and the MS fragmentation pattern can be found in the supplementary material (Table S1–S5). In all the regions, flavonoids were more abundant than simple phenolics (Fig. 3a) with pinocembrin, chrysin, pinobanksin-3-*O*-acetate and galangin as major compounds. The difference between these two phenolic groups is even more evident in samples from

Table 2
Flavonoids identified by LC/DAD/ESI-MS^n in *Cistus ladanifer* exudates.

Nr	t_R (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	MS^n (% base peak)	Compound
1	19.3	265, 331	447	MS^2[447]: 284 (100), 285 (66)	Kaempferol-3-*O*-glucoside[a]
2	30.9	265, 313	593	MS^2[593]: 285	Kaempferol derivative[b]
3	43.4	268, 337	269	MS^2[269]: 225 (100), 151 (69)	Apigenin[a]
4	47.4	265, 352	299	MS^2[299]: 284	Kaempferol-methyl-ether[b,c]
5	63.7	268,331	283	MS^2[283]: 269	Acacetin[a]
6	65.7	265, 346	313	MS^2[313]: 299 (10), 298 (100), 165 (<1); MS^3[298]: 283 (100), 269 (8), 255 (41), 241 (2); MS^4[283]: 255 (100), 151 (<1)	Kaempferol-3,7-dimethyl-ether[b,c]

[a] Confirmed with standard.
[b] Confirmed with MS^n fragmentation.
[c] Confirmed with reference (Falcão et al., 2013).

Table 3
Composition of phenolic classes present in Portuguese propolis and its plant sources (mg/g of extract).

Sample	Phenolic acids	Phenolic esters	Total simple phenolics	Flavonols	Flavones	Dihydroflavonols	Flavanones	Flavonoid esters	Flavonoid glycosides	Total flavonoids
Propolis										
N1	95.5	161.0	256.5	102.9	94.83	35.9	74.6	150.2	nd	458.4
N2	39.6	115.1	154.7	109.5	98.41	36.3	73.3	102.2	nd	419.7
N3	53.6	157.2	210.8	92.2	70.99	23.5	45.9	67.7	nd	300.2
N4	72.1	118.2	190.3	114.7	86.45	47.3	64.0	147.7	nd	460.0
N5	44.3	100.7	145.0	81.1	63.41	26.9	53.8	80.9	nd	306.2
N6	35.3	84.7	120.1	45.4	38.36	18.2	43.4	76.3	nd	221.6
N7	47.1	98.1	145.2	90.6	66.01	22.3	46.4	71.3	nd	296.6
N8	40.9	93.2	134.1	112.2	84.37	39.0	93.4	128.3	nd	457.3
N9	83.5	32.3	115.9	68.7	36.39	47.8	25.4	25.1	nd	203.4
N10	nq	76.8	76.8	62.5	60.26	6.8	27.7	55.1	nd	212.3
N11	12.9	28.1	41.1	49.1	35.65	3.6	8.3	12.4	23.2	132.1
N12	1.5	6.4	8.0	47.1	30.16	2.7	7.8	9.6	12.6	109.9
N13	1.0	nq	1.0	45.5	19.49	1.9	nq	1.5	19.4	87.8
N14	89.9	171.4	261.3	55.9	74.97	61.2	55.5	42.6	nd	290.2
CI1	43.6	nq	43.6	167.8	96.22	10.6	17.9	14.4	227.7	534.6
CI2	nq	nq	nq	50.2	20.59	2.0	nq	nq	nd	72.8
CI3	4.9	19.8	24.7	123.3	35.19	4.5	24.9	16.0	nd	203.9
CI4	15.5	27.1	42.6	99.9	33.12	5.5	15.9	25.7	nd	180.1
CC1	58.4	91.8	150.1	79.3	53.39	50.1	53.4	90.4	nd	326.6
CC2	30.4	36.4	66.9	100.8	50.85	75.2	60.1	96.6	nd	383.5
CC3	59.7	97.7	157.4	85.2	62.70	24.4	52.5	72.8	nd	297.6
CC4	24.9	103.1	128.0	100.7	71.55	23.3	60.5	94.3	nd	350.4
S1	42.5	88.3	130.8	99.6	74.48	38.4	61.4	91.9	nd	365.8
S2	nq	nq	nq	90.4	22.21	4.0	nq	nq	nd	116.6
S3	nq	nq	nq	121.1	24.56	6.2	nq	nq	nd	151.9
S4	36.8	6.8	43.6	82.3	22.41	2.7	nq	nq	11.5	118.9
A1	51.8	129.8	181.6	99.1	84.48	33.2	64.0	88.5	nd	369.3
A2	67.5	171.7	239.3	56.2	67.73	11.9	36.4	65.1	nd	237.4
A3	56.4	130.8	187.2	41.4	63.43	11.4	45.0	50.2	nd	211.5
A4	47.9	110.0	157.9	39.8	52.87	10.3	38.9	41.4	nd	183.2
A5	55.0	98.4	153.4	40.6	47.66	10.8	32.9	43.2	nd	175.1
A6	63.5	142.6	206.1	47.7	59.54	10.7	39.2	63.0	nd	220.1
A7	60.9	139.9	200.8	55.4	53.08	10.3	31.8	56.1	nd	206.6
A8	35.8	85.4	121.2	50.7	45.44	7.8	25.3	44.1	nd	173.3
A9	38.5	73.7	112.3	52.1	48.84	12.5	30.2	47.2	nd	190.9
A10	52.7	92.2	144.8	56.7	47.70	13.1	25.9	20.9	nd	164.3
A11	60.0	123.2	183.2	82.6	69.36	23.6	40.5	31.7	nd	247.7
M1	nq	nq	nq	nd	14.15	nd	nq	nq	nd	14.1
M2	nq	nq	nq	91.4	4.64	8.4	nq	nd	14.7	119.1
M3	64.2	28.4	92.6	61.0	25.22	8.6	32.1	34.1	nd	160.9
Plant sources										
PM	29.1	59.1	88.2	41.8	35.38	6.0	21.5	23.4	nd	128.1
PF	29.4	64.5	93.8	30.9	46.95	3.8	36.8	41.7	nd	160.1
C	nd	nd	nd	26.9	10.57	nd	nd	nd	27.4	64.8

