DOI:10.1021/jf1034866

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Propolis Specimens from Different Locations of Central Italy: Chemical Profiling and Gas Chromatography−Mass Spectrometry (GC−MS) Quantitative Analysis of the Allergenic Esters Benzyl Cinnamate and Benzyl Salicylate

Andrea Aliboni,* Armando D'Andrea, and Paolo Massanisso

Italian National Agency for New Technologies, Energy, and Sustainable Economic Development (ENEA), CR Casaccia, Via Anguillarese 301, 00123 Santa Maria di Galeria, Rome (RM), Italy

Propolis is a beehive product popular in natural medicine thanks to its noteworthy properties. Propolis is non-toxic but is responsible for allergic reactions in sensitive individuals. In this paper, we propose a new gas chromatography−mass spectrometry (GC−MS) analytical methodology for the quantitative analysis of two allergenic esters in propolis specimens, benzyl salicylate and benzyl cinnamate, and test it on specimens from different locations of central Italy. We also present the results obtained in the chemical characterization of the same specimens. The characterization showed that the resin fractions of all of the specimens are of poplar origin.

KEYWORDS: Propolis; poplar; cinnamate; salicylate; Italy

INTRODUCTION

Propolis is a beehive product that bees manufacture by mixing their own wax with materials of vegetal origin, bud exudates and resins from different species of trees. Insoluble matter, debris and polymers, is also present (*1*). Wax content ranges between 2 and 30% (*2−6*), and vegetable resins range between 40 and 80% (*4*, *6*, *7*). The bees employ propolis to finish the hive internal walls, close and limit the entrances, cover holes, and prevent floodings. Its antiseptic properties maintain the healthy hive (*1*).

In temperate regions, bees collect the material for propolis manufacture from different poplar species (*8−10*). Poplar propolis is a very complex mixture. It is characterized by a high content of flavonoids and phenolics (*10*) and the presence of many other different classes of compounds, among them cinnamic and benzoic acids and their esters, aldehydes and ketones, wax esters, alcanes, alcohols, and terpenoids (*1*, *8*).

Italian propolis is generally believed to be of poplar origin (*11*). Recently, a propolis specimen from southern Italy (Sicily) has been characterized and found to be rich in diterpenic species (*12*). This report has anticipated a wider work where Greek propolis specimens have been characterized and turned out to be rich in diterpenic species as well. The botanical origin of this type of propolis, called Mediterranean, has not yet been identified (*13*). These works indicate the presence of an area in the middle of the Mediterranean Sea where propolis has a common origin. The borders of this area are yet to be traced but should encompass at least a part of southern Italy.

Ultraviolet (UV) spectra of poplar propolis resin show a characteristic profile, with a maximum around 290 nm and a minimum around 250 nm (*14*, *15*). The UV profiles in reverse-phase gradient HPLC analysis also display common features. Cinnamic acids dominate the low retention times. Their peaks have relatively low intensities and are well-separated. Flavonoids and caffeates dominate the central part. Their peaks tend to be crowded in places and have the highest intensities. At the highest retention times, fewer peaks of low intensity are visible (*15−20*).

Propolis is accredited of many beneficial properties that are the object of extensive reviews (*1*, *21*, *22*). Propolis has a very low toxicity but may start allergic reactions in sensitive individuals that may also be severe (*21*). The major allergens contained in poplar propolis are some caffeic acid esters, 1,1-dimethylallyl caffeate isomers and phenylethyl caffeate (*23−27*). Other species are involved in the sensitization process, among them the esters benzyl cinnamate and benzyl salicylate (**Figure 1**) (*25*). These two esters are known allergens present in other matrices as well (*28*, *29*).

In this work, we propose the results of the chemical characterization of propolis specimens coming from different locations of central Italy. Collected data have been compared to literature, and all specimens turned out to be of poplar origin. We have also tested a new method based on GC−MS analysis for the quantitative determination of two allergenic esters, benzyl salicylate and benzyl cinnamate, using hexyl salicylate and hexyl cinnamate as internal standards (**Figure 1**). The content of these two allergens in propolis specimens are proposed for the first time.

MATERIALS AND METHODS

Chemicals. Acetonitrile was "HPLC-gradient-grade" from Sigma-Aldrich Chemie, Buchs, Switzerland. All other solvents, inorganic and organic reagents and mineral and organic acids, were analytical-grade from worldwide recognized firms.

Standards for gas chromatography−mass spectrometry (GC−MS), high-performance liquid chromatography (HPLC), and ultraviolet−visible (UV−vis) spectrophotometry were as follows: hexyl salicylate, ≥99%;

*To whom correspondence should be addressed. Telephone: +390630486807. Fax: +390630484554. E-mail: andrea.aliboni@enea.it.

Published on Web 12/02/2010
© 2010 American Chemical Society

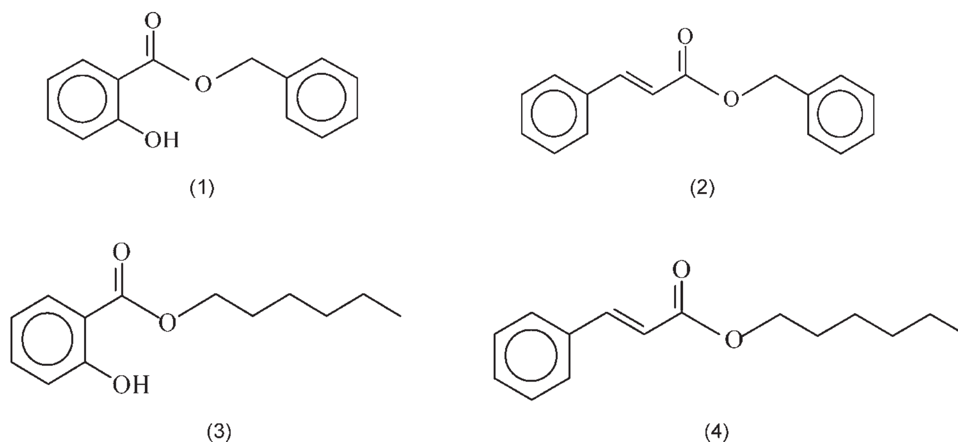

Figure 1. Allergenic esters: (1) benzyl salicylate and (2) benzyl cinnamate and the esters used as internal standards for their quantitative analysis: (3) hexyl salicylate and (4) hexyl cinnamate.

