Propolis from Northern California and Oregon: Chemical Composition, Botanical Origin, and Content of Allergens

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Propolis is a beehive product that bees manufacture by mixing their own wax with vegetable resins collected from different species of trees and bushes. The chemical composition of propolis is very variable because it depends on the flora locally available, and specimens from different geographical and climatic areas display unique properties. In this paper, the results of the chemical characterization of some propolis specimens collected in northern California and in Oregon are presented. Their chemical compositions show that all specimens contain resins from poplars of the *Tacamahaca* section (balsam poplars) – characteristic of the western part of the North American continent. Nevertheless, some of the specimens are of mixed origin because they also contain resins from poplars of the *Aigeiros* section (cottonwoods) – also present in this part of the world. Propolis causes allergies in sensitive human individuals, which are due to the presence of certain esters. The contents of known propolis allergenic esters – phenylethyl caffeate, 1,1-dimethylallyl caffeate, benzyl cinnamate, and benzyl salicylate – have been investigated in these specimens and found to depend on the botanical origin.

Key words: Poplar, *Tacamahaca*, *Aigeiros*, Propolis

Introduction

Propolis is a resinous product that bees manufacture by mixing their own wax with vegetable resins. The bees use it to finish the hive, close and limit entrances, avoid flooding, embalm dead invaders, and prevent their rot. Thanks to its antiseptic properties, propolis keeps the hive environment healthy and limits the spread of infections. Bees gather the vegetable resins for the manufacture of propolis from bud exudates and resins of different species of trees and bushes (Marcucci, 1995). This fraction of propolis is generally called balsam, and can be separated from waxes and debris by extraction with ethanol or water/ethanol mixtures (Park and Ikegaki, 1998). In temperate areas, poplar propolis is the most widespread type. Nevertheless, many other propolis are known that have very different botanical origins, i.e. they are made up of completely different balsams and thus show very different chemical profiles (Bankova *et al.*, 2000).

Propolis displays several beneficial properties and is very popular in folk medicine (Burdock, 1998; Banskota *et al.*, 2001). Nevertheless, it may cause allergic reactions that can be severe. Up to 2.5 % of the population may potentially suffer from this side effect (Hausen, 2005). Some caffeic acid esters – 1,1-dimethylallyl caffeate and phenylethyl caffeate – are the major allergens found in propolis from *Aigeiros* poplars (Hausen *et al.*, 1987a, b; Hausen, 2005). The esters benzyl salicylate and benzyl cinnamate also play a role in allergy development (Hausen and Wollenweber *et al.*, 1988) – albeit a minor one – and are minor components of propolis from *Aigeiros* poplars (Aliboni *et al.*, 2011). Allergic reactions that are induced by non-poplar propolis have also been reported. The compounds that are the cause of these reactions are not always known (Hausen, 2005).

The western part of the Northern American continent displays a variety of ecological environments, ranging from desert to temperate rain forest. Poplars are common, and in California and Oregon species from both the *Tacamahaca* [*Populus trichocarpa* (USDA-NRCS, 2013a), *Populus balsamifera* L. (USDA-NRCS, 2013b)] and the *Aigeiros* sections [*Populus fremontii* (USDA-NRCS, 2013c) and *Populus alba* (USDA-NRCS, 2013d)] are present. The poplars

from these two sections are similar and are not easily distinguishable by a morphological analysis. Nevertheless, the genetic fingerprinting analysis of microsatellite loci can effectively discriminate between them (Liesebach *et al.*, 2010). They can also be differentiated by the chemical analysis of their respective resins and bud exudates that have been the object of thorough studies and that are characteristic for the presence of certain compounds (English *et al.*, 1991; Greenaway *et al.*, 1989a, b, 1990; Mattes *et al.*, 1987). Few reports are available on the composition and the botanical origin of propolis from this part of the world. The chemical composition of the balsamic fraction of a propolis specimen from Vancouver Island (BC, Canada) – rain forest environment – has been characterized. Its origin was from *Populus trichocarpa* (Christov *et al.*, 2005). Specimens from the Sonoran Desert (AZ, USA) were also characterized. Some were from *Populus fremontii*, but others had a completely different origin – the balsamic material came from typical desert bushes belonging to the Asteraceae family (Wollenweber and Buchmann, 1997).

In an effort to increase and consolidate knowledge on the composition of North American propolis, some specimens have been collected from different locations in Oregon and northern California and characterized for the first time. The balsamic fractions of all specimens contained resins from *Tacamahaca* poplars. Nevertheless, some specimens also unequivocally contained resins from poplars of the *Aigeiros* section and were thus of mixed origin. The two different types showed similar chemical profiles, but the difference in their botanical origin had a considerable impact on the content of allergens.

Materials and Methods

Chemicals

HPLC (high-performance liquid chromatography) eluents were acetonitrile "CHROMASOLV® gradient grade for HPLC" (Sigma-Aldrich Corporation, St. Louis, MO, USA) and water (18 MΩ) both acidified with 0.1% (v/v) formic acid (Baker, Deventer, Holland). Absolute ethanol, *n*-octane, hydrochloric acid, anhydrous sodium sulfate, and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma-Aldrich, *n*-hexane and isooctane from Panreac (Castellar del Vallès, Spain) were all analytical grade.