nq – not quantified; nd – not detected.

central interior, south and Madeira Island, where flavonoids represented more than 85% of the total phenolic content.

The contribution of the individual chemical classes was not identical for each region: for the simple phenolics, the esters derivatives were more abundant than the corresponding acid compounds, with the propolis from north (N) of Portugal and Azores Archipelago (A) revealing the highest content, with 89 and 117 mg/g, respectively (Fig. 3b). Samples from the central interior region, south and Madeira Island were exceptions, with an evident low content in simple phenolics, where phenolic acids were prevailing.

The flavonoid content was also distinct between regions and highly abundant in propolis from the north (N) and central coast (CC) (Fig. 3c), although some samples can be seen as outliers in their region, particularly those from central interior CI1 with a abnormal high content, 534.6 mg/g and the samples N11–N13 from north, CI2 from central interior, S2 and S4 from south and M1–M2 from Madeira island, with values below 132 mg/g of extract (Table 3). Looking at each individual flavonoid sub-classes, the concentrations profile changed between regions, which must stem from the different vegetation sources around the hive: in the north (N), central coast (CC) and Azores (A), propolis was richer in flavonols, flavones and flavonoid esters, while on the south (S) and in Madeira (M) the propolis flavonoid composition was mainly flavonols (above 50%). The propolis from the central interior (CI) fitted on a different level due to the huge presence of flavonoid glycosides, observed also in the north (N), south (S) and Madeira (M) but only at very low concentration (Fig. 3c).

