ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 10:2 (2016) 176-188

records of natural products

The Volatile Composition of Portuguese Propolis Towards its Origin Discrimination

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(Received January 06, 2014; Revised November 22, 2014; Accepted April 06, 2015)

Abstract: The volatiles from thirty six propolis samples collected from six different geographical locations in Portugal (mainland, Azores archipelago and Madeira Island) were evaluated. *Populus x canadensis* Moenchen leaf-buds and *Cistus ladanifer* L. branches essential oils were comparatively analysed. The essential oils were isolated by hydrodistillation and analysed by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). Cluster analysis based on propolis samples volatiles chemical composition defined three main clusters, not related to sample site collection. Cluster I grouped 28 samples with high relative amounts of oxygen-containing sesquiterpenes (20-77%), while cluster II grouped 7 samples rich in oxygen-containing monoterpenes (9-65%) and the only sample from cluster III was monoterpene hydrocarbons rich (26%). Although *Populus x canadensis* and *Cistus ladanifer* were associated as resin sources of Portuguese propolis, other *Populus* species as well as plants like *Juniperus* genus may contribute to the resin in specific geographical locations.

Keywords: Propolis; volatiles; *Populus x canadensis*; *Cistus ladanifer*; GC; GC-MS.

1. Introduction

Over the last thirty years, propolis has been attracting researcher's interest all over the world. This fascinating bee product is the result of the collection of resins, bud exudates and part of plants by bees [1]. Propolis is a multifunctional material used by bees in the construction and defence of their hives [2]. It has been proposed that it has a role in the immunity of honeybees, reducing the risk of disease and parasite transmission through the colony [3]. Nowadays propolis is extensively used in food, cosmetic and pharmaceutical industry due to its wide range of biological properties presented

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 08/01/2015 EISSN:1307-6167

[4,5].

Propolis is composed by an extremely complex mixture of natural substances, with several hundred of different chemical compounds and highly dependent on the plants available around the hive, and therefore on the geography and climatic conditions of the site [3,6]. Overall, the so called bee glue is composed by a balsamic part derived from the collected plant parts which contains 40-70% of resin (mainly phenolic compounds) and 3-5% of essential oils and a non-balsamic part which is added by bees and contains 20-35% of wax, 5% of pollen and 5% of other compounds (minerals, polysaccharides, proteins, etc.) [1]. According to the botanical origin and consequently its composition, propolis was typified worldwide: in temperate zones the bud exudates of *Populus* species and their hybrids are the main source of the bee glue [2]. The typical components of poplar propolis are the phenolics: flavonoid aglycones (flavones and flavanones), phenolic acids and their esters [2,7]. On the other hand, in tropical regions of the world, where poplars are not native, plant sources are much diversified. The highest commercial valorised propolis type is known as ‘‘green propolis’’, which predominates in the southeast of Brazil, it has is origin in the leaves of *Baccharis dracunculifolia* and is mainly composed by prenylated *p*-coumaric acids and caffeoyl quinic acids [2]

Even though volatile compounds are found in smaller concentrations, they play an important role in propolis characterization and can enhance the potential uses due to their aroma and significant biological activity. Furthermore, their composition can give valuable information about plant sources in the origin of propolis. Mono- and sesquiterpenes were identified as the major components of propolis although the diversity of volatile compounds present is very high [2]. Research studies concerning European propolis volatile composition revealed a predominance of sesquiterpenes in Bulgarian propolis [8], monoterpenes in Dalmatian [9] and Greek propolis [10] and organic compounds like benzyl alcohol, benzoic acid and benzyl benzoate in Slavonia propolis [9]. Recently the volatile profile of propolis samples from acaricide-treated and–untreated beehives in the south Portugal was evaluated, the samples presenting an intense rock-rose aroma supported by the presence of characteristic *Cistus* and labdanium oil volatile components [11].

Portugal is a country of botanical diversity which is reflected in propolis different compositions. Our former studies allowed the establishment of two distinct groups of propolis based on the phenolic profile [12]. In this work we characterize the volatile composition of propolis samples from the different regions of Portugal, mainland and islands, with the purpose of creating a pattern for origin discrimination of the samples and contributing to standardize this bee product. Additionally, the volatiles of two plant extracts, mentioned as propolis floral sources in temperate areas [2], *Populus x canadensis* buds and *Cistus ladanifer*, were used for comparison with the propolis profile.

2. Materials and Methods

2.1. Propolis samples

This work was performed in thirty six propolis samples from different regions of continental Portugal (north - N; central interior - CI; central coast - CC; south - S), Azores archipelago (A) and Madeira Island (M) accordingly to the availability and the beekeeping activity within the region (see supplementary material, Figure. S1). Table 1 shows the general status of propolis samples: year, geographical collection sites and collection method. They were obtained after the honey harvesting season, randomly by conventional scraping (1) or through plastic screens boards (2), depending on the method used by the local beekeeper. After the removal of debris, wood and bees, these propolis samples were then stored at -20 ºC until analysis.

