Study of Propolis by High Temperature High Resolution Gas Chromatography-Mass Spectrometry

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The underivatized hexane and acetone extracts of two propolis samples (from Brazil's southwest) were analyzed by HT-HRGC (high temperature high resolution gas chromatography) and HT-HRGC coupled to mass spectrometry (HT-HRGC-MS). Several compounds, including flavonoid aglycones, phenolic acids and high molecular weight compounds could be readily characterized in the crude extracts by HTHRGC-MS. HTHRGC and HTHRGC-MS were shown to be quick and informative tools for rapid analysis of crude extracts without need for prior derivatization and purification.

Introduction

Propolis (bee glue) is a resinous hive product, formed from resinous and balmy material, collected by bees from different parts of plants (branch, flowers, pollen and buds) and modified in the beehive by addition of salivate secretions and beeswax (Vanhaelen and Vanhaelen-Fastré, 1979 a; Ghisalberti, 1983; Bankova *et al.,* 1987). Bees use it as a sealer for their hives and, more importantly, to prevent the decomposition of insects which have been killed by the bees after attempted invasion of the hive (Marcucci, 1995).

Propolis presents several biological properties, the most important of which are: antimicrobial, antiparasitic, immunostimulating, antiinflammatory as well as cytostatic and hypoglycemic activities *in vitro.* Propolis is also widely employed in folk medicine. Some reports were published about successful clinical use of propolis to aid the healing of wounds, ulcers, tuberculosis, in treatment of mycotic infections and eczems, in stomatology, etc. (Bankova *et al.,* 1983; Marcucci, 1995).

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These valuable properties of propolis generated interest in its chemical composition. Alcohols, aldehydes, aliphatic and aromatic acids, aliphatic and aromatic esters, chalcones, terpenoids, steroids, sugars, amino acids, as well as a large number of flavonoids, were identified in propolis (Greenaway *et al.,* 1991; Marcucci, 1995). The relative proportion of these compounds varies and depends, among many other variables, on the place and time of collection (Bankova *et al.,* 1992).

The flavonoids, aromatic acids and phenolic derivatives are believed to be the principal components responsible for the therapeutic effects of propolis (Vanhaelen and Vanhaelen-Fastre, 1979 b; Grange and Davey, 1990; Millet, 1992; Serkedjieva *et al.,* 1992). Several flavonoids were shown to inhibit the replication of picornaviruses such as poliomyelitis and rhinoviruses. Compounds such as 4′-ethoxy-2′-hydroxy-4,6′-dimethoxy-chalcone interact directly with specific sites on the viral capsid proteins, producing thereby uncoating of the virus with consequently liberation of viral RNA (De Meyer *et al.,* 1991).

Characterization of flavonoids is usually accomplished by classic phytochemical techniques, comprising a step of isolation before identification by

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the usual spectroscopic methods (UV, IR and NMR). As a result, identification of multiple components by classical phytochemistry is extremely slow. An alternative method, would be to analyze whole plant extracts or fractions beforehand by HRGC and HRGC-MS, to obtain the distribution profiles and identities of as many compounds as possible, and subsequently to decide convenience and strategy for isolation and unambiguous identification of any unknown substances (Godoy *et al.,* 1987). Unfortunately, direct analysis by HRGC of several classes of bioactive compounds is difficult or impossible, because such components frequently possess high boiling points and are, in many cases, thermolabile.

High Temperature High Resolution Gas Chromatography (HT-HRGC) and HT-HRGC-Mass Spectrometry (MS) are established techniques for separation of complex mixtures and identification of high molecular weight (HMW) compounds, many of which do not elute when analyzed on ordinary HRGC columns (Pereira *et al.,* 1996). HT-HRGC may be an excellent alternative to classical analytical phytochemistry and a potent tool for the rapid evaluation of crude natural products and medicinal plants (Pereira *et al.,* 1998).

Experimental

Materials

Two propolis samples (A and B) were collected in 1992, from bee hives at Sapucaia, Rio de Janeiro, Brazil with predominant local flora of *Citrus* spp. and *Vernonia polyanthes.* A third sample (C) was from bee hives at Guarapari, collected in 1996, Espírito Santo, Brazil, with local flora probably of gender *Cambará.*

Fractionation of extracts

Powdered propolis (A, B and C) were extracted with 75 ml of the hexane (1:25, w/v), at room temperature; the extraction residue was submitted to further extraction with 75 ml of acetone at room temperature. The solvents were removed under vacuum, and these crude extracts were separately analyzed by HT-HRGC.