The flavonols concentration varied from the highest average value of 110 mg/g found in the central interior (CI) to 51 mg/g in Madeira (M) propolis. With exception of propolis from Azores (A), this class was the most relevant for the total flavonoid content (25% in Azores to 52% in the south).

The contribution of flavones was also very significant in the north (N), central coast (CC) and Azores (A) propolis ranging from a maximum of 61 mg/g in the north (22%) down to a minimum of 15 mg/g in Madeira propolis (15%), Table 3. In Azores (A), the flavones content in propolis was even higher than flavonols (Fig. 3c).

Dihydroflavonols did not played the same role in the propolis flavonoid composition and its input can be down to 2% in samples from the central interior (CI). The maximum amount was observed

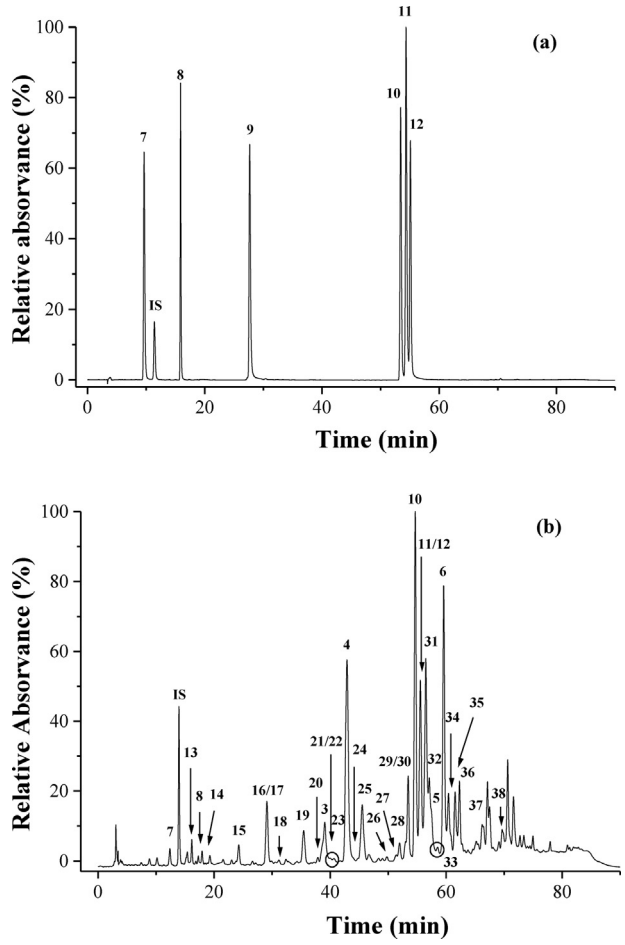

Fig. 2. Chromatographic profiles at 280 nm. (a) Standards compounds: 7 – caffeic acid (0.2 mg/mL); IS – internal standard (salicylic acid; 0.2 mg/mL); 8 – ferulic acid (0.2 mg/mL); 9 – quercetin (0.6 mg/mL); 10 – pinocembrin (0.3 mg/mL); 11 – chrysin (0.3 mg/mL); 12 – caffeic acid phenylethyl ester (0.3 mg/mL). (b) Propolis ethanolic extract of sample CI3 (10 mg/mL): 7 – caffeic acid; IS – internal standard (salicylic acid); 13 – *p*-coumaric acid; 8 – ferulic acid; 14 – isoferulic acid; 15 – 3,4-dimethyl-caffeic acid; 16 – pinobanksin-5-methyl-ether; 17 – cinnamic acid; 18 – *p*-coumaric acid methyl ester; 19 – pinobanksin; 20 – pinocembrin-5-methyl-ether; 3 – apigenin; 21 – chrysin-5-methyl-ether; 22 – kaempferol; 23 – isorhamnetin; 4 – kaempferol-methyl-ether; 24 – quercetin-dimethyl-ether; 25 – cinnamyldenacetic acid; 26 – rhamnetin; 27 – quercetin-dimethyl-ether (isomer); 28 – caffeic acid isoprenyl ester; 29 – caffeic acid isoprenyl ester (isomer); 30 – caffeic acid benzyl ester; 10 – pinocembrin; 11 – chrysin; 12 – caffeic acid phenylethyl ester; 31 – pinobanksin-3-*O*-acetate; 32 – galangin; 5 – acacetin; 33 – chrysin-6-methyl-ether; 6 – kaempferol-3,7-dimethyl-ether; 34 – *p*-coumaric acid isoprenyl ester; 35 – caffeic acid cinnamyl ester; 36 – pinobanksin-3-*O*-propionate; 37 – pinobanksin-3-*O*-butyrate or isobutyrate; 38 – pinobanksin-3-*O*-pentanoate or 2-methylbutyrate.