Analytical standards were as follows: caffeic acid (95%), *p*-hydroxy benzoic acid (99%), ferulic acid (99%), *p*-hydroxy acetophenone (98%), *p*-coumaric acid (98%), *t*-cinnamic acid (99%), 1,1-dimethylallyl caffeate (98%), cinnamyl cinnamate (95%, mixture of isomers), *p*-hydroxy benzaldehyde (98%), ethyl salicylate (99%), methyl cinnamate (99%), ethyl cinnamate (98%), benzyl benzoate (99%), benzyl salicylate (98%), benzyl cinnamate (98%), hexyl salicylate ($\geq$ 99%), phenylethyl salicylate (97%), phenylethyl cinnamate (96%), α-pinene (98%), eucalyptol (99%), γ-terpinene (98.5%), terpinen-4-ol (95%), α-terpineol (96%), *t*-nerolidol (85%) were from Sigma-Aldrich. Phenylethyl caffeate (98%) was from Biotrend (Destin, FL, USA). Isosakuranetin (99%), pinocembrin (95%), chrysin (90%), galangin (99%) were from Extrasynthese (Lyon, France).

Hexyl cinnamate was synthetized by direct reaction of 1-hexanol and *t*-cinnamic acid using sulfuric acid as catalyst as reported elsewhere (Aliboni *et al.*, 2011).

Propolis specimens

Raw propolis specimens were collected in Oregon and California (Fig. 1). Specimen Or1 was from Klamath Falls, Klamath County, OR, USA, and do-

Fig. 1. A map of the area where specimens were collected. The dots represent major cities in the area, the stars the propolis harvesting locations.

nated by Mrs. Carol and Mr. Alistair Mowat of Mowat Apiaries (Weed, CA, USA). Specimen Or2 was from Shady Cove, Jackson County, OR, USA, and donated by Mr. Henrique Mori. Specimen Or3 was from Corvallis, Benton County, OR, USA, and donated by Prof. Ramesh Sagili of Oregon State University, Corvallis, OR, USA. Mr. Seth Rick donated three specimens collected in different areas from Humboldt Redwoods State Park, Humboldt County, CA, USA: specimen Ca1 near Rio Dell, specimen Ca2 near Phillipsville (inside the forest), and specimen Ca3 also from apiaries located inside the forest itself.

Propolis mother solutions (about 5 g/L) in ethanol were prepared by sonication. The mass fractions of ethanol-soluble matter (balsamic fraction), *n*-hexane-soluble (waxes), and insoluble matter were determined following the treatment scheme reported elsewhere (Aliboni *et al.*, 2011).

UV spectra were recorded in ethanol with an Evolution 201 UV-VIS spectrophotometer from Thermo Fisher Scientific (Waltham, MA, USA). The mother solutions were diluted to about 25 mg/L in 25-mL volumetric flasks, and spectra were measured in the 400 – 230 nm interval against pure ethanol. The points of maxima and minima were registered along with their specific absorption $E_{1\%}$ (cm^{-1}).

GC-MS analyses

The GC-MS (gas chromatography-mass spectroscopy) instrument was from Thermo Fisher Scientific: oven, Trace GC; detector, ion trap, Polaris Q; autosampler, Triplus, compatible with both liquids and headspace; injector, PTV, split-splitless; carrier gas, He, constant flow of 1 mL/min. The column used in all runs was an SLB™-5 ms (30 m × 0.25 mm, 0.25 μm film thickness) from Supelco (Bellefonte, PA, USA).

Derivatization of ethanolic propolis extracts was carried out as follows. Twenty μL of a 500 mg/L ethanolic propolis solution (about 7 μg of propolis ethanol-soluble matter) were pipetted into an 1-mL conical vial. The solvent was evaporated with a gentle flow of nitrogen, and 20 μL of BSTFA were added to the residue. The resulting solution was heated at 60 °C for 5 min. Fifty μL of isooctane were added, and the solution was again heated at 60 °C. After 25 min, 130 μL of isooctane were added, and the solution was analysed without further treatments. Chromatographic parameters for the analysis of these solutions were as follows: elution program, 80 °C for 1 min, at 25 °C/min to 110 °C, at 10 °C/min to 320 °C, hold for 10 min; injection, CT splitless, 1 min hold, 300 °C, 1 μL injected. Detection was as follows: MS transfer line, 250 °C; ion source, 250 °C; fragmentation, EI, 70 eV; full scan, m/z^+ 50 – 750.