benzyl salicylate, 98%; ethyl cinnamate, 98%; benzyl cinnamate, 98%; galangin, 96%; chrisin, 96%; pinocembrin, 95%; *p*-coumaric acid, 98%; caffeic acid, 95%; ferulic acid, 99% (from Sigma-Aldrich Chemie); phenylethyl caffeate, 98% (from Biosynth Chemistry and Biology, Switzerland); quercetin dihydrate; kaempferol; apigenin; and naringenin (from Extrasynthese, Lyon, France).

Hexyl cinnamate (**Figure 1**), a non-commercial product, was prepared following the procedure reported in the literature for methyl cinnamate synthesis (*30*), with modifications: (i) a mixture of 1.02 g of *E*-cinnamic acid in 8.50 mL of 1-hexanol (molar ratio of 1:10) in a 50 mL flask is heated under reflux (165−175 °C) on a sand bath for 5 h with 100 μL of 96% sulfuric acid; (ii) the yielded organic phase is poured over ice water and then purified 3 times with 10 mL of 1 M sodium carbonate; and (iii) the purified mixture is reduced with a rotary evaporator (75 °C) until no more solvent is actually removed and dried over anhydrous sodium sulfate. This product was characterized as follows. Infrared (IR) spectrum and GC−MS analysis showed that desired esterification actually took place. HPLC showed that *E*-cinnamic acid was not present. UV−vis analysis was used to determine the actual content of the ester. To this end, we assumed that UV−vis molar extinction coefficients on the maximum (270 nm) of hexyl and ethyl cinnamate (for the latter, $\varepsilon = 21\,900 \pm 100\ M^{-1}\ cm^{-1}$ in hexane, measured from standard solutions) are the same. The ester content in the product was 51 ± 1% of the total mass. In the text, we refer to hexyl cinnamate as the name of this characterized solution of the ester in 1-hexanol that has been used without further purification.

Propolis Specimens. We collected the following raw propolis specimens during the winter 2007 in different locations of central Italy: L2 from Apicoltura Maria Fiorentini, Fiumicino (RM), Lazio; L3 from Mr. Romolo Proietti, Arsoli (RM), Lazio; A1 from Azienda Apistica Tommaso de Arcangelis del Forno, Opi (AQ), Abruzzo; T1 from Prof. Colombini, San Giuliano Terme (PI), Toscana; and T2 from Apicoltura Rocchi, Trassilico (LU), Toscana. Work solutions of the propolis resin fraction were prepared by dissolution of raw material in absolute ethanol. A lump of 3 g of propolis is homogenized and divided into four portions. Aliquots of about 350 mg are taken from each piece, reunited, and blended. This lump is again broken down into four pieces. An aliquot from each one is placed in a weighted centrifuge tube (100 × 15.50 mm), so that the final amount of propolis is about 125 mg. A total of 4 mL of absolute ethanol is added, and the system is treated in an ultrasonic bath for 10 min at maximum power (Transsonic Digital Elma, 320 W). The solution is separated from the residue by centrifugation at 4000 rpm (Hettich Universal 320). The operation is repeated 3 more times. All of the extracts were reunited in a 25 mL volumetric flask and brought to volume with ethanol. The concentration of propolis is about 5000 mg/L. The mass fractions of resin, wax, and insoluble, as defined by Ghisalberti (*31*), are determined following the scheme reported in **Figure 2**.

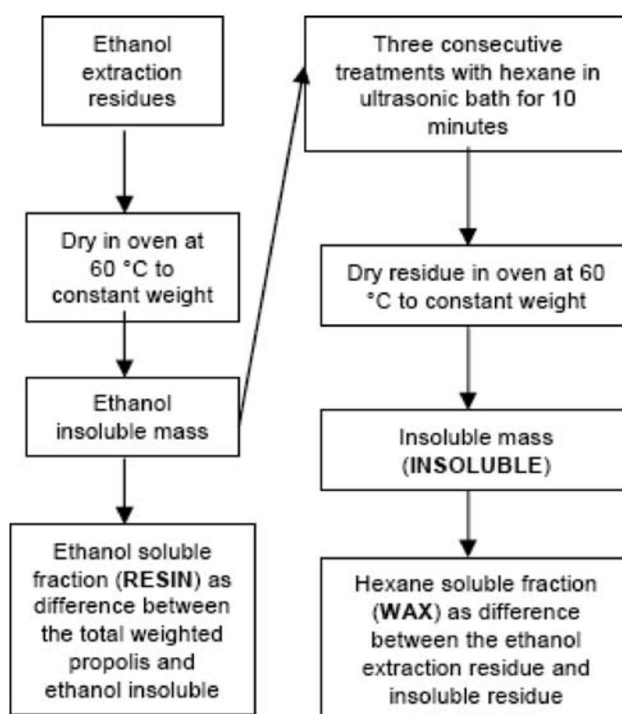

Figure 2. Treatment scheme for the determination of mass fractions of wax, resin, and insoluble in propolis specimens.