2.2 Plant Material

The study was performed on plant samples available in the hive neighborhood that were reported as propolis floral sources [2]. *Populus x canadensis* Moenchen (P) leaf-buds and *Cistus ladanifer* L. (C) branches, in the floral stage, were collected from wild growing plants in the Bragança region, northeast Portugal, in the spring of 2009, Table 1. Voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança under voucher number

BRESA 5174 and BRESA 5355, for C and P, respectively. All plant material were stored at -20ºC until oil extraction and analysis.

Table 1. Propolis and plant material samples.

Sample Code	Region	Geographical location	Year	Collection method
Propolis				
N1	North	Bragança	2007	1
N2		Bragança	2007	2
N3		Bragança	2008	2
N4		Bragança	2009	1
N5		Bragança	2009	1
N6		Bragança	2009	1
N8		Mirandela	2009	2
N9		Chaves	2009	2
N10		Chaves	2009	2
N11		Montalegre	2009	1
N12		Boticas	2009	1
N13		Boticas	2009	1
N14		Barcelos	2010	1
CI4		Nisa	2009	2
CC1	Central coast	Figueira da Foz	2009	2
CC2		Leiria	2009	1
CC3		Coruche	2009	2
CC4		Ramada	2009	1
S1	South	Aljezur	2009	1
S2		Aljezur	2009	1
S3		Aljezur	2009	1
S4		Moncarapacho	2009	1
A1	Azores Archipelago	Terceira Island	2009	1
A2		S. Miguel Island	2009	1
A3		S. Miguel Island	2009	1
A4		S. Miguel Island	2009	1
A5		S. Miguel Island	2009	1
A6		S. Miguel Island	2009	1
A7		S. Miguel Island	2009	1
A8		S. Miguel Island	2009	1
A9		S. Miguel Island	2009	1
A10		S. Miguel Island	2009	1
A11		S. Miguel Island	2009	1
M1	Madeira Island	Funchal	2009	1
M2		Funchal	2009	1
M3		Funchal	2009	1
Plant material				
P	North	Bragança	2009	-
C	North	Bragança	2009	-

1-Conventional scraping; 2-Plastic screen boards

2.3. Volatiles extraction

Prior to the extraction, the samples were grounded and homogenized. Propolis and the plant material (approximately 8 g) were submitted to hydrodistillation for 3 h using a Clevenger-type apparatus according to the European Pharmacopoeia method [13]. The volatile isolation procedure was run at a distillation rate of 3 mL min^{-1}, and the oils were recovered in distilled *n*-pentane, concentrated at room temperature under a slight nitrogen flux, and stored at -20ºC in the dark until analysis.

2.4. Volatiles analysis

2.4.1. Gas Chromatography (GC)

Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a

vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (100% polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column [(50% phenyl)-methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc.]. Oven temperature was programmed, 45-175°C, at 3°C.min^{-1}, subsequently at 15°C.min^{-1} up to 300°C, and then held isothermal for 10 min; injector and detector temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm.s^{-1}. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.1 μL of a pentane-volatiles solution (1:1). The percentage composition of the volatiles was computed by the normalization method from the GC peak areas, calculated as mean values of two injections from each sample, without using correction factors.

2.4.2. Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS unit consisted on a Perkin Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm.s^{-1}; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_8-C_{25} *n*-alkane indices and GC-MS spectra from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesised components and commercial available standards.

2.5. Statistical analysis

The percentage composition of the isolated volatiles was used to determine the relationship between the different samples by cluster analysis using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc software, version 2.2, Exeter Software, Setauket, New York) [14]. For cluster analysis, correlation coefficient was selected as a measure of similarity among all accessions, and the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to the previously described [15] and classified as very high (0.9-1), high (0.7-0.89), moderate (0.4-0.69), low (0.2-0.39) and very low (<0.2).

3. Results and Discussion

3.1 Portuguese propolis volatile composition

All propolis volatiles were obtained in a yield <0.05% (v/w). The volatile component isolated from each individual propolis sample was a complex mixture in which two hundred and one components were identified. Each identified volatile component is listed in Table 2, following their elution order on the DB-1 column, and arranged according to the three types of volatile oils defined by agglomerative cluster analysis. The table includes the lowest and the highest percentages found for each component in each volatile oil type.

Despite the major chemical variability, the sesquiterpene fraction was dominant in all thirty six samples analysed (17-83%), while the monoterpene fraction ranged from 1 to 66%, Table 2. A fraction named as others ranged from 4 to 36%. This fraction corresponds to components that were neither terpenes nor phenylpropanoids or fatty acids, comprising aliphatic and aromatic alcohols, carbonyl compounds and hydrocarbons.