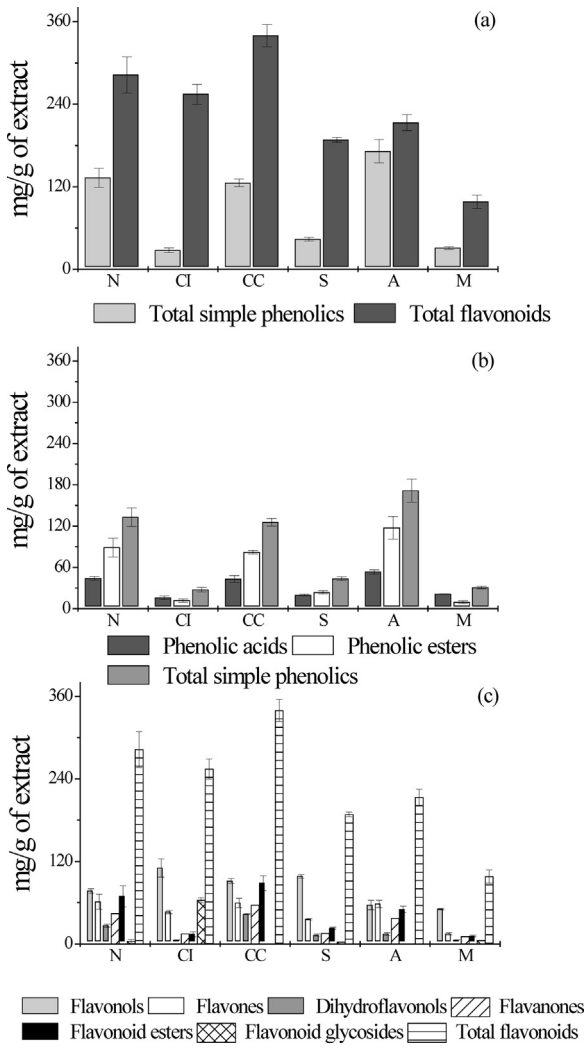

Fig. 3. Composition for the main classes of phenolic compound present in propolis samples from: north (N), central interior (CI), central coast (CC), south (S), Azores Archipelago (A) and Madeira Island (M) regions. (a) Total simple phenolics and total flavonoids. (b) Phenolic acids, phenolic esters and total simple phenolics. (c) Flavonols, flavones, dihydroflavonols, flavanones, flavonoid esters, flavonoid glycosides and total flavonoids.

in the samples from the north (N) and coast center (CC), but never above 13% (43 mg/g).

In this study, only two flavanones were detected, pinocembrin-5-methyl-ether (20) and pinocembrin (6), and only the last one was quantifiable. The generality of samples from north (N) and coast center (CC) showed a high content in pinocembrin, 25–93 mg/g and 52–61 mg/g of extract, respectively (Table 3). Exceptions within the north region were found in samples N11–N12 presenting low values, around 8 mg/g, or sample N13 where pinocembrin was even below the quantification limit. Other samples such as CI1–CI3, S2–S4 or MI–M2 revealed also a poor content in flavanones.