Spectrophotometric Assays and UV Spectra. All spectrophotometric measurements for antioxidant power and class content were carried out with the Gilson UV-7500 single-beam spectrophotometer. The Folin−Ciocalteau tests for the determination of total phenol content, the assays for total flavones and flavonols, and the assays for total flavanones and dihydroflavonols were carried out following methods reported in the literature specifically for poplar propolis (*11*). All calibrations were traced with four solutions of the appropriate standard. The Folin−Ciocalteau test was calibrated with a mixture of pinocembrin and galangin (2:1, w/w) (linear range, 0.65−6.5 mg/L; *R*, 0.9981). The assay for total flavones and flavonols was calibrated with galangin (linear range, 1.2−12 mg/L; *R*, 0.9998). The assay for total flavanones and dihydroflavonols was calibrated with pinocembrin (linear range, 0.35−3.5 mg/L; *R*, 0.9941). The assays were tested on four different aliquots of A1 propolis resin solution. The relative standard deviation (RSD) was 5% for total phenols, 7% for flavanone and dihydroflavonol tests, and 2% flavone and flavonol tests. The measures on the other specimens have been carried out in single experiments. The RSD on these measures was posed equal to the one determined on the A1 specimen.

The antioxidant power was determined as the ability of propolis to reduce Fe^{III} to Fe^{II} in acetate buffer. The assays were carried out following the method reported in the literature (*32*), with modifications. Briefly, 240 μL of deionized water and 2.4 mL of Fe^{III} reactive mixture [2.1 mL of 20 mM Fe^{III} chloride hexahydrate and 2.1 mL of 10 mM 2,4,6-tri-(2-pyridil)-*s*-triazine (TPTZ), both in 40 mM HCl, to volume in a

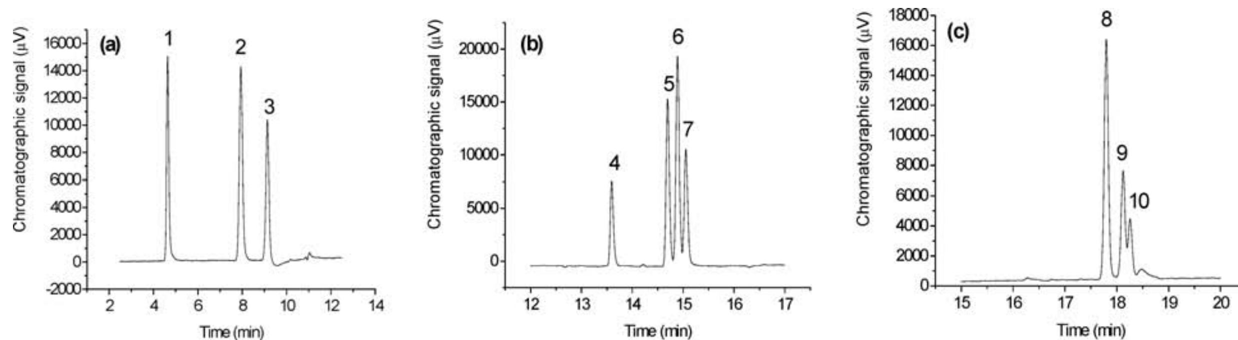

Figure 3. HPLC chromatogram of standards of interest for the propolis specimens, injecting about 2 mg/L each in a 4:1 H_2O/ACN mixture containing 0.1% formic acid: (a) 1, caffeic acid; 2, *p*-coumaric acid; and 3, ferulic acid (monitored at 320 nm); (b) 4, quercetin; 5, apigenin; 6, naringenin; 7, kaempferol (monitored at 250 nm); and (c) 8, chrysin; 9, pinocembrin + phenylethyl caffeate; and 10, galangin (monitored at 250 nm).

25 mL volumetric flask with 300 mM acetate buffer, with a molar ratio of CH_3COONa/CH_3COOH = 0.084, at pH 3.7] were mixed in two spectrophotometric cells. The cells were placed in an oven at 40 °C to equilibrate for 10 min. An appropriate volume (20−80 μL) of a 500 mg/L propolis ethanolic solution was then added to the first cell, and an equal volume of ethanol was added in the second cell (blank). The cells were left in the oven at 40 °C for 20 min, and then the absorbance of the solutions were measured at 593 nm. The amount of Fe^{II} formed was determined as the Fe^{II}−TPTZ chelate complex (ε_{593} = 22 200 ± 100 M^{-1} cm^{-1}). The antioxidant power was expressed as the mass ratio of generated Fe^{II} and added propolis. The method has been tested with four different aliquots of the solution of L3 propolis and was linear in the 10−43 μg range (*R*, 0.9980; RSD, 5%). The measures on the other specimens have been carried out in single experiments. The RSD on these measures was posed equal to the one determined on the L3 specimen.

The UV spectra of propolis resin solutions were registered in the 400−230 nm spectral interval using a Varian "Cary 50" double-beam spectrophotometer. The ethanolic propolis solutions were diluted with ethanol in a 25 mL volumetric flask to a final concentration of about 25 mg/L. The spectra were normalized for the fraction of matter actually dissolved in ethanol and reported as specific absorption $E_{1\%}$ (cm^{-1}), the absorbance of a 10 000 mg/L solution.

HPLC Analyses. The HPLC apparatus was made up of the following parts: a quaternary pump "Perkin Elmer series 200 pump", an injection group with a 2 μL loop, a UV−vis detector "Perkin Elmer series 200 UV−vis detector" with a 2.4 μL cell, and an electronic interface "Perkin Elmer PE 600 series link". The data were acquired on a personal computer (PC) with Turbochrom software, version 4. The ethanolic propolis solutions were diluted in a 10 mL volumetric flask with a 4:1 (v/v) water/acetonitrile mixture containing 0.1% formic acid, for a final concentration of about 60 mg/L. These solutions were eluted with the following method: (i) column, Varian Pursuit XRs C18, 150 × 2.1 mm, 3 μm particles; (ii) eluents, (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile; (iii) elution, 2 min for 20% B isocratic, 26.5 min for 100% B gradient, and 0.5 min for 100% B isocratic; and (iv) flow, 200 μL/min. Each solution was analyzed 4 times, monitoring at four different UV signals: 320, 293, 275, and 250 nm. Peaks were preliminarily assigned by retention times and co-injection with pure standard solutions (**Figure 3**). These assignments were further tested. The signal ratios at different wavelengths in real specimens were compared to ones registered with pure standard solutions (tolerance, ±50%). Pinocembrin and phenylethyl caffeate coeluted. Calibration lines were traced using the area signal, monitored at 250 nm for flavonoids and 320 nm for cinnamic acids. The parameters of analytical interest are in **Table 1**. The analyses were tested on four different aliquots of A1 propolis resin solution, and RSD was 15% for the determination of all of the species. The measures on the other specimens have been carried out in single experiments. The RSD on these measures was posed equal to the one determined on the A1 specimen.