Quantitative analysis of the allergenic esters benzyl cinnamate and benzyl salicylate was carried out following a GC-MS analytical protocol reported elsewhere (Aliboni *et al.*, 2011). Briefly, 15 mL of ethanolic propolis solution (5 g/L) were suspended in 90 mL of 17 mM HCl along with known amounts of the internal standards (about 15 μg each of hexyl cinnamate and hexyl salicylate from a standard ethanolic solution) in a 250-mL separating funnel. The suspension was extracted thrice with *n*-hexane, the combined extracts filtered over anhydrous sodium sulfate, and concentrated in *n*-octane for GC-MS analysis. Chromatographic parameters for the analysis of these solutions were as follows: elution program, 100 °C for 1 min, at 30 °C/min to 130 °C, at 10 °C/min to 280 °C, hold for 2 min; injection, CT splitless, 1 min hold, 260 °C, 1 μL injected. Detection was as follows: MS transfer line, 250 °C; ion source, 250 °C; fragmentation, EI, 70 eV; mass range, SIM using ions typical of the fragmentation of both target esters and of internal standards reported elsewhere (Aliboni *et al.*, 2011). The untreated *n*-hexane extracts were analysed by the same method, but in the TIC mode and a range of m/z^+ 50 – 450.

For headspace analysis, 80 – 110 mg of solid propolis were placed in an autosampler vial (pure standards, 20 μL of each compound). In a first series of analyses, 1,000 μL of vapours (200 μL for the analyses of pure standards) were drawn, after heating the sample for 2 min at 70 °C, with an injection syringe at 85 °C. Chromatographic parameters for these analyses were as follows: elution program, 55 °C for 1 min, at 10 °C/min to 220 °C; injection, CT split, 1 min hold, split ratio 10 mL/min (200 mL/min for the analyses of pure standards), 260 °C. Detection was as follows: MS transfer line, 250 °C; ion source, 250 °C; fragmentation, EI, 70 eV; full scan, m/z^+ 34 – 300. In a second series of analyses, 1,000 μL of vapours (200 μL for the analyses of pure standards) were drawn, after heating the sample for 2 min at 110 °C, with an injection syringe at 115 °C. Chromatographic parameters for these analyses were as follows: elution program: 120 °C for 1 min, at 10 °C/min to 240 °C; injection, CT split, 1 min hold, split ratio 10 mL/min (200 mL/min for the analyses of pure standards), 260 °C. Detection was as follows: MS transfer line, 250 °C; ion source, 250 °C; fragmentation, EI, 70 eV; full scan, m/z^+ 34 – 300.

Table I. Analytical parameters for HPLC determination of flavonoids, aromatic acids, and esters. The sensitivities are reported relative to that of caffeic acid, set as 1. All calibrations were linear in the 3 – 42 ng (0.5 – 7 mg/L) range, r^2 values are the respective coefficients of determination of the least squares interpolations. Retention times (R_t) are in minutes – all ±0.2 min. The limits of detection (LOD) are given in injected pg of a compound producing a signal three times higher than the average background noise of the baseline.

Compound	R_t [min]	Sensitivity	r^2	LOD [pg]
Caffeic acid	4.93	1.00	0.9990	3
p-Hydroxy benzoic acid	6.11	1.07	0.9974	3
Ferulic acid	6.70	1.02	0.9985	3
p-Hydroxy acetophenone	7.15	1.02	0.9996	3
p-Coumaric acid	8.22	1.13	0.9985	4
t-Cinnamic acid	13.88	1.56	0.9991	3
1,1-Dimethylallyl caffeate	21.23	0.792	0.9938	5
Phenylethyl caffeate	22.12	0.755	0.9892	5
Isosakuranetin	22.59	0.501	0.9992	6
Pinocembrin	23.18	0.478	0.9991	7
Chrysin	25.45	0.850	0.9984	4
Galangin	27.04	0.423	0.9834	8
Cinnamyl cinnamate	28.45	1.31	0.9928	3

Where possible, peak assignments were carried out using reference mass spectra and retention times measured by injection of solutions of pure standards. Other peaks were assigned by comparison of the registered mass spectra with those reported in either mass spectra libraries (NIST search 2.0) or the scientific literature. All mass spectra of peaks assigned using either NIST or literature had to obey the following parameters to be acceptable: The five major mass peaks had to have the same order of intensity in both reference and recorded spectrum, respectively, and ratios of the intensities of these peaks to that of the highest one should not differ by more than 20%. Some peaks could only be detected and identified following deconvolution of GC-MS profiles with AMDIS software. Under experimental fragmentation conditions, sesquiterpenes and sesquiterpenoids yielded mass spectra that were very similar to each other. Many peaks were therefore assigned to these classes of compounds rather than to a single specific compound. The same was true for mass spectra resulting from the fragmentation of diterpenes and diterpenoids.

HPLC analyses

The HPLC instrument was from Perkin-Elmer (Waltham, MA, USA) and consisted of the following parts: a binary pump "Series 200"; an injection group with a 6-μL loop; a UV-VIS detector "Series 200 UV/VIS detector"; an electronic interface "NCI 900". The data were acquired on a PC with Turbochrom software, version 4.

For the analysis, the ethanolic propolis solutions were diluted in 10-mL volumetric flasks with the initial eluent to a final concentration of about 200 mg/L (about 1.2 μg injected). The chromatographic method was the following: column, Ascentis Amide RP®, 250 mm × 4.0 mm, 5 μm particle size (Supelco); eluents, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); elution program, 35% B/65% A isocratic for 2 min, within 27.5 min to 100% B, 100% B isocratic for 3.5 min; flow, 750 μL/min.