Cluster analysis based on the volatiles chemical composition grouped the 36 samples in three main clusters (Figure 1, Table 2). Cluster I was poorly correlated with cluster II ($S_{corr} < 0.4$), and both showed a very low correlation with cluster III ($S_{corr} < 0.2$) (Figure 1). With 28 of the samples, cluster I represented 78% of the sampling, including samples from the Azores Archipelago, the central coast and the majority of the samples from north, south and Madeira Island (Figure 1). Cluster I samples showed high relative amounts of oxygen-containing sesquiterpenes (20-77%), sesquiterpene

hydrocarbons (1-35%) and compounds from the group of others (4-36%). Monoterpenes, phenylpropanoids and fatty acids were found in relative amounts <10%. γ-Eudesmol (3-18%), β-eudesmol (2-26%), α-eudesmol (24%) and α-bisabolol (48%) were the most abundant oxygen-containing sesquiterpenes. Nevertheless, not all these compounds were found in every sample, in fact α-bisabolol was detected in just four out of the twenty eight samples of this cluster, A1 from Azores Archipelago, CC1 and CC2 from central coast and M3 from Madeira Island.

Table 2. Percentage of volatiles of Portuguese propolis and plant sources. (*Samples for each cluster are defined according to Figure 1*).

Components	RI_E	RI_L	Propolis							Plant Sources	
			Cluster I			Cluster II			Cluster III		
			Min	Max	Aver	Min	Max	Aver		P	C
n-Heptanal	897						t				
n-Nonane	900						t				
Tricyclene	921	927[16]					0.1				0.2
α-Thujene	924	929[16]		t	t		0.1				
Benzaldehyde	927	960[17]		0.4	t		0.2			t	
α-Pinene	930	931[16]		4.7	0.5	t	1.3	0.3	18.8		1.6
Camphene	938	938[16]		0.2	t		0.7	0.1	0.4		1.3
Thuja-2,4(10)-diene*	940			0.6	t		1.2	0.2	0.5		0.4
n-Heptanol	952						t	t			
tert-Butyl valerate	956			t	t						
Sabinene	958	959[16]		t	t		0.1	t			
6-Methyl-5-hepten-2-one	960	966[16]		0.3	t		0.1	t			0.4
1-Octen-3-ol	961	972[16]					t	t			
β-Pinene	963	962[16]		0.5	t		0.1	t	5.5		
Hexanoic acid (= Caproic acid)	968			t	t						
2-Pentyl furan	973	975[16]		t	t		t	t			0.2
n-Octanal	973			1.0	0.2	t	0.8	0.3			
β-Myrcene	975	991[17]		0.2	0.1						
1,2,4-Trimethylbenzene	978	978[16]					t	t			
p-Cresol methyl ether	987			0.2	t						0.3
α-Phellandrene	995	986[16]		t	t		t	t			
Verbenene*	998			t	t		t	t			
Benzyl alcohol	1000	1004[16]		0.1	t						
Salicyaldehyde*	1000			1.9	0.1						
Benzene acetaldehyde	1002	1006[16]		t	t						
α-Terpinene	1002	1017[16]		0.1	t		0.6	0.1			0.4
p-Cymene	1003	1004[16]		t	t		0.5	0.1	0.1		
2,6,6-Trimethyl cyclohexanone	1003			2.0	0.2		5.4	0.9		t	29.8
1,8-Cineole	1005	1010[16]		t	t						
β-Phellandrene	1005	1030[17]		0.2	t		0.1	t	t		0.1
Limonene	1009	1014[16]		1.1	0.1		0.1	t	0.2		0.1
cis-β-Ocimene	1017	1015[16]		t	t						
Acetophenone	1017	1026[16]		0.8	0.1		0.1	t		0.1	0.8
trans-β-Ocimene	1027	1026[16]		t	t						
γ-Terpinene	1035	1060[17]		0.2	t		0.9	0.1			0.2
cis-Linalool oxide	1045			t	t						0.2
n-Octanol	1045	1068[17]		t	t						
Fenchone	1050	1087[17]					0.4	0.1			
Methyl benzoate	1053	1093[16]		t	t						
2-Nonanone	1058			t	t						
2,5-Dimethyl styrene	1059	1099[17]		0.2	t		0.3	0.1			0.3
Terpinolene	1064	1089[17]		t	t		0.2	t			
6-Methyl-3,5-heptadien-2-one	1064			0.1	t		0.3	t			0.2