GC−MS Characterization of Benzyl Salicylate and Benzyl Cinnamate. The GC−MS apparatus was made up of the following parts: a HP 7890A GC, a single quadrupole mass spectrometer HP 5975C, and EI fragmentation with energies up to 70 eV. Data were acquired on a PC with the Agilent MSD ChemStation software, E.01.01.335 version.

Table 1. Analytical Parameters for HPLC Determination of Cinnamic Acids Monitored at 320 nm and Flavonoids Monitored at 250 nm[a]

	linearity range (ng)	*R*	LOD (pg)	LOQ (pg)	retention time (min)
caffeic acid	0.1−3.7	0.9984	10	30	4.9
p-coumaric acid	0.1−4.0	0.9999	2	7	8.6
ferulic acid	0.1−4.3	0.9947	3	10	9.8
quercetin	0.11−0.44	0.9999	7	24	13.7
apigenin	0.12−0.48	0.9999	8	28	14.8
kaempferol	0.14−0.54	0.9997	5	17	15.2
chrisin	0.2−10	0.9997	3	10	18.2

[a] The limits of detection (LOD) and quantitation (LOQ) are the amounts that yield a signal 3 and 10 times higher than the average background noise of the baseline, respectively. Retention times are all ±0.1 min.

Propolis ethanolic solutions were treated before the analysis (**Figure 4**). The octanic solutions were analyzed by GC−MS with the following method. A total of 1 μL was injected, in splitless mode, with an injector temperature of 280 °C. Separations were carried out on a Hewlett-Packard, 30 m capillary column, HP-5 ms (5% methyl on phenyl silicone), with 0.20 mm inner diameter and 0.11 μm thickness. The elution program was as follows: 120 °C, 2 min; 200 °C, 5 °C/min; 200 °C, 5 min; 280 °C, 10 °C/min; 280 °C, 1 min; 300 °C, 20 °C/min; and 300 °C, 20 min. The carrier gas was He, flowing at 1.00 mL/min. Selected ion monitoring (SIM) detection was carried out choosing fragments typical of the two target esters and their respective internal standards, three per ester: hexyl salicylate, m/z^+ 120, 138, and 222; benzyl salicylate, m/z^+ 85, 91, and 228; hexyl cinnamate, m/z^+ 131, 148, and 232; and benzyl cinnamate, m/z^+ 91, 131, and 192. Qualitative analysis was carried out by retention times and analysis of the mass spectra fragment ratio (tolerance, ±20%). The chromatogram of standards is reported in **Figure 5**.

Calibration lines were traced using the area signal. The parameters of analytical interest are in **Table 2**. The analytical concentrations of benzyl salicylate and benzyl cinnamate were corrected for the measured yields of the respective hexyl ester. The assays were tested on A1 propolis 4 times. RSD of benzyl cinnamate and benzyl salicylate determinations were 15 and 10%, respectively. The measures on the other specimens have been carried out in single experiments. The RSD on these measures was posed equal to the one determined on the A1 specimen.

RESULTS

Physicochemical Characterization. Mass fractions of resin, wax, and insoluble in propolis specimens are reported in **Table 3**. The contents of wax are on the low side of literature values, comprised between 2 and 30% (*2−6*). The contents of resin are on the high side of literature values, comprised between 40 and 80% (*4*, *6*, *7*).

UV spectra of propolis resin fractions all display a similar profile (**Figure 6**), and specific absorption values are reported in **Table 3**. They are characterized by a maximum (292.5 ± 1 nm), a

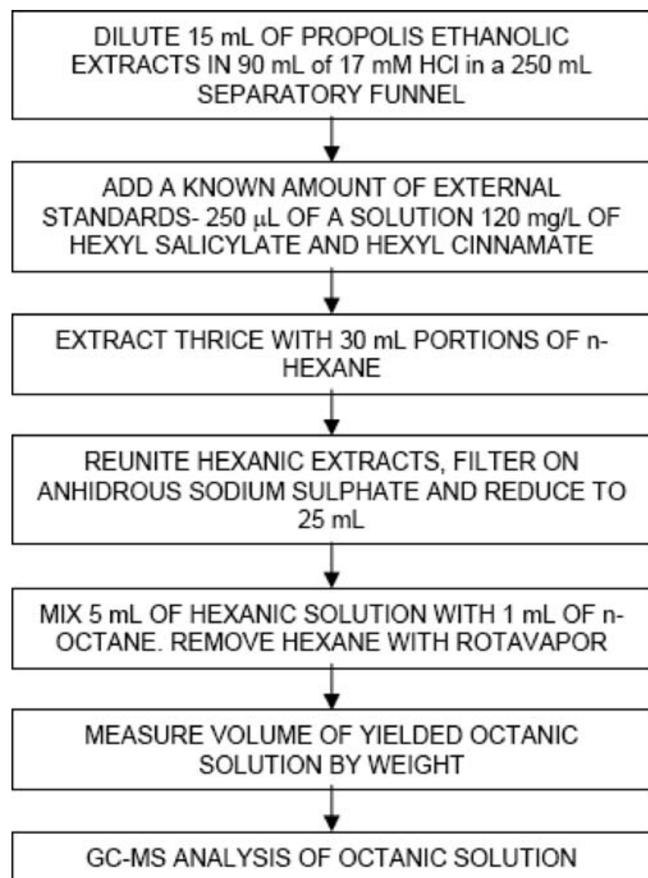

Figure 4. Treatment used to prepare specimens for the GC−MS analysis of benzyl salicylate and benzyl cinnamate using hexyl salicylate and hexyl cinnamate as internal standards.