Chromatographic analysis

HT-HRGC analyses were performed on a HP 5890-II gas chromatograph with flame ionization detector (FID, Hewlett Packard, Palo Alto, USA), using a cold on-column injector (Carlo Erba, Milano, Italy). Gas chromatography was performed on borosilicate capillary columns (18 m×0.30 mm i.d.; Duran-50 glass, Vidrolex, Brazil) coated with 0.1 μm of OV-1701-OH (5%-phenyl-7%-cyanopropyl-88%-methylpolysiloxane; Ohio Valley Specialty Chemical, Co., USA). The columns were prepared, according to a literature procedure (Blum, 1985). Column performance was checked prior to use by the Grob test (Grob Jr. *et al.,* 1978; Grob *et al.,* 1981). Sample volumes were 0.2 μl, with the injector at room temperature and the detector at 380 °C. Column temperature was programmed as follows: analysis of crude extracts: 40 °C, 10 °C/min to 370 °C (10 min). Hydrogen was used as carrier gas, at a linear velocity of 50 cm/s. The data were acquired and processed on a HP 3390 integrator.

The Kovats retention indices, were determined by injection of Polywax 655; an even number series of *n*-alkanes ranging from 20 to 80 carbons (Petrolite Specialty Polymers Group, Tulsa, USA) doped with *n*-C_{40} (Aldrich, USA).

Mass spectrometric analysis

HT-HRGC-MS analysis was performed on a HP 5972 MSD (Hewlett Packard, Palo Alto, USA), under electron impact ionization (70 eV). MS scan range was 40 to 700 a.m.u. During the analysis by HRGC-MS, the end of the capillary column was connected to a 2 m piece of (0.25 mm i.d.) High Temperature Fused Silica (HTFS, J&W, USA) which served as interface. The HTFS was purged with hydrogen at 180 °C for 15 min and deactivated by flushing with hexamethyldisilazane (HMDS)/1,3-diphenyl-1,1,3,3-tetramethyldisilazane (DPTMDS) 1:1 v/v (Sigma, USA), sealing the capillary, and heating at 400 °C for 12 h. The tubing was then rinsed with hexane, methanol and diethyl ether.

The GC-MS interface was at 350 °C and the ion source temperature at 300 °C. Column temperature program and injection mode were as for chromatographic analysis.

Compound characterization

The compounds were characterized by mass spectral interpretation and automatic comparison

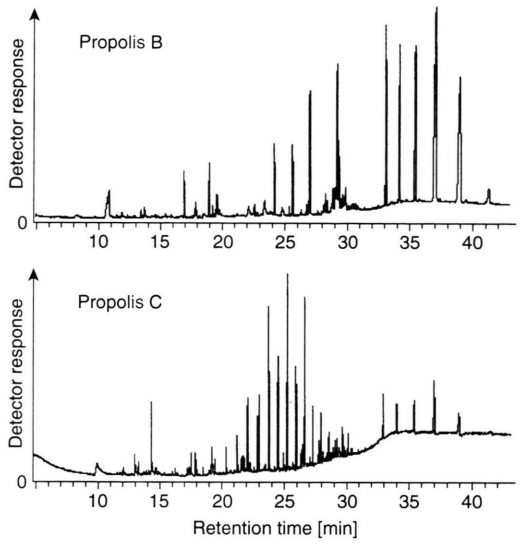

Fig. 1. Chromatograms of the hexane crude extracts of propolis B and C. The chromatograms of the hexane crude extracts of propolis A were similar to B (see Table I). For HT-HRGC conditions: see Experimental.

with library spectra. The library searches were of relatively limited help in the case of the HMW compounds, because many of these compounds had not been previously analyzed by GC-MS.

Results and Discussion

Crude extracts of propolis are complex samples, containing several acids and phenolic compounds, and are, in many instances, difficult to analyze, due, among other factors, to their strong tendency to overload the apolar (or medium polar) stationary phases needed for high temperature work (Stuckenbruck and Aquino Neto, 1990). The utilization of thin films of the stationary phase ($\leq 0.1\,\mu m$), favors the more rapid elution (at lower temperatures) of compounds with high molecular mass and/or boiling point, but limit even more the sample capacity of the capillary column. Despite this fact, the acetone crude extracts of propolis could be analyzed by HT-HRGC, with no clean-up, affording an informative chromatogram (see below).

Analysis of the hexane fractions of propolis by HT-HRGC and HT-HRGC-MS (Fig. 1) showed the presence of several high molecular weight compounds (Table I). Their composition is source dependent mainly in relation to the relative concentration of the *n*-alkanes and the high molecular weight compounds (see Table I). These high molecular weight compounds, comprise an homologous series with fragment ions at *m/z* 257 (base peak, Fig. 2), and were characterized as fatty acid esters of long chain fatty alcohols (FAWE) with more than 676 Daltons.