The second class of flavonoids observed in high quantities in the north (N), central coast (CC) and in Azores (A) propolis were the flavonoid esters, representing approximately 25% of the total flavonoid content. Some propolis samples in the north (N) reveal amounts of the pinobanksin esters up to 150 mg/g of extract. For other side, in the south (S) and in Madeira (M) the content of these ester derivatives in propolis was low and sometimes even absent (Table 3).

Portuguese propolis has been recently described to present an uncommon composition rich in flavonoid glycosides (Falcão et al., 2013). This was detected in samples N11–N13 from north, CI1 from central interior, S4 from south and M2 from Madeira Island. On those, fourteen flavonoid glycosides, mainly quercetin and kaempferol glycosides were identified. Due to the complexity of the resulting chromatograms and the proximity in the retention time, the flavonoid glycosides were quantified as one. Sample CI1 from the central interior (Guarda) is clearly different from all the others with almost 228 mg/g, representing 43% of the flavonoids in this sample and contribute to the highest value observed in all the samples under study (535 mg/g). The other propolis samples with flavonoid glycosides had a much lower content, between 12 and 23 mg/g (Table 3).

As described, not all the samples within a region present the same phenolic profile or content. The statistic analysis with hierarchical cluster (Fig. 4), clearly identified three groups and an outlier sample (CI1): the first group contain most of the samples

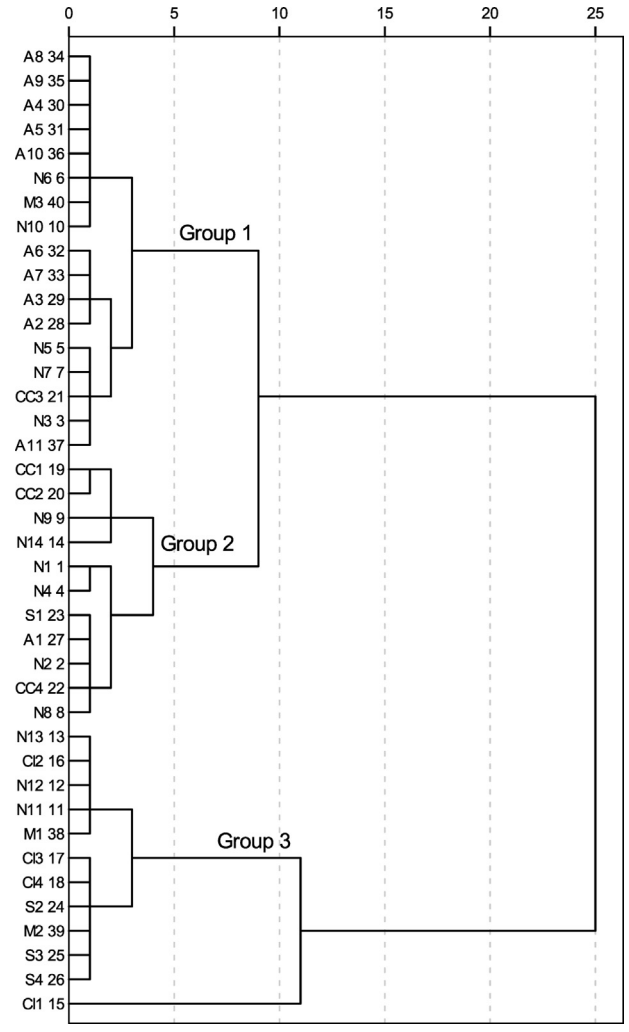

Fig. 4. Dendrogram obtained by hierarchical cluster analysis of the propolis phenolic content from: north (N), central interior (CI), central coast (CC), south (S), Azores Archipelago (A) and Madeira island (M).

from Azores (A), five samples from the north (N), one sample from Madeira (M) and other from center coast; the second group is mainly composed by the north (N) and central coast (CC) samples; the third group contains most of the samples from central interior (CI), south (S) and Madeira (M), and include samples N11–N13 from the north. This later group is distinctive from the above due to the low content on simple phenolics and the presence of flavonoid glycosides. The distinction between the first two groups is not as evident and relies in the total amount of flavonoids: group 2 is richer, particularly in respect to flavonols and dihydroflavonols. These differences can only be explained by the phyto-diversity provided within those Portuguese regions.