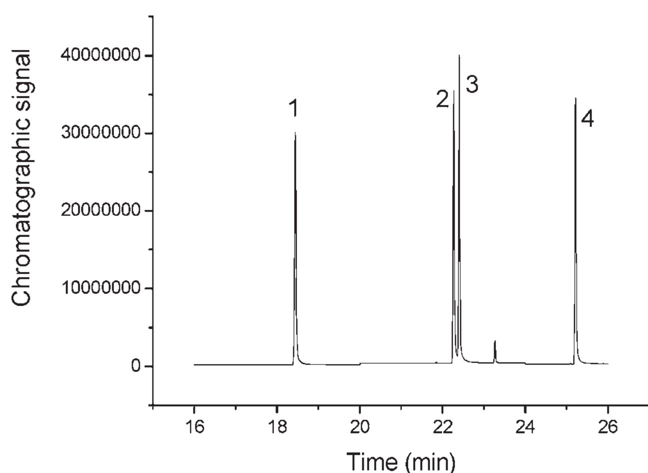

Figure 5. GC chromatogram of standards of esters: 1, hexyl salicylate (t_{ret} = 18.4 min); 2, benzyl salicylate (t_{ret} = 22.3 min); 3, hexyl cinnamate (t_{ret} = 22.4 min); and 4, benzyl cinnamate (t_{ret} = 25.2 min), at 2 mg/L each in *n*-octane. All retention times are ±0.1 min.

minimum (253 ± 1 nm), and two shoulders on either side of the maximum (318 ± 2 and 274.5 ± 0.5 nm, respectively). The RSD of coefficients is quite reduced. These observations point to a common botanical origin of all examined specimens. The profiles of the spectra are very similar to the ones reported for poplar propolis from Argentina (*15*) and Brazil (*14*). The specific absorption values on the maximum are comparable to but higher than the absorption values of Japanese poplar propolis that are comprised between 54 and 288 cm^{-1} (*20*).

Table 2. Analytical Parameters for the GC−MS Quantitative Determination[a]

	linearity range (ng)	LOD (pg)	LOQ (pg)	R
benzyl salicylate	1−8	17	57	0.9980
benzyl cinnamate	2−19	17	57	0.9932
hexyl salicylate	1−6	16	54	0.9930
hexyl cinnamate	1−6	20	70	0.9927

[a] The limits of detection (LOD) and quantitation (LOQ) are the amounts yielding a signal 3 and 10 times higher than the average background noise of the baseline, respectively.

Table 3. Physicochemical Parameter Characteristics of Propolis Specimens[a]

specimen	resin (EtOH soluble)	waxes (hexane soluble)	insoluble	UV maximum	UV minimum
A1	89.0	8.3	2.7	364	203
T1	84.5	9.2	6.2	288	184
T2	76.0	8.0	15.8	291	169
L2	69.0	11.5	19.4	250	165
L3	81.5	8.5	10.2	300	176
average	80.0	9.1	10.9	299	179
RDS	10	15	63	14	8

[a] Mass fractions (%) of resin, wax, and insoluble in solid propolis, measured following the scheme reported in **Figure 2**. Specific absorption values $E_{1\%}$ (cm^{-1}) on both the maximum and minimum points of UV−vis spectra of the resin fraction of propolis specimens, with the uncertainty of UV coefficients ±1%.

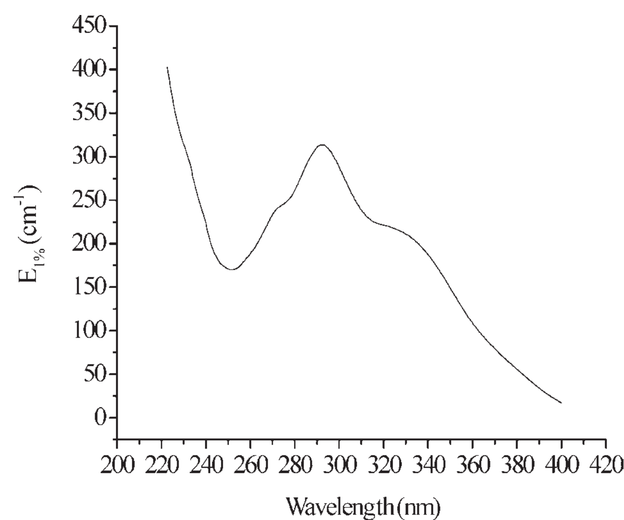

Figure 6. UV−vis spectra of the ethanolic solution of propolis resin from specimen A1. The other specimens display a similar profile.

Composition Profiles. The chromatograms of all specimens were registered 4 times, each time monitoring a different UV signal: 250, 275, 293, and 320 nm. The chromatograms of specimens A1 and T2 registered at 293 nm (maximum signal) are shown in panels **a** and **b** of **Figure 7**. The profiles of other specimens are comparable to the one yielded by the A1 specimen. T2 propolis displays a higher density of peaks. Peak assignments in specimen A1 are visible in panels **c**−**e** of **Figure 7**. Similar assignments were possible in all of the examined specimens. Preliminary assignments made by co-injection were tested by comparing intensity ratios of signals registered at different wavelengths to intensity ratios determined with standard injections. All of the assignments were confirmed with the exception of the peak at 18.70 min. It was assigned by co-injection to galangin, but intensity ratios were significantly different from expected intensity ratios. This implies that either galangin is not present or its peak in the proposed HPLC method coelutes with some other species. Pinocembrin and phenylethyl caffeate coelute (**Figure 7e**) with the proposed