Each solution was analysed four times, recording the absorption of the eluates at four different wavelengths: 320, 293, 275, and 250 nm. The peaks were preliminarily identified by comparing their retention times with those determined by injection of pure standard solutions and by co-injecting pure standards. The peak assignments were then confirmed by comparing the measured signal intensity ratios at different wavelengths with those determined with pure standards as explained elsewhere (Aliboni, 2010). Quantitative analysis was carried out with calibration curves established with signals recorded at 320 nm for caffeates, phenolic cinnamic acids, and galangin, with signals recorded at 275 nm for all other compounds. All calibrations were linear in the 0.5 – 7 mg/L range (Table I).

Results and Discussion

The specimens were extracted with ethanol to give balsam solutions, and the relative masses of the balsamic, wax, and insoluble fractions were determined

Table II. Physicochemical parameters of examined propolis specimens. The wavelengths of the maxima and the minima in the UV spectra are all ±1 nm. Mass fractions are expressed as percentage [(w fraction/w propolis) · 100]. RSD of all determinations are from measurements on four different solutions prepared from different portions of Ca1 specimen: UV coefficients, ±5 %; EtOH-soluble fraction, ±1 %; n-hexane-soluble fraction, ±8 %; insoluble, ±10 %.

Specimen	UV spectra parameters				Mass fractions by solubility (%)		
	Maximum [nm]	$E_{1\%}$ [cm^{-1}]	Minimum [nm]	$E_{1\%}$ [cm^{-1}]	Resin (EtOH-soluble)	Waxes (n-hexane-soluble)	Insoluble
Ca1	285.0	380	248.0	230	85	11	3.0
Ca2	288.0	290	248.5	160	76	17	7.0
Ca3	273.5	390	245.5	220	90	5.0	5.0
Or1	291.0	310	251.0	220	80	14	6.0
Or2	275.0	280	248.0	160	64	31	5.0
Or3	275.0	250	248.0	160	61	37	2.0
Average		317		192	76	19	4.7
RSD (%)		18		18	15	64	40

(Table II). The reported values were in ranges generally accepted in the literature (Aliboni *et al.*, 2011). The UV spectra of all specimens were characterized by a maximum and a minimum (Table II). The values of the $E_{1\%}$ coefficients at the maxima and the minima were comparable with those recorded for other poplar propolis (Aliboni *et al.*, 2011; Hamasaka *et al.*, 2004; Miyataka *et al.*, 1997) and were considerably homogeneous (see their average and RSD in Table II). These results indicate a common poplar origin for all examined specimens. The positions of the maxima and the minima were slightly shifted towards the violet region when compared with those reported elsewhere for poplar propolis of different geographical origin (Aliboni *et al.*, 2011; Hamasaka *et al.*, 2004; Miyataka *et al.*, 1997). This observation indicates that the specimens examined here have a botanical origin that is different from that of poplar propolis from Asia and Europe which have been characterized so far.

The contents of phenolics, cinnamic acids, and esters measured by HPLC are reported in Table III. All specimens displayed high contents of galangin, p-hydroxy acetophenone and t-cinnamic acid – all compounds that are typical of resins of poplars of the *Tacamahaca* section (English *et al.*, 1991). It is remarkable that ferulic acid and caffeates were below their limit of detection in the Ca2 and Ca3 specimens. From the limits of detection by this analytical protocol (Table I) the maximally possible contents of ferulic acid and caffeic acid esters in the balsamic fractions of these specimens were estimated to be 2.5 μg/g and 5.0 μg/g, respectively. The contents of these compounds were also very reduced in the Or2 specimen. Ferulic acid and caffeic acid esters are typical of resins

Table III. Content (μg/g) of aromatic acids, esters, and flavonoids in the ethanol-soluble fraction from propolis specimens determined by HPLC and of benzyl salicylate determined by GC-MS. RSD, 10 % for all values; BDL, below limit of detection.

Compound	Ca1	Ca2	Ca3	Or1	Or2	Or3	Average	RSD (%)
Caffeic acid	1750	355	490	5400	880	2150	1838	102
p-Hydroxy benzoic acid	300	360	390	BDL	800	550	480	42
Ferulic acid	1900	BDL	BDL	7900	146	420	2592	140
p-Hydroxy acetophenone	35,000	16,200	24,700	6050	11,000	30,100	20,508	55
p-Coumaric acid	8100	11,700	14,800	11,200	15,500	3950	10,875	40
t-Cinnamic acid	18,000	12,100	58,000	25,500	17,700	21,500	25,467	65
1,1-Dimethylallyl caffeate	9900	BDL	BDL	20,000	980	8700	9895	79
Phenylethyl caffeate	690	BDL	BDL	6900	800	3150	2885	101
Isosakuranetin	8500	9500	7500	9500	15,000	8000	9667	28
Pinocembrin	12,000	6000	5300	19,000	10,000	8500	10,133	49
Chrysin	11,700	11,900	8100	25,000	11,400	11,200	13,217	45
Galangin	25,000	30,000	28,000	31,000	32,000	13,500	26,583	26
Cinnamyl cinnamate	4500	1500	3500	3600	3100	3200	3233	30
Benzyl salicylate	6100	4800	8500	200	2300	12,000	5650	75

Table IV. Compounds detected in the GC-MS profiles of silylated propolis specimens. Names are given without TMS ester diction. ID mode refers to the use of either a pure standard (St), a spectrum from NIST library (NIST), or a literature reference mass spectrum for the peak identification (references in table notes). 1 refers to a compound displaying a peak that is 10% or more of the intensity (by height) of the major peak in the TIC profile; 2 refers to a peak that is below 10% of the intensity of the major peak and can be observed in the TIC profile; 3 refers to a peak that can be detected only in the SIM mode and/or following AMDIS deconvolution; 4 refers to a peak below detection limit.