Components	RI_E	RI_L	Propolis							Plant Sources	
			Cluster I			Cluster II			Cluster III		
			Min	Max	Aver	Min	Max	Aver		P	C
Phenyl ethyl alcohol	1064			0.1	t						
n-Nonanal	1073	1073[16]		2.0	0.6	t	1.2	0.5	0.3		0.7
Linalool	1074	1082[16]		2.7	0.7	t	1.5	0.6	0.3	0.3	0.6
Chrysanthenone	1081								t		
cis-Rose oxide	1083			t	t		0.2	t			
α-Fenchol (= *endo*-fenchol)	1085			0.1	t		0.1	t	t		
α-Campholenal	1088			t	t		0.8	0.1	0.6		0.9
trans-*p*-menth-2-en-1-ol	1095						t	t			
trans-Rose oxide	1100			0.3	t						
Camphor	1102	1107[16]		t	t		0.2	t			0.5
trans-Pinocarveol	1106			0.5	0.1		1.1	0.3	2.7		1.9
cis-Verbenol	1110			t	t		0.4	0.1	0.6		0.4
trans-Verbenol	1114						1.2	0.3	1.1		1.5
trans-Tagetone	1116			t	t						
trans-Pinocamphone	1121							t	0.2		0.5
Pinocarvone	1121	1165[17]					0.8	0.1	0.2		0.5
cis-Tagetone	1123			t	t						
Benzyl acetate	1123			1.0	0.1		0.5	0.1		t	
2-*trans*-Nonen-2-al	1124			0.1	t						
Nerol oxide	1127			0.4	t		0.3	t			
α-Phellandrol	1134						0.6	0.1			
β-Mentha-1,5-dien-8-ol*	1134			0.4	t		1.7	0.2			3.4
Borneol	1134	1137[16]		0.5	0.1		1.7	0.3	1.1	t	
p-Methylacetophenone	1143				t		t	t			t
Terpinen-4-ol*	1148	1177[16]		1.2	0.2		1.0	0.2	0.6		0.9
Octanoic acid	1152	1180[16]		0.4	t		t	t		t	
Myrtenal	1153						0.6	0.1	0.4		0.4
Methyl salycilate	1159			0.2	t						
α-Terpineol	1159	1157[16]		0.6	0.1		0.2	0.1	2.5	t	0.1
Safranal	1160			t	t						
Verbenone	1164	1170[16]					0.3	t			
2-Decanone	1166			0.1	t						1.6
Myrtenol	1168	1196[17]		0.6	t		1.0	0.2	1.9		
cis-*p*-Menthan-2-one*	1172			0.1	t						
n-Decanal	1180			1.2	0.2	t	1.0	0.6			
trans-Carveol	1189	1217[17]					0.3	0.1	0.5		0.7
cis-Ocimenone	1200			0.4	t		0.2	0.1			0.9
Carvone	1206	1243[17]					t	0.0			0.2
trans-Ocimenone	1207			0.1	t		t	t			0.4
Carvacrol methyl ether	1224			t	t						
2-Decenal	1224			0.2	t		0.1	t			
2-Phenyl ethyl acetate	1228			0.7	0.1		0.3	t		t	0.2
Geraniol	1236	1253[17]		0.7	0.2		0.2	0.1		0.1	t
Ethyl guaiacol*	1242						t	t			
Nonanoic acid (= pelargonic acid)	1263			0.8	0.1		0.4	0.1			t
Bornyl acetate	1265	1259[16]		0.3	0.1		0.7	0.1	t		1.8
trans-Cinnamyl alcohol	1268			0.2	t						
Thymol	1275	1290[17]		6.5	0.6	8.2	64.3	34.6	t	0.5	
Carvacrol	1286	1299[17]		0.4	0.1		0.4	0.1			t
Dihydrolinalyl acetate*	1217						0.3	t			0.2

Components	RI_E	RI_L	Propolis							Plant Sources	
			Cluster I			Cluster II			Cluster III		
			Min	Max	Aver	Min	Max	Aver		P	C
2-Phenyl ethyl propionate	1321			0.5	t						
Eugenol	1327	1327[16]		0.3	t		t	t		1.0	0.1
Octyl isobutyrate	1338								t		
α-Cubebene	1345	1351[16]		0.6	t		0.5	0.1	0.1		
7-Acetyl-2,6,6-trimethylbicyclo [4.2.0] octane*	1346						0.2	t			0.4
Decanoic acid (= capric acid)	1350			2.7	0.4		0.3	0.2			t
Cyclosativene	1363	1378[18]									0.2
Geranyl acetone	1370	1426[16]					t	t			
α-Ylangene	1371			0.6	0.1		0.1	t		0.1	
α-Copaene	1375	1377[16]		3.9	0.2		1.0	0.1		0.9	
β-Bourbonene	1379			2.5	0.1		1.3	0.3	0.5		
Ylang-2,4(15)-diene*	1398			0.3	t		0.2	t			
Longifolene	1399			0.5	t		0.2	t	1.5		
α-Ionone	1399						t	t			
α-Cedrene	1400			0.8	0.1						
trans-Cinnamyl acetate	1414			t	t		t	t			
β-Cedrene	1414			0.2	t						
β-Caryophyllene	1414	1415[16]		1.2	0.2		0.5	0.2	1.5	0.1	
β-Copaene	1426	1430[18]		0.2	t		0.3	0.1			
trans-α-Bergamotene	1434	1434[18]		0.5	0.1		t	t			
β-Ylangene	1435			0.3	t						
Guaia-6,9-diene	1447			0.8	t		t	t			
α-Humulene	1447	1439[16]		1.2	0.1		0.3	0.1	t	t	
allo-Aromadendrene	1456	1460[16]		2.6	0.4		1.5	0.4		1.5	t
Geranyl propionate	1461										t
trans-Cadina-1(6),4-diene	1469	1472[18]		2.1	0.2		0.2	0.1		0.2	
γ-Muurolene	1469	1474[18]		2.3	0.4		1.7	0.6		1.3	
α-Amorphene	1469			0.5	t						
(10,11)-Epoxycalamenene*	1469						t	t		0.7	
Germacrene D	1474	1474[16]		t	t		0.4	0.1			
ar-Curcumene	1475			1.3	0.1						
γ-Curcumene	1475			1.7	0.1						
β-Selinene	1476	1486[18]		0.2	t					0.3	
Valencene	1484			0.9	0.2		0.4	0.1			
Viridiflorene	1487						1.1	0.2			0.3
α-Muurolene	1494	1494[16]		1.7	0.3		0.7	0.2		1.9	
γ-Cadinene	1500	1507[18]		3.5	1.1		2.2	0.8		4.9	
n-Pentadecane	1500			1.5	0.2		t	t	t		
β-Bisabolene	1500	1506[17]		1.4	0.1						
trans-Calamenene	1505	1517[18]		1.6	0.4		0.7	0.2	t	1.7	t
δ-Cadinene	1505	1513[16]	0.3	7.6	1.8	0.4	3.5	1.6	1.1	6.8	0.2
α-Calacorene	1525	1525[16]		2.4	0.3		0.8	0.3		0.7	t
Isocaryophyllene	1528			0.3	t		t	t		0.2	
α-Cadinene	1529			0.5	0.1		0.1	t		0.8	
Elemol	1530			t	t						
1-*epi*-3,4-Dehydroviridiflorol*	1533						1t	2.7			
Hexenyl benzoate*	1533			0.1	t						
trans-α-Bisabolene	1536			0.2	t						
trans-Nerolidol	1549	1547[16]		8.8	0.4		0.5	0.1	2.0		
β-Calacorene*	1550			t	t						
β-Caryophyllene alcohol	1550						0.3	t			0.1