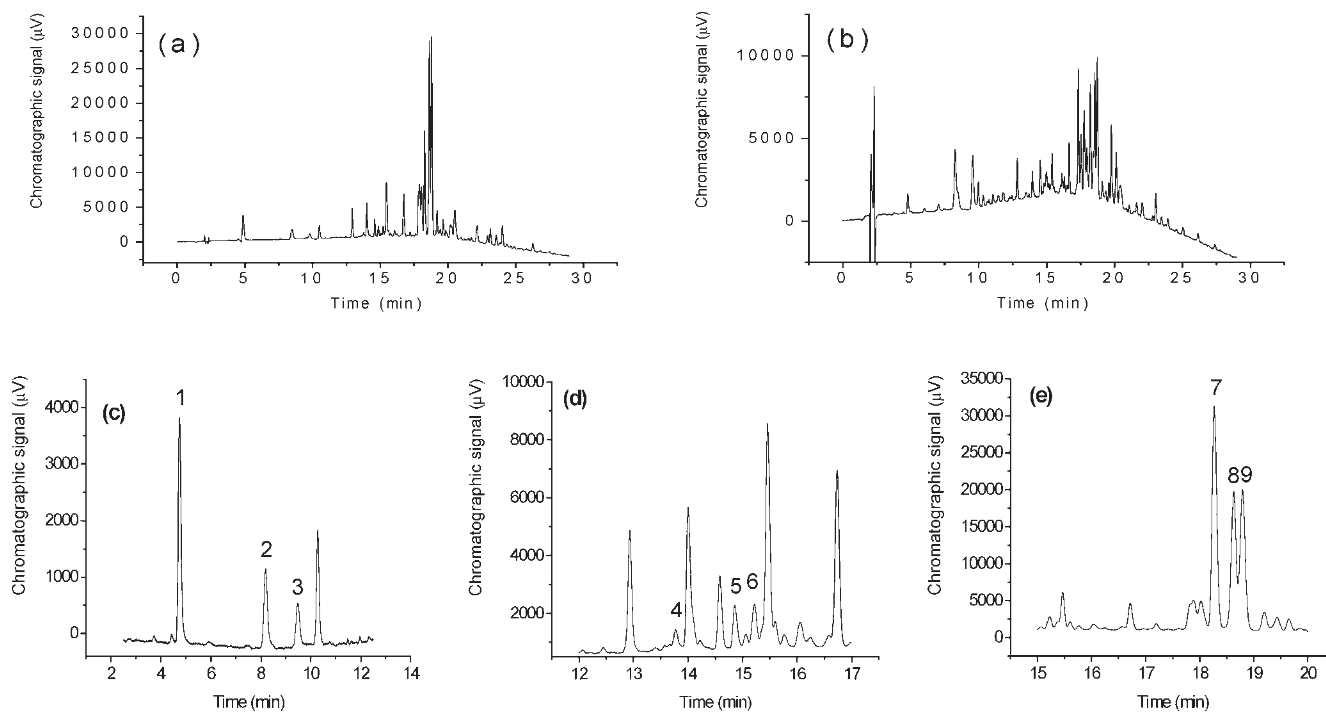

Figure 7. HPLC profiles of (a) A1 and (b) T2 propolis ethanolic extracts, diluted to about 60 mg/L, monitored at 293 nm (maximum signal). T1, L2, and L3 display HPLC profiles that are comparable to the HPLC profile from the A1 specimen. Particulars of the HPLC chromatograms of A1 propolis ethanolic extracts showing peaks assigned by co-injection. (c) Peaks of 1, caffeic acid; 2, *p*-coumaric acid; and 3, ferulic acid are shown (monitored at 320 nm). (d) Peaks of 4, quercetin; 5, apigenin; and 6, kaempferol are shown (monitored at 250 nm). (e) Peak of 7, chrisin; 8, caffeic acid phenyl ester (CAPE) + pinocembrin; and 9, galangin are shown (monitored at 250 nm). The co-injection of other specimens brought the same assignments.

Table 4. Content Values in μg/g of Cinnamic Acids and Flavonoids in Solid Propolis Specimens Determined by HPLC, with RSD of 15%, and Content Values of Benzyl Cinnamate and Benzyl Salicylate in Solid Propolis Specimens (μg/g) Determined by GC−MS, with RSD of 10% for Benzyl Salicylate and 15% for Benzyl Cinnamate

specimen	caffeic acid	*p*-coumaric acid	ferulic acid	quercetin	apigenin	kaempferol	chrysin	galangin	benzyl salicylate	benzyl cinnamate
A1	25000	2800	4200	8500	8000	8500	50000	30000	20	55
T1	19000	2400	3100	4000	3500	3500	34000	18000	40	75
T2	2600	1900	3500	600	1300	1400	6000	5000	70	310
L2	22000	5400	5200	5200	5000	6000	39000	29000	15	20
L3	7000	3600	3300	4900	1100	3300	30000	4700	80	1025
average	15000	3200	3900	4600	3800	4500	32000	ND[a]	45	300
RSD	65	43	22	61	76	61	51	ND[a]	65	142

[a] ND = not determined.

elution method. All HPLC profiles are compatible to the HPLC profiles typical of poplar propolis (*15−20*).

The concentrations of cinnamic acids and flavonoids are reported in **Table 4**. We assigned the entire peak with a retention time of 18.70 min to galangin, calculating concentrations that are in excess of real concentrations. The attempt to carry out quantitative analysis of pinocembrin and phenylethyl caffeate using a system of two equations failed, probably because of the presence of additional species contaminating the peak.

The specimens look rather homogeneous, with caffeic acid being the prominent cinnamic acid and chrysin being the most abundant flavonoid. It also turns out that galangin, if at all present, is minor.