Compound	ID mode	R_t [min]	Ca1	Ca2	Ca3	Or1	Or2	Or3
Succinic acid	NIST	7.26	2	2	2	1	2	3
p-Hydroxy benzaldehyde	St	8.18	2	2	2	2	2	2
3-Phenyl propanoic acid	NIST	8.66	2	2	2	2	2	3
Cinnamyl alcohol	NIST	8.79	4	3	2	3	4	3
Decanoic (caprinic) acid	NIST	9.08	2	2	2	2	2	2
p-Hydroxy acetophenone	St	9.35	1	1	1	2	1	1
1,6-Hexandioic acid	NIST	9.63	2	1	2	2	2	2
Methoxy benzoic acid[a]	NIST	9.93	4	3	4	3	3	3
t-Cinnamic acid	St	10.26	1	1	1	1	1	1
2-Hydroxy-3-phenyl propanoic acid	NIST	10.68	2	2	2	3	3	2
p-Hydroxy acetophenone enol	St	10.73	2	2	2	2	3	2
Methoxy benzoic acid[a]	NIST	10.73	3	3	3	4	4	4
p-Hydroxy benzoic acid	St	11.14	2	2	2	2	2	2
Dodecanoic (lauric) acid	NIST	11.40	2	2	2	2	2	2
p-Methoxyphenyl propanoic acid	NIST	11.53	2	2	2	3	3	2
4-Methyl cinnamic acid	NIST	11.60	3	2	2	2	3	2
1,8-Octandioic acid	NIST	11.88	2	2	2	2	2	2
Vanillic acid	NIST	12.65	3	3	4	3	3	3
1,9-Nonanoic acid	NIST	12.96	2	2	2	2	2	2
Methoxy *t*-cinnamic acid[a]	NIST	13.45	2	2	2	1	2	2
Tetradecanoic (myristic) acid	NIST	13.54	2	2	2	1	2	2
Phenylethyl benzoate	NIST	13.86	4	4	2	4	4	4
p-Coumaric acid	St	14.51	1	1	1	1	1	1
Methoxyphenyl benzoate[a]	NIST	15.16	2	2	2	4	3	2
3,4-Dimethoxy *t*-cinnamic acid	NIST	15.39	2	3	4	1	2	2
Hexadecanoic (palmitic) acid	NIST	15.53	1	1	1	1	1	1
4-Methoxy-3-hydroxy *t*-cinnamic acid	NIST	15.84	2	2	3	1	2	2
Ferulic acid	St	15.97	2	4	4	1	2	2
Caffeic acid	St	16.35	2	2	2	1	2	2
Oleic acid	NIST	17.09	3	3	3	1	2	1
Ottadecanoic (stearic) acid	NIST	17.42	1	2	2	1	2	2
1,1-Dimethylallyl caffeate	St	18.33	2	4	4	1	2	2
2′,6′-Dihydroxy-4′-methoxy dihydrochalcone	[b]	18.69	1	1	1	2	1	2
2′,4′,6′-Trihydroxy dihydrochalcone	[b]	18.90	1	1	2	3	2	2
Eicosanoic (arachic) acid	NIST	18.98	2	4	2	4	2	2
2′,6′-Dihydroxy-4′-methoxy chalcone	[b]	19.42	1	2	2	2	2	2
2′,4′,6′-Trihydroxy chalcone	[b]	19.58	1	1	2	2	2	2
Docosanoic (beenic) acid	NIST	20.53	3	3	4	4	4	4
2′,6′-Dihydroxy-4′,4-dimethoxy dihydrochalcone	[b]	20.55	1	1	2	3	4	2
2′,4′,6′-Trihydroxy-4-methoxy dihydrochalcone	[b]	20.70	1	1	2	2	2	2
Pinobanskin 3-acetate	[c]	20.78	3	3	3	1	2	2
2′,6′,4-Trihydroxy-4′-methoxy dihydrochalcone	[b]	21.03	2	2	2	4	4	2
Galangin	St	21.33	2	2	2	2	2	2
Chrysin	St	21.45	2	2	2	1	2	2
Phenylethyl caffeate	St	21.78	2	4	4	3	2	2
Isosakuranetin	St	21.86	2	2	2	3	2	2
Tetracosanoic (lignoceric) acid	NIST	21.96	2	2	2	2	2	2

[a] The details of the mass spectrum did not permit to indicate the exact position of the methoxy substituent in the phenyl ring.
[b] Mass spectra from Greenaway *et al.* (1989a).
[c] Mass spectrum from Greenaway *et al.*(1989b).

Table V. Compounds detected by GC-MS in *n*-hexane extracts of propolis specimens. For legend, see Table IV.