Components	RI_E	RI_L	Propolis							Plant Sources	
			Cluster I			Cluster II			Cluster III		
			Min	Max	Aver	Min	Max	Aver		P	C
Dodecanoic acid	1551			2.0	0.1		t	t			
Spathulenol	1551	1557[16]		1.2	0.2	0.2	1.3	0.9		0.1	0.2
Caryophyllene oxide	1561	1565[16]					0.9	0.2	6.3		0.1
Globulol	1566	1571[16]					2.5	0.7			
Viridiflorol	1569			8.1	1.3	t	10.2	2.1		0.1	7.0
Cedrol	1574	1601[17]		9.6	0.6		t	t	2.4		
Guaiol	1575			3.4	0.6		t	t			
Anhydrooplopanone	1576						2.1	0.6			
β-Oplopenone	1576			3.7	0.6		1.2	0.2		0.9	
Ledol	1580	1590[16]		3.0	0.3		4.7	1.5			2.4
Humulene epoxide II	1580	1608[17]							1.0		
1,2-Dehydroviridiflorol*	1582						3.5	0.9			
n-Tetradecanal	1596			t	t		t	t			
n-Hexadecane	1600			1.1	t						
1-*epi*-Cubenol	1600	1629[17]		1.1	0.2		0.2	0.1		0.5	
γ-Eudesmol	1609		3.1	18.0	8.4	2.1	9.0	3.8		7.6	0.1
T-Cadinol	1616	1616[16]	1.0	8.8	4.0	1.5	7.2	3.2		8.9	0.4
δ-Cadinol	1618			3.4	0.1						
α-Muurolol	1618	1627[16]		t	t		5.1	1.3			
β-Eudesmol	1620		2.1	25.5	11.2	1.0	12.7	4.4	1.3	20.4	0.4
1,2-Dehydroglobulol*	1623						2.6	0.7			
Valerianol	1623			13.5	1.2		2.1	0.3		t	0.3
α-Cadinol	1626	1637[16]					8.9	2.5			
α-Bisabolol oxide B*	1630			9.5	0.4						
α-Eudesmol	1634			24.5	12.7		12.6	4.1	0.9	20.6	0.3
Cadalene*	1640			0.5	t		0.3	t		0.1	0.1
cis,*trans*-Farnesol	1648			0.9	0.2						
α-Bisabolol	1656	1656[16]		44.7	2.1						
epi-α-Bisabolol	1658			0.8	0.1		t	t			
n-Heptadecane	1700		0.2	4.4	1.2	0.2	3.1	1.1	1.1	t	0.1
Benzyl benzoate	1701			4.3	0.2				1.4		0.1
α-Bisabolol oxide A*	1702			t	t						
Tetradecanoic acid	1723	1749[16]									t
Phenylethyl octanoate *	1764			4.4	0.2		t	t			t
n-Hexadecanal	1776			t	t						
Benzyl salicylate	1790			9.5	0.4						
n-Octadecane	1800			0.8	t		0.4	0.1			
n-Hexadecanol	1821			7.6	0.4						
UI Labdanes 1	1821			8.2	0.4						0.3
UI Labdanes 2	1829			1.1	t						
n-Heptadecanal	1894			1.1	0.1						
n-Nonadecane	1900			8.9	2.1	0.7	5.1	2.0	2.9		0.3
Hexadecanoic acid	1908			2.9	0.2		t	t		1.1	
Ethyl hexadecanoate	1936									0.1	
8-*epi*-13-*nor*-Ambreinolide*	1965			1.0	0.1						
n-Eicosane	2000			0.8	0.1		0.4	0.1	0.8	t	
2-Phenyl ethyl phenyl acetate*	2017										3.6
Abietatriene	2027	2057[17]		t	t						
Phytol acetate	2047			1.1	0.2						
Manool*	2047			7.6	0.3				1.6		