In **Figure 8**, the GC−MS profile of a treated propolis specimen is shown. The peaks of both target analytes and internal standards are well-resolved. The concentrations of benzyl salicylate and benzyl cinnamate in the specimens are reported in **Table 4**.

The contents of compound classes are reported in **Table 5**, along with the antioxidant power. The content of flavones and flavonols and polyphenols are quite greater than values reported for poplar propolis specimens from Japan (*20*), Korea (*19*), China, and Uruguay (*4*, *16*).

DISCUSSION

The specimens used in the experimentation are characterized by a high content of resin material. The intense UV-specific absorption values and the results of the Folin−Ciocalteau test let us conclude that the resin fractions are unusually rich in phenolic-conjugated compounds.

The profiles of the UV spectra, the form of the HPLC profiles, and the high content of flavonoids and cinnamic acids let us conclude that all of the specimens from central Italy characterized here are of poplar origin. Thus far, two distinct types of Italian propolis have been characterized: poplar propolis (*11*) and Mediterranean propolis (*12*). These propolis types are probably from different geographical areas of Italy. The data that we present here provide a contribution to better define these two areas.

The HPLC profile of the T2 balsam fraction displays a number of peaks that are much higher. Furthermore, the concentration of the species that characterize other specimens are considerably

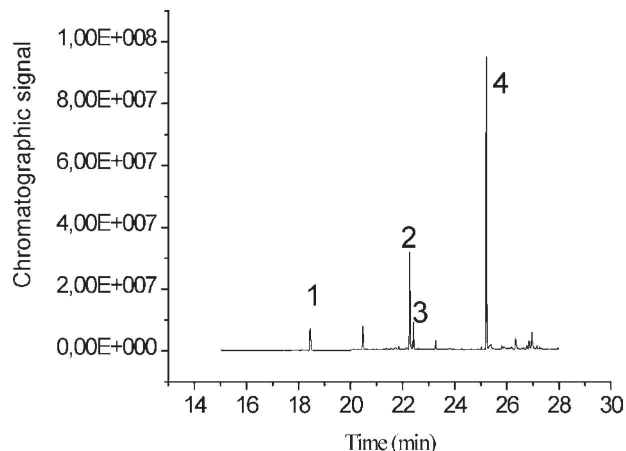

Figure 8. GC−MS chromatogram of L3 propolis-treated solution. The chromatograms of other propolis specimens are comparable. The following peaks were assigned by mass spectra analysis and retention times: 1, hexyl salicylate; 2, benzyl salicylate; 3, hexyl cinnamate; and 4, benzyl cinnamate.

Table 5. Total Flavones and Flavonols (1) as mg of Galangin/g of Solid Propolis, with RSD of 2%, Total Flavanones and Dihydroflavonols (2) as mg of Pinocembrin/g of Solid Propolis, with RSD of 7%, Total Phenols (3) as mg of a 1:2 Mixture of Galangin/Pinocembrin/g of Solid Propolis, with RSD of 5%, and Antioxidant Power (4) as Mass of Fe^{2+}/Propolis Mass, with RSD of 5%

specimen	1	2	3	4
A1	118	140	500	0.17
T1	80	95	380	0.150
T2	25	30	150	0.052
L2	108	110	450	0.16
L3	96	60	310	0.145
average	85.4	87	358	0.135
RSD	43	50	39	35

lower in T2. This observation cannot be explained with a significantly lower content of balsam (**Table 3**). In the T2 specimen, resin material must therefore be of mixed origin, containing only a part of poplar resins. This difference in composition does not seem to affect UV spectra significantly.

The proposed GC−MS method for the quantitative analysis of benzyl salicylate and benzyl cinnamate turned out to be valid. The use of hexyl salicylate and hexyl cinnamate as internal standards accounted very well for the preparation process and made quantitative analysis possible. Using the HPLC method proposed for flavonoids and phenolics, these esters would be below the LOD. To the best of our knowledge, this is the first report of the quantitative content of these esters. The content of benzyl cinnamate is very variable among our specimens, but it is a minor compound. This is in contrast to what happens in another allergenic natural resin, balsam of Peru, where it is over 10% of the total mass (*25*). The amounts used in sensitizing tests are generally much higher (*25*−*29*). Benzyl salicylate is a minor compound as well, and the amount used in sensitization tests is usually much higher (*25*). Because many synergic effects are active in a complex mixture, such as propolis, the analysis of potentially allergenic species, even if present in tiny concentrations, should be an important part of the quality control. The method tested here poses as an efficient instrument to this end.

ACKNOWLEDGMENT

Thanks to Prof. Maria Perla Colombini from Università di Pisa (Pisa, Italy), who tutored Andrea Aliboni's Ph.D. research with patience and enthusiasm.