Compound	ID mode	R_t [min]	Ca1	Ca2	Ca3	Or1	Or2	Or3
Ethyl salicylate	St	5.31	4	3	3	2	3	3
Cinnamyl alcohol	NIST	5.66	2	2	2	2	2	2
3-Phenyl propanoic acid ethyl ester	NIST	6.04	2	2	2	2	2	4
Eugenol	NIST	6.13	4	4	4	2	4	4
Methoxy acetophenone[a]	NIST	6.17	2	2	2	4	2	2
(4-Phenyl)-2-buten-3-one	NIST	6.22	3	3	3	2	4	3
Methyl cinnamate	St	6.49	4	4	3	2	4	4
Ethyl cinnamate	St	7.39	3	3	3	2	3	4
3-(4-Methoxyphenyl) propanoic acid ethyl ester	NIST	8.81	2	2	2	3	2	4
Isoledene	NIST	8.91	1	4	2	1	2	2
Benzophenone	NIST	9.30	4	4	4	4	3	4
1,1,3a-Trimethyl-7-methylendecahydro-1*H*-cyclopropa-[a]-naphthalene	NIST	9.34	1	1	1	1	3	2
α-Eudesmol	NIST	9.63	1	1	1	1	2	1
Cinnamic acid ester[b]	NIST	10.67	4	3	3	4	3	3
Benzyl benzoate	St	10.77	1	1	1	1	1	1
Phenylethyl benzoate	NIST	11.69	3	1	1	2	1	2
3,4-Dimethoxy *t*-cinnamic acid methyl ester	NIST	11.81	4	4	4	1	3	2
Benzyl salicylate	St	11.89	1	1	1	2	1	1
Phenylethyl salicylate	St	12.77	2	2	2	4	2	2
Methoxy benzoic acid benzyl ester	NIST	13.00	1	1	1	2	1	1
Phenylethyl cinnamate	St	14.86	2	2	2	2	2	2
Cinnamic acid ester[b]	NIST	14.94	3	4	2	4	4	2
Cinnamic acid ester[b]	NIST	15.44	3	4	2	4	2	3
Cinnamic acid ester[b]	NIST	16.18	2	4	2	4	4	4
Cinnamyl cinnamate	St	16.89	1	1	1	1	1	1
Benzyl *p*-coumarate	NIST	17.37	2	2	2	1	2	2
Pinocembrin	NIST	17.52	2	1	2	2	2	2
5-Hydroxy-7,4′-dimethoxy flavone	NIST	17.86	4	3	3	4	3	3
5-Hydroxy-7-methoxy flavone	NIST	18.58	3	3	4	1	1	2
3-Phenyl propanoic acid cinnamyl ester	NIST	18.78	1	1	1	3	2	2

[a] The position of the methoxy substituent could not be ascertained.
[b] The mass spectrum of the peak was that of an ester of cinnamic acid, but it was not possible to assign the alcohol moiety.

from poplars of the *Aigeiros* section and are absent in resins from poplars of the *Tacamahaca* section (Greenaway *et al.*, 1990; English *et al.*, 1991).

The GC-MS profiles of silylated propolis (Table IV) were dominated by the peaks of the trimethylsilyl esters of *t*-cinnamic acid, *p*-hydroxy acetophenone, and *p*-coumaric acid. Another remarkable characteristic was the presence of the trimethylsilyl esters of five dihydrochalcones and two chalcones, all displaying major peaks that were easily distinguishable in the TIC profiles. Dihydrochalcones and chalcones are distinctive of resins from poplars of the *Tacamahaca* section (Greenaway *et al.*, 1989a). The trimethylsilyl esters of saturated fatty acids ($C_{10}-C_{24}$) were present in all analysed specimens and displayed prominent peaks. The trimethylsilyl esters of caffeic acid esters and ferulic acid trimethylsilyl ester were below the detection limits in the Ca2 and Ca3 specimens, confirming the results of the HPLC analyses.

n-Hexane extracts were analysed in both the SIM and the TIC mode, respectively. In the SIM mode, the quantitative analysis of the esters benzyl cinnamate and benzyl salicylate, two allergenic esters that are sometimes present in propolis (Hausen and Wollenweber, 1988), was carried out. Benzyl cinnamate was below its detection limit in all specimens. From the limits of detection by this analytical protocol (Aliboni *et al.*, 2011), the maximum content of this ester in the balsamic matter was estimated to be 2.0 μg/g. The contents of benzyl salicylate in all specimens were noteworthy, with the exception of that of Or1 (Table III). This is in sharp contrast to what has been reported for Italian propolis (Aliboni *et al.*, 2011) in which benzyl salicylate is a very minor component. Benzyl salicylate is a characteristic component of the

Table VI. Compounds detected in profiles of headspace GC-MS (vapours drawn at 70 °C). For legend, see Table IV. DT stands for a diterpene, SQT for a sesquiterpene.