Components	RI_E	RI_L	Propolis							Plant Sources	
			Cluster I			Cluster II			Cluster III		
			Min	Max	Aver	Min	Max	Aver		P	C
Abietadiene	2060	2088[17]		0.9	t						
Musk ambrette*	2061			0.3	t		0.2	0.1			
n-Octadecanol (= Steryl alcohol)	2071			0.9	t		t	t			
cis,trans-Farnesol tiglic ester	2098						t	t			
n-Heneicosane	2100		0.2	7.1	1.8	0.5	1.5	1.0	2.2	0.7	
Linoleic acid	2125									0.2	
Ethyl linoleate	2137									0.5	
n-Docosane	2200			1.7	0.5		0.9	0.4	1.1	0.1	
Labd-7-en-15-ol	2201			5.1	0.3						
Sandaracopimarinol*	2219			0.3	t						
trans-Totarol	2259			6.9	0.3						
n-Eicosanol	2265			0.8	0.1		0.4	0.1			
n-Tricosane	2300			6.1	1.5		2.3	0.7	3.0		
n-Tetracosane	2400			8.3	0.7				2.0		
n-Pentacosane	2500			2.9	0.9		1.2	0.5	1.6	0.3	
Grouped Components											
Monoterpene hydrocarbons				5.8	0.8	t	5.5	1.1	25.5		4.3
Oxygen-containing monoterpenes			0.3	8.4	2.2	8.6	65.2	38.0	12.7	0.9	16.0
Sesquiterpene hydrocarbons			0.6	35.0	6.5	1.0	13.5	5.6	4.7	21.5	0.8
Oxygen-containing sesquiterpenes			20.4	76.9	44.6	16.3	49.3	30.5	13.9	59.8	11.3
Diterpene hydrocarbons				9.2	0.5						0.3
Oxygen-containing diterpenes				14.8	0.9				1.6		
Phenylpropanoids				0.3	0.0		t	t		1.0	0.1
Fatty acids				4.0	0.8		0.6	0.2		1.3	t
Others			3.6	36.2	12.2	4.8	13.8	8.7	16.4	1.8	39.0

RI_E – Experimentally obtained retention index relative to C_8-C_{25} *n*-alkanes on the DB-1 column; RI_L – Retention Index from literature; Min – Minimum value; Max – Maximum value; Aver – Average value; P- *Populus x canadensis*; C – *Cistus ladanifer*; t - trace (<0.05%); * Identification based on mass spectra only; unless otherwise stated, an empty cell means that the compound was not detected in the sample; UI –Unidentified.

Cluster II, with seven samples, comprised some from the north (N5, N11-14), one from the south (S4) and the sample CI4 from central interior (Figure 1). Cluster II was mainly characterized by high oxygen-containing monoterpenes content (9-65%), oxygen-containing sesquiterpenes (16-49%) and the fraction designated as others (5-14%) (Table 2, Figure 1). Cluster II was sub-divided in two moderately correlated sub-clusters (S_{corr} = 0.44). The sub-cluster comprising samples N5 and N14 (North), S4 (South) and CI4 (central interior) was richer in oxygen-containing monoterpenes while N11, N12 and N13 (North) showed a higher content in oxygen-containing sesquiterpenes. Thymol (8-64%), 1-*epi*-3,4-dehydroviridiflorol (10%), viridiflorol (t-10%), β-eudesmol (1-13%), α-eudesmol (13%) were the main compounds present in this cluster samples volatiles. The high thymol content in most of these samples is probably linked with the long-term use of this compound as acaricide in the treatment against the ectoparasitic mite *Varroa destructor,* as pointed out by Miguel *et al*. [11]. These authors also found a predominance of viridiflorol, *n*-tricosane and *n*-nonadecane in the majority of propolis samples volatiles from the Algarve region. Comparing Miguel *et al*. [11] data with the present results, from samples obtained within the same region, but from different geographical locations, all samples volatiles had in common a high content in viridiflorol (5-8%) and *n*-nonadecane (3-7%), with exception for sample S1, from the east coast in which β-eudesmol (14%) and α-eudesmol (18%) were the main components.

Cluster III, with only one sample from Madeira Island (M2), was mainly composed by monoterpene hydrocarbons (26%), the others fraction (16%), oxygen-containing sesquiterpenes (14%) and oxygen-containing monoterpenes (13%) (Table 2, Figure. 1). Unlike the other propolis clusters, cluster III was α-pinene rich (19%) followed by β-caryophyllene oxide (6%) and β-pinene (6%), and

showed lower relative amounts of α-eudesmol and β-eudesmol. α-Pinene dominance was also detected in propolis from Greek regions [10]. The abundance of the monoterpene fraction, with a high α-pinene content, was described in the species of the genus *Juniperus*, particularly *Juniperus cedrus* [19,20], which is endemic to Madeira Island and could therefore be a possible contributor for this propolis sample volatiles.