LITERATURE CITED

(1) Marcucci, M. C. Propolis: Chemical composition, biological properties and therapeutic activity. *Apidologie* **1995**, *26*, 83−99.
(2) Salatino, G.; Negri, G.; Marcucci, M. C.; Salatino, M. L. F. Hydrocarbons and monoesters of propolis waxes from Brazil. *Apidologie* **1998**, *29*, 305−314.
(3) Salatino, A.; Woisky, R. G. Analysis of propolis: Some parameters and procedures for chemical quality control. *J. Apicol. Res.* **1998**, *37*, 99−105.
(4) Bonvehi, J. S.; Ventura Coll, F. Study on propolis quality from China and Uruguay. *Z. Naturforsch.* **2000**, *55C*, 778−784.
(5) Salatino, G.; Negri, G.; Marcucci, M. C.; Salatino, M. L. F. Comb and propolis waxes from Brazil (states of São Paulo and Paraná). *J. Braz. Chem. Soc.* **2000**, *11*, 453−457.
(6) Bonvehi, J. S.; Ventura Coll, F.; Escolà Jordà, R. The composition, active components and bacteriostatic activity of propolis in dietetics. *J. Am. Oil Chem. Soc.* **1994**, *71*, 529−532.
(7) Park, Y. K.; Ikegaki, M.; Alencar, S. M. Classificação das própolis brasileira a partir de suas caracteristicas fisico-quimicas e propriedades biológicas. *Mensagem Doce* **2000**, *58*, 2−7.
(8) Greenaway, W.; Scaysbrook, T.; Whatley, F. R. The composition and plant origins of propolis: A report of work at Oxford. *Bee World* **1990**, *71*, 107−118.
(9) Greenaway, W.; Scaysbrook, T.; Whatley, F. R. Identification by gas chromatography−mass spectrometry of 150 compounds in propolis. *Z. Naturforsch.* **1991**, *46* (*c*), 111−121.
(10) Bankova, V. S.; De Castro, S. L.; Marcucci, M. C. Propolis: Recent advances in chemistry and plant origin. *Apidologie* **2000**, *31*, 3−15.
(11) Popova, M. P.; Chinou, I. B.; Marekov, I. N.; Bankova, V. S. Terpenes with antimicrobial activity from Cretan propolis. *Phytochemistry* **2009**, *70*, 1262−1271.
(12) Trusheva, B.; Popova, M.; Bankova, V.; Tsvetkova, I.; Naydensky, H.; Sabatini, A. G. A new type of European propolis, containing bioactive labdanes. *Riv. Ital. EPPOS* **2003**, *36*, 3−7.
(13) Bankova, V. S.; Popova, M. P.; Graikou, K.; Chinou, I. GC−MS profiling of diterpene compounds in Mediterranean propolis from Greece. *J. Agric. Food Chem.* **2010**, *58*, 3167−3176.
(14) Park, Y. K.; Alencar, S. M.; De Aguiar, C. L.; Scamparini, A. R. P.; Gonzales, M.; Molina, M. A. A. Comparação das caracteristicas fisico-quimicas das própolis produzidas na região sub-tropical da América do Sul: Evidência fitoquimica de sua origem botânica. *Mensagem Doce* **2001**, *61*, 2−10.
(15) Park, Y. K.; Isla, M. I.; Paredes-Guzman, J. F.; Nieva Moreno, M. I.; Koo, H. Some chemical composition and biological activity of northern Argentine propolis. *J. Agric. Food Chem.* **2005**, *53*, 1166−1172.
(16) Bonvehi, J. S.; Ventura Coll, F. Phenolic composition of propolis from China and from South America. *Z. Naturforsch.* **1994**, *49C*, 712−718.
(17) Markham, K. R.; Mitchell, K. A.; Wilkins, A. L.; Daldy, J. A.; Lu, Y. HPLC and GC−MS identification of the major organic constituents in New Zealand propolis. *Phytochemistry* **1996**, *42*, 205−211.
(18) Park, Y. K.; Alencar, S. M.; De Aguiar, C. L. Botanical origin and chemical composition of Brazilian propolis. *J. Agric. Food Chem.* **2002**, *50*, 2502−2506.
(19) Kumazawa, S.; Ahn, M. R.; Hamasaka, T.; Bang, K. S.; Nakayama, T. Antioxidant activity and constituents of propolis collected in various areas of Korea. *J. Agric. Food Chem.* **2004**, *52*, 7286−7292.
(20) Hamasaka, T.; Kumazawa, S.; Fujimoto, T.; Nakayama, T. Antioxidant activity and constituents of propolis collected in various areas of Japan. *Food Sci. Technol. Res.* **2004**, *10*, 86−92.
(21) Burdock, G. A. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem. Toxicol.* **1998**, *36*, 347−363.
(22) Kadota, S.; Banskota, A. H.; Tezuka, Y. Recent progress in pharmacological research of propolis. *Phytother. Res.* **2001**, *15*, 561−571.
(23) Hausen, B. M.; Wollenweber, E.; Senff, H.; Post, B. Propolis allergy. (I). Origin, properties, usage and literature review. *Contact Dermatitis* **1987**, *17*, 163−170.
(24) Hausen, B. M.; Wollenweber, E.; Senff, H.; Post, B. Propolis allergy. (II). The sensitizing properties of 1,1-dimethylallyl caffeic acid ester. *Contact Dermatitis* **1987**, *17*, 170−177.

(25) Hausen, B. M.; Wollenweber, E. Propolis allergy. (III). Sensitization studies with minor constituents. *Contact Dermatitis* **1998**, *19*, 296−303.
(26) Hausen, B. M. Evaluation of the main contact allergens in propolis (1995 to 2005). *Dermatitis* **2005**, *16*, 127−129.
(27) Walgrave, S. E.; Warshaw, E. M.; Glesne, L. A. Allergic contact dermatitis from propolis. *Dermatitis* **2005**, *16*, 209−215.
(28) Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP). *Fragrance Allergy in Consumers, SCCNPF/0017/98 Final*; European Commission (EC): Brussels, Belgium, Dec 8, **1999**.
(29) Bhatia, S. P.; Wellington, G. A.; Cocchiara, J.; Lalko, J.; Letizia, C. S.; Api, A. M. Fragrance material review on benzyl cinnamate. *Food Chem. Toxicol.* **2007**, *45*, S40−S48.
(30) Vogel, A. I. Composti aromatici. *Chimica Organica Pratica*, 2nd ed.; Casa Editrice Ambrosiana: Milan, Italy, 1998; Chapter 4, p 879 (in Italian).
(31) Ghisalberti, E. L. Propolis: A review. *Bee World* **1979**, *2*, 59−84.
(32) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay. *Anal. Biochem.* **1996**, *23*, 70−76.

Received for review September 9, 2010. Revised manuscript received November 8, 2010. Accepted November 15, 2010. Thanks to Dr. Roberto Balducchi director of the Biotec-Agro Unit of ENEA, who strongly supported this research with institutional funds of his Unit.