Compound	R_t [min]	ID mode	Ca1	Ca2	Ca3	Or1	Or2	Or3
Acetic acid	2.09	NIST	3	2	2	1	4	2
4-Penten-1-ol acetate	4.90	NIST	3	4	4	1	4	2
Styrene	5.11	NIST	2	2	2	2	1	1
DT	5.56	NIST	4	4	4	2	4	4
DT	5.60	NIST	4	4	2	2	4	4
α-Pinene	5.76	St	2	2	2	1	2	1
DT	5.99	NIST	2	3	2	3	4	4
Camphene	6.04	NIST	2	2	2	2	4	4
DT	6.09	NIST	4	4	4	2	4	4
DT	6.51	NIST	3	2	2	2	4	4
DT	6.56	NIST	4	4	2	4	4	4
DT	6.73	NIST	2	1	2	2	1	1
DT	6.89	NIST	3	4	2	4	4	4
DT	6.95	NIST	4	4	4	2	1	4
DT	7.06	NIST	2	2	2	2	4	4
p-Cymene	7.18	NIST	2	2	1	2	2	4
DT	7.27	NIST	3	2	1	2	2	1
Eucalyptol	7.33	St	1	1	1	2	1	1
γ-Terpinene	7.71	St	2	2	2	2	4	4
DT	8.15	NIST	2	2	2	2	4	4
DT	8.30	NIST	2	2	2	2	1	1
DT	9.52	NIST	2	4	2	4	4	4
Terpinen-4-ol	9.64	St	2	4	2	4	4	4
α-Terpineol	9.85	St	2	2	2	2	4	4
β-Cyclocitral	10.24	NIST	2	2	2	2	4	1
$C_{12}H_{18}$	10.70	NIST	2	4	2	4	4	4
$C_{12}H_{18}$	11.26	NIST	2	2	2	4	4	4
$C_{12}H_{18}$	11.48	NIST	4	4	2	4	4	4
SQT	11.74	NIST	3	4	2	4	4	4
SQT	11.86	NIST	2	3	2	4	4	4
SQT	12.39	NIST	2	4	2	4	4	4
SQT	12.47	NIST	2	2	2	2	2	2
SQT	12.83	NIST	2	2	2	2	2	4
SQT	13.10	NIST	3	4	4	2	4	4
SQT	13.23	NIST	2	2	2	2	1	4
SQT	13.41	NIST	2	4	2	4	4	4
SQT	13.56	NIST	2	3	2	2	4	4
SQT	13.76	NIST	2	2	2	4	4	2
SQT	13.87	NIST	2	4	2	2	4	4
SQT	14.00	NIST	3	3	2	2	4	4
SQT	14.05	NIST	3	3	2	4	4	4
SQT	14.26	NIST	2	2	2	3	4	2
SQT	14.30	NIST	2	2	2	2	2	2
SQT	14.70	NIST	2	4	4	4	4	4
SQT	14.72	NIST	3	4	4	4	4	4
SQT	15.28	NIST	2	4	4	2	4	1
SQT	15.73	NIST	2	2	2	2	4	4
SQT	16.02	NIST	2	2	2	2	2	2

resins from poplars of the *Tacamahaca* section (English *et al.*, 1991). It was possible to assign a number of peaks in the TIC chromatogram of these solutions (Table V). Benzyl benzoate is also typical of the bud extracts of the *Tacamahaca* poplars (English *et al.*, 1991) and displayed major peaks in all profiles.

Two different analyses of the volatile fraction were carried out using headspace GC-MS. The profiles of the headspace vapours drawn at 70 °C (Table VI) were

Table VII. Compounds detected in profiles of headspace GC-MS (vapours drawn at 110 °C). For legend, see Table IV and Table VI.

Compound	R_t [min]	ID mode	Ca1	Ca2	Ca3	Or1	Or2	Or3
2-Phenyl ethanol	3.22	NIST	2	2	2	1	2	1
Benzoic acid	3.44	NIST	2	4	1	1	4	1
DT	3.82	NIST	1	1	1	2	4	4
DT	3.92	NIST	1	1	1	1	3	2
$C_{12}H_{18}$	4.55	NIST	2	4	2	4	4	4
$C_{12}H_{18}$	4.95	NIST	2	2	1	2	4	4
$C_{12}H_{18}$	5.12	NIST	4	4	2	4	4	4
o-Anisic acid methyl ester	5.29	NIST	2	2	2	2	2	1
SQT	5.41	NIST	2	2	2	2	1	4
SQT	5.84	NIST	2	2	2	2	4	2
SQT	5.92	NIST	2	2	2	1	2	2
SQT	6.15	NIST	4	4	2	3	2	4
SQT	6.25	NIST	1	1	1	1	1	4
SQT	6.35	NIST	3	4	3	2	4	4
SQT	6.48	NIST	3	2	3	2	4	4
SQT	6.60	NIST	1	1	1	1	1	4
SQT	6.64	NIST	2	3	2	3	3	4
SQT	6.75	NIST	2	2	2	2	4	2
SQT	6.85	NIST	2	2	3	3	4	4
SQT	6.90	NIST	2	2	1	1	2	4
SQT	6.96	NIST	2	2	2	2	4	2
SQT	7.01	NIST	3	2	2	4	4	4
SQT	7.06	NIST	1	1	1	1	2	1
SQT	7.11	NIST	3	3	3	3	3	4
SQT	7.17	NIST	2	2	2	1	2	3
SQT	7.25	NIST	4	3	2	3	4	3
SQT	7.29	NIST	4	1	1	1	1	1
SQT	7.33	NIST	3	2	1	1	2	2
SQT	7.36	NIST	2	2	2	2	4	2
SQT	7.43	NIST	2	3	2	3	4	4
SQT	7.52	NIST	2	2	2	2	2	2
SQT	7.56	NIST	1	1	1	1	1	1
Cadina-1,3,5-triene	7.61	NIST	2	2	2	1	1	2
SQT	7.79	NIST	2	3	2	2	2	4
α-Calacorene	7.86	NIST	2	2	2	2	2	2
t-Nerolidol	7.93	St	1	2	3	3	3	1
SQT	8.49	NIST	1	4	4	2	4	1
SQT	8.76	NIST	2	3	2	2	1	4
SQT	8.86	NIST	2	2	2	2	1	4
SQT	8.92	NIST	1	1	1	1	4	1
SQT	9.02	NIST	2	2	2	3	2	2
SQT	9.18	NIST	4	3	2	2	2	4
SQT	9.22	NIST	1	1	1	1	4	1
SQT	9.29	NIST	1	3	3	2	4	1