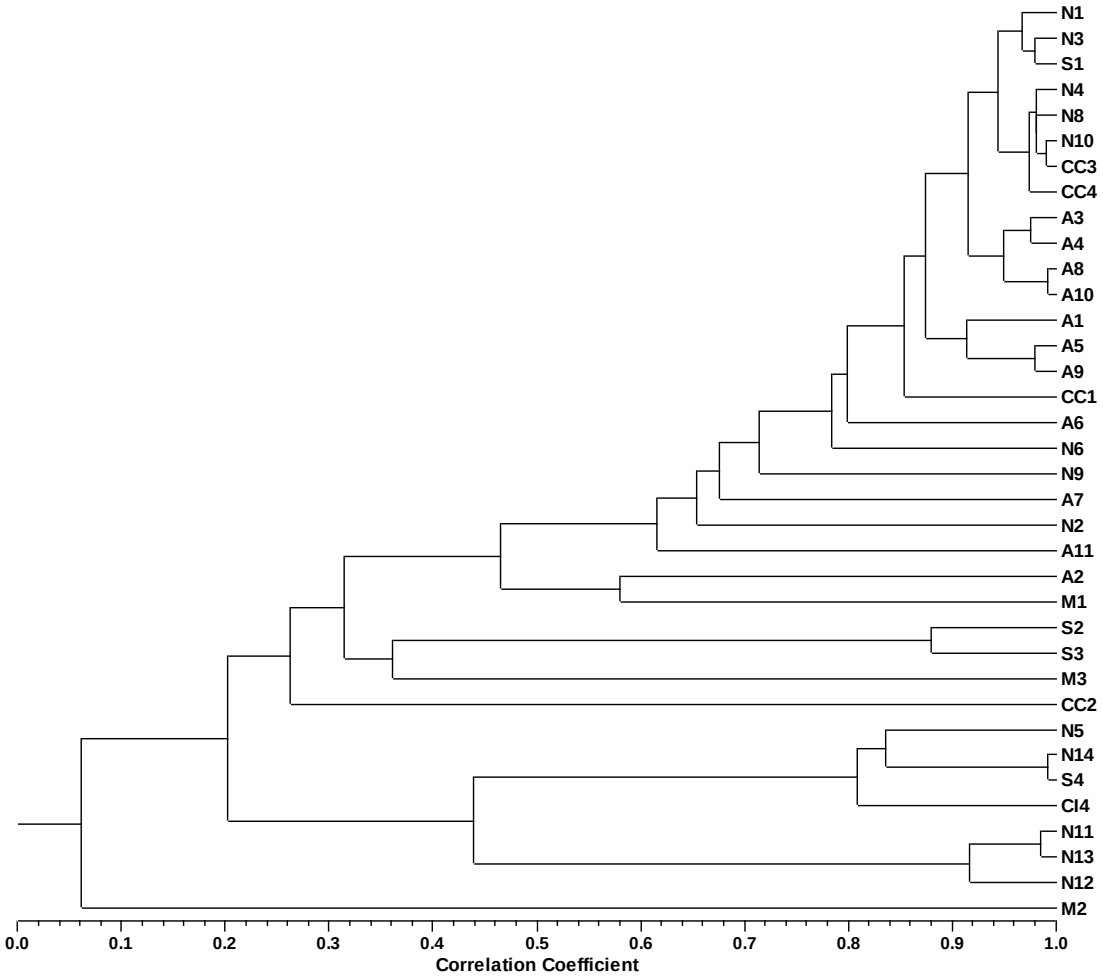

Figure 1. Dendrogram obtained by cluster analysis of the percentage composition of volatiles isolated from propolis based on correlation and using unweighted pair-group method with arithmetic average (UPGMA). Twenty-eight samples constituted cluster I, seven cluster II and one cluster III. For abbreviations see section 2.1.

The composition of the volatile oils isolated from the 36 propolis samples did not allow grouping them according to their geographical collection site. Moreover, no relationship could be established between the chemical composition of the volatiles and the collection year. In this context, the chemical polymorphism recorded could be mainly a result of the local flora characteristics at the bee harvesting site, which is discussed in the following section.

3.2. Essential oil composition of plant sources

The odour of propolis is empirically used by beekeepers to assess its floral origin, which reflects the floral sources present around the hive. To address the floral origin of propolis, the evaluation of the

volatile composition of the leaf-buds of the hybrid *Populus x canadensis* and branches of *Cistus ladanifer* was included in the study, as two potential floral sources referred by propolis producers. *Populus x canadensis* is a hybrid species very common in Portugal and therefore a potential source of resin for the honeybees. *Cistus ladanifer* (rock rose), an wild odorous shrub widespread in the mediterreanean region [21], was also studied as potential plant source due to its great abundance in some hive neighbourhoods but also based on the typical rock rose aroma presented by some of the propolis samples.