Fig. 2 shows as the mass spectrum of the tetraicosyl hexadecanoate as an example of this FAWE series (see Table I). The interpretation of the mass spectra of these compounds clearly indicates a FAWE structure: this is based on consideration of the molecular ions, the fragmentation patterns and retention indices and also that several FAWE compounds have been reported previously in propolis (Marcucci, 1995).

Analysis of the acetone crude extract by HTHRGC-MS, confirmed the presence of several flavonoids. The mass spectral characteristics of three of these compounds are presented in Fig. 2, and indicate the presence of three positional isomers of a trihydroxymethoxyflavanone.

The identification of this class of natural products by mass spectral analysis alone is rather difficult, because of the number of possible isomers and, in several cases, minor differences between

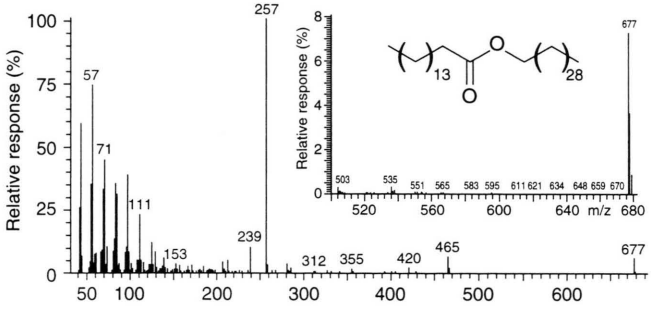

Fig. 2. Mass spectra of octaicosylhexadecanoate, representative of the homologous series of palmitic acid esters found in propolis.

Table I. Composition of propolis A, B and C as assessed by HT-HRGC-MS. Relative concentrations (%) were determined for FID.

Compound	Retention time [min]	Retention indices [I]*	Relative concentration [%]					
			Hexane fraction			Acetone fraction		
			A	B	C	A	B	C
3-Hexen-2-one	4.9	Not calculated	–	<0.1	–		–	–
Acetophenone	6.6	Not calculated	–	–	–	–	<0.1	–
2-Butyl-1-octanol	6.9	Not calculated	–	–	–	–	<0.1	–
Benzoic acid	8.2	Not calculated	–	0.1	–	–	–	–
Hydrocinnamic methyl ester	9.6	1333	–	<0.1	–	1.8	–	–
Hydrocinnamic acid	10.8	1475	<0.1	0.3	0.3	–	–	–
4-Hydroxy-3-methoxy-benzaldehyde	11.4	1580	0.2	0.7	–	–	–	–
7-Methoxy-2,2-dimethyl-2H-1-benzopyran	12.1	1655	–	0.1	–	–	–	–
2,4-Bis(1,1-dimethylethyl)-phenol	12.8	1782	–	–	0.2	–	–	–
Cadinene (isomer)	12.8	1782		<0.1	–	–	–	–
Octyl phenol isomer	14.0	1951	–	–	0.2	–	–	–
Naphathalene, 1,6-dimethyl-4-(1-methylethyl)	15.3	2148	–	–	0.6	–	–	5.0
Benzyl benzoate	15.8	2200	<0.1	1.1	–	–	–	–
Tetracosane	17.2	2400	<0.1	<0.1	0.3	–	–	–
Hexadecanoic acid	17.9	2425	0.1	0.2	0.9	8.5	0.5	0.5
Hexadecanoic acid, ethyl ester	18.2	2451	–	<0.1	<0.1	–	–	–
Pentaicosane	19.2	2500	<0.1	<0.1	2.8	–	–	–
Benzyl cinnamate	19.3	2510	0.8	0.4	–	0.8	0.4	–
9-Octadecenoic acid	19.6	2552	<0.1	<0.1	–	–	–	–
9-Octadecenoic acid (Z), methyl ester	19.7	2575	–	–	–	–	<0.1	–
Octadecanoic acid	19.8	2585	<0.1	<0.1	–	–	3.7	–
9-Octadecenoic acid (Z), ethyl ester	19.9	2592	–	–	0.6	–	–	–
Heneicosane	20.1	2600	<0.1	<0.1	3.3	–	–	–
Octadecanoic acid, isopropyl ester	20.2	2610	–	–	–	–	–	0.4
Heptaicosane	21.0	2700	0.1	<0.1	6.3	–	–	–
2-Propenoic acid-3-(4-methoxy-phenyl)-2-ethylhexyl ester	21.2	2720	0.2	0.2	–	–	–	0.8
Dihydroxymethoxyflavone	21.2	2720	–	0.1	–	–	–	–
Octaicosane	21.9	2800	0.3	0.4	0.2	–	–	–
Trihydroxymethoxyflavanone (isomer)	22.1	2820	–	–	<0.1	–	–	7.3
Hydroxydimethoxy-β-methyl-chalcone	22.1	2820	–	–	–	2.1	–	–
Hydroxydimethoxyflavone	22.2	2835	–	–	–	2.8	0.9	–
Trihydroxymethoxyflavanone (isomer)	22.2	2835	–	–	<0.1	–	–	7.9
Trihydroxymethoxyflavone	22.5	2870	–	–	<0.1	–	–	–
Nonaicosane	22.7	2900	0.2	0.2	6.9	–	–	–
Trihydroxymethoxyflavanone (isomer)	22.8	2910	–	–	<0.1	–	–	10.1
Triacontane	23.5	3000	3.7	4.1	4.5	–	–	–
Tetrahydroxymethoxyflavone	24.0	3080	–	–	–	–	0.3	–
Untriacontane	24.1	3100	0.1	0.3	6.8	–	–	–
Dotriacontane	24.8	3200	1.8	1.0	3.5	–		–
Tetraicosanoic acid	24.9	2210	–	0.1	–	–	–	–
Tritriacontane	25.5	3300	0.1	<0.1	2.7	–		–
Nonacosanol	25.5	3300	1.7	3.5	2.0	–	–	–
Tetratriacontane	26.2	3400	0.1	9.1	1.6	–	–	–
Pentatriacontane	26.9	3500	0.1	1.0	0.8	–	–	–
Trihydroxydimethoxyflavone	27.0	3512	–	–	–	2.2	1.7	–