characterized by the presence of a total of 21 different diterpenes and diterpenoids among the six examined specimens, while the analysis of the profiles of the headspace vapours drawn at 110 °C (Table VII) revealed the presence of a total of 36 different sesquiterpenes and sesquiterpenoids. Terpenes and terpenoids are characteristic of the volatile fraction of resins from poplars of the *Tacamahaca* section (Mattes *et al.*, 1987; Greenaway *et al.*, 1989c).

The resins and bud exudates of the two poplar sections characteristic of North America – *Tacamahaca* and *Aigeiros* – are distinctive. All specimens investigated in this study contained the chemicals that are typical of resins and bud exudates from poplars of the *Tacamahaca* section, *i. e.* terpenes, chalcones and dihydrochalcones, *p*-hydroxy acetophenone, *t*-cinnamic acid, and benzyl salicylate. Nevertheless, only the specimens collected inside the forest of the Humboldt

Redwoods State Park – Ca2 and Ca3 – had a pure *Tacamahaca* origin. All other specimens – Ca1, Or1, Or2, Or3 – were of mixed origin, *i. e.* from both *Tacamahaca* and *Aigeiros* origin, because they contained measurable amounts of caffeic acid esters and ferulic acid – typical of resins from *Aigeiros* poplars and not present in those from *Tacamahaca* poplars.

1,1-Dimethylallyl caffeate and phenylethyl caffeate are described as the main poplar propolis allergens and as strong sensitizers (Hausen *et al.*, 1987b; Hausen and Wollenweber,1988). The mixed *Aigeiros-Tacamahaca* propolis characterized here contained these esters (Table III). Benzyl salicylate has been described as a moderately sensitizing allergen in European propolis (Hausen and Wollenweber, 1988), where it is a minor component (Aliboni *et al.*, 2011). It has been classified as a contact allergen in cosmetics by the EU (SCCNPF, 1999), although recently some authors argued that it actually displays a very limited sensitizing activity (Schnuch *et al.*, 2007). The two specimens of pure *Tacamahaca* origin – Ca2 and Ca3 – contained noteworthy amounts of this ester (Table III), well over the threshold recommended by the EU for its content in cosmetics (Mondello *et al.*, 2007). The specimens of mixed origin also contained comparable amounts of this ester, with the exception of Or1. Finally, some compounds detected in all GC-MS profiles, but not quantified, are classified as contact allergens in cosmetics (SCCNPF, 1999), but have not been reported so far as propolis allergens: benzyl benzoate (Table V) and cinnamyl alcohol (Tables IV and V).

The conscious use of pharmacologically active natural products requires the knowledge of all possible side effects. Propolis induces allergic reactions in a significant fraction of the population, and reports on many cases are found in the literature, but to our knowledge, none on propolis from this area. Yet, it is known from the literature that all propolis are allergenic, independent of their botanical origin (Hausen, 2005). On the basis of literature data, it is reasonable to expect that the caffeates contained in the mixed type propolis will cause the known allergic reactions in sensitive individuals that are well described in the literature (Hausen *et al.*, 1987a). On the other hand, nothing can be said for pure *Tacamahaca* propolis, but based on the high content of benzyl salicylate, care in its use is recommended. The reports on the actual sensitizing power of this ester are not univocal (SCCNPF, 1999; Schnuch *et al.*, 2007), but reactions may be individual, and many factors play a role in the sensitizing process, *e. g.* the geographical origin of the user (Larsen *et al.*, 1996).

Conclusions

Specimens of propolis from northern California and Oregon, USA, have been characterized here for the first time. The results confirm that in temperate areas bees gather resins for propolis manufacture from locally available poplars. Nevertheless, the genus *Populus* encompasses a wide number of species and consequently many different poplar propolis are actually possible (Bankova *et al.*, 2000). To our knowledge, this report is the first one on a propolis specimen made up of two different poplar resins. The specimens studied here originate from a relatively limited geographical area, and their composition profiles share many common features in the composition profile, but differ in their allergen content.

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