The essential oil isolated from the poplar buds was clear, colourless and attained a yield <0.05% (v/w). Forty eight volatile components were identified, representing 86% of the overall essential oil, Table 1. Oxygen-containing sesquiterpenes were the main group of constituents (60%), followed by sesquiterpene hydrocarbons (22%). Monoterpenes, diterpenes and others were found only in very low percentage (≤2%), Table 2, Figure 1. Among the main groups, the sesquiterpene alcohols α-eudesmol (21%), β-eudesmol (20%), γ-eudesmol (8%), T-cadinol (9%), the sesquiterpenes hydrocarbons δ-cadinene (7%) and γ-cadinene (5%) were predominant. The high level of eudesmol isomers found in the present study was also described in the volatile composition of other poplar species leaf-buds, particularly *Populus nigra* and *Populus balsamifera* [22,23].

Populus species are described to be the main source of propolis in temperate regions of the world [2]. *Populus x canadensis* essential oil composition was comparable with the one obtained for cluster I samples, with a dominance of oxygen-containing sesquiterpenes, especially α- and β-eudesmol. The presence of high levels of bisabolol in some propolis samples within this cluster is not consistent with the profile observed for the poplar specie under study; however, we must consider that other poplar species may show different volatile profiles. Indeed, the presence of bisabolol was previously described in bud exudates of *Populus generosa* and *Populus candicans* [24,25]. Thus, besides the contribution of *Populus x canadensis* as a source of resin for propolis samples of cluster I, other poplar species can be as well potential contributors and so, creating the diversity found in the samples. Moreover, in cluster II, α- and β-eudesmol are within the main compounds (at less extent), which are indicative of some contribution of the poplar resin to this type of propolis.

On rock rose essential oil seventy three components were identified, representing 79% of the overall volatiles (Table 2). The fraction named as others (39%), oxygen-containing monoterpenes (16%) and oxygen-containing sesquiterpenes (11%) were the most abundant groups (Table 2). The main components identified in *Cistus ladanifer* volatiles were the aromatic ketone 2,6,6-trimethylcyclohexanone (20%), viridiflorol (7%), 2-phenyl ethyl acetate (4%) and β-mentha-1,5-dien-8-ol (3%). Other compounds like α-pinene, *trans*-pinocarveol, *trans*-verbenol, 2-decanone, bornyl acetate and ledol, were found in lower percentages. The main characteristic compounds associated in the literature with *Cistus ladanifer* and labdanum oil were present [21,26,27], namely: 2,6,6-trimethylcyclohexanone, acetophenone, viridiflorol, ledol and labdane. Nevertheless, there were some minor differences between our results and previous ones [21,26,27], which are probably linked to the diverse geographical origin of the plants analysed, to different plant parts analysed, or to the fact that under the common name rock rose reported by beekeepers, different *Cistus* species can be included.

Comparing the rock rose volatile profile and to that from propolis samples, only samples CI4 from central interior and S2-4 from the south showed a predominance of viridiflorol (10% and 5-8% respectively). Additionally, these samples also revealed the presence of ledol (2 to 5%) and other components associated with the typical *Cistus* aroma, like, 2,6,6-trimethyl cyclohexanone, acetophenone and labdane compounds [28]. Due to the lack of other resin source in the surroundings of the apiary, *Cistus ladanifer* is likely to be a strong contributor for Portuguese propolis volatiles, particularly in central interior and south regions.

4. Conclusions

Volatiles composition, isolated by hydrodistillation from 36 propolis samples from different provenances in Portugal (mainland and islands), were evaluated. Cluster analysis showed a major chemical variability in the volatile profile, displaying three poorly correlated main clusters. *Populus x canadensis* and *Cistus ladanifer* essentials oils chemical composition was compared to those of propolis samples and some correlation was found among them. *Populus x canadensis* essential oil composition was comparable with the one obtained for cluster I samples, with a dominance of oxygen-

containing sesquiterpenes, especially α- and β-eudesmol. Samples CI4 from central interior and S2-4 from the south showed a predominance viridiflorol and also presence of minor compounds such ledol, 2,6,6-trimethyl cyclohexanone, acetophenone and labdane compounds associated with typical *Cistus* aroma. Thus, in the absence of other resin sources, *Cistus ladanifer* is likely to be a strong contributor for Portuguese propolis volatiles, particularly in central interior and south regions.

The propolis samples volatiles diversity seems to be more dependent on the variability, as well as on the availability, of the flora sources at the site of bee collection, rather than the geographical origin.

Acknowledgments

Soraia I. Falcão thanks Fundação para a Ciência e a Tecnologia (FCT) for the PhD grant SFRH/BD/44855/2008, financially supported by POPH-QREN and FSE. This study was partially funded by FCT, under Pest-OE/EQB/LA0023/2011 and PTDC/CVT-EPI/2473/2012. Thanks to National Federation of Portuguese Beekeepers for propolis samples.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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