3.3. Phenolic quantification in the plant sources – botanical origin

The ethanolic extracts of *Populus x Canadensis*, male (PF) and female (PM), showed a comparable composition of simple phenolics, with higher values for the esters derivatives, up to 65 mg/g (approximately 70% of the total phenolics) (Table 3). Nevertheless, the total phenolic content is lower in the poplar buds than in propolis, indicating that bees collect resin from other floral sources, thus enriching the phenolic composition of the bee glue. For *C. ladanifer* exudates, phenolic acids and esters were not detected at all using this experimental approach. This class of compounds is unusual in *C. ladanifer*, and in the literature only vanillic acid has been described at low concentrations in the methanol: water (80:20) extract of fresh leaves (Barros et al., 2013).

The amount of flavonoids in the plant sources under study is higher in comparison with the simple phenolics. The exudates of PM were richer in flavonols (42 mg/g) and flavones (35 mg/g of extract) whereas PF had more flavones (47 mg/g) and flavonoid esters (42 mg/g). Both poplars extracts presented a low content in dihydroflavonols, 4–6 mg/g (Table 3). These patterns are consistent with the general observation found in the propolis samples, particularly those from the north (N), central cost (CC) and Azores (A), however, in the latter region, the high quantity of flavones in comparison to flavonols reveal some deviation. It is interesting to notice that the exudates of female poplar reveal the same behavior, what let us consider that the variability in the poplar species around the hive could contribute to those minor differences in quantities within the phenolic composition. In fact, poplar bud exudates were reported to contain a great variety of phenolic compounds, which were dependent on the species studied (Vardar-Ünlü et al., 2008).

Through the analysis of the exudates of *C. ladanifer*, only flavonols, flavones and flavonoid glycosides were detected in a concentration of 27, 11 and 27 mg/g of extract, respectively. The rich composition in kaempferol derivatives present in this plant source was also found in samples CI2–CI4 from central interior and samples S2–S4 from the south, standing out the compound kaempferol-3,7-dimethyl-ether (6) which was absent in the other propolis samples with a poplar type composition.

In conclusion, the Portuguese propolis samples presented a similar phenolic composition, with significant differences found in their concentrations. The samples from north (N), central coast (CC), Azores Archipelago (A) and sample S1, from south, revealed the higher phenolic content, up to 261 mg/g in simple phenolics and 460 mg/g in flavonoids, with a profile similar to the one observed in the bud exudates extracts of *Populus x Canadensis*. The other samples, which include propolis N11–N13 from north regions, CI2–CI3 from central interior, S2–S4 from south and samples M1–M2 from Madeira Island presented a poor composition in the generality of the phenolic compounds, with less than 44 mg/g of simple phenolics and 204 mg/g of flavonoids. Moreover, the phenolic profile for this propolis type is clearly different, suggesting other floral contributions for the resin rather than poplar. The phenolic constituents found in samples CI2–CI4 and S2–S4, rich in kaempferol derivatives, particularly kaempferol-3,7-dimethyl-ether, resembles the *C. ladanifer* exudates. This plant source, very common in those regions, is probably the origin of the resin and the compound kaempferol-3,7-dimethyl-ether, absent in the poplar type propolis, can be regard as a marker substance for floral origin discrimination between these two types of propolis. Central interior sample, CI1, is clearly an outsider: the exclusive presence of quercetin-tetramethyl-ether, luteolin and chrysoeriol-methyl-ether, all described before in *Labiatae*, particularly in *Origanum* spp. (Tomás-Barberán and Wollenweber, 1990; Melpomene et al., 2008), and its high content in flavonoid glycosides, 228 mg/g, with a probable origin in the conifer plants (Falcão et al., 2013), confirm the difficulty to unequivocally assign the floral composition of each propolis sample.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2013.07.021.

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