Table I. (cont.).

Compound	Retention time [min]	Retention indices [I]*	Relative concentration [%]					
			Hexane fraction			Acetone fraction		
			A	B	C	A	B	C
Hexatriacontane	27.6	3600	0.1	0.2	0.5	–	–	–
Untriacontanol	28.3	3623	<0.1	0.1	0.2	–	–	–
β-Amyrin	29.0	3790	0.1	0.1	–	–	–	–
β-Amyrin, methyl ether	29.2	3825	–	–	<0.1	–	–	–
Dotriacontanol	29.4	3875	0.1	0.2	–	–	–	–
α-Amyrin	29.5	3890	–	–	<0.1	–	–	–
Nonatriacontane	29.6	3900	<0.1	–	–	–	–	–
Tetraicosylhexadecanoate	33.1	4270	5.3	3.8	2.3	1.1	–	–
Heneicosylhexadecanoate	34.2	4410	5.3	3.8	2.2	1.0	–	–
Octaicosylhexadecanoate	35.4	4550	6.7	4.7	3.1	1.1	–	–
Triacontylhexadecanoate	37.0	4700	26.1	16.1	6.8	2.0	–	–
Dotriacontylhexadecanoate	38.9	4865	11.1	7.6	4.8	1.5	–	–
Tetratriacontylhexadecanoate	41.1	5035	3.5	3.1	1.1	<0.1	–	–
Hexatriacontylhexadecanoate	43.2	5196	1.6	1.4	0.3	–	–	–
Octatriacontylhexadecanoate	45.0	5360	0.7	<0.1	–	–	–	–
Tetracontylhexadecanoate	47.0	5530	0.4	<0.1	–	–	–	–
Dotetracontylhexadecanoate	49.2	5710	0.2	<0.1	–	–	–	–

* Calculated according to E. Kovats, (Grob, 1995), using as reference compounds a mixture of even carbon number saturated hydrocarbons (Polywax 655).

their mass spectra. Usually only probable structures can be advanced using exclusively mass spectral data together with biogenetic arguments. For example, a methoxy group is more common at the 4′ position, based on the biogenetic formation of the flavonoids from shikimic acid (Dewick, 1995). For a discussion of the fragmentation characteristics of flavonoids see Porter (Porter, 1985).

The results of HT-HRGC and HT-HRGC-MS analyses of the crude extracts exhibited the main advantages of the techniques: fast simultaneous characterization of a great number of different classes of natural products in a single sample, including highly functionalized compounds, such as flavonoids. As the chemical composition of propolis, depends on the place and time of the collection, collecting bee species, source quality/type and abundance, its accessibility and attractive power, among other variables, rapid and efficient analytical screening is mandatory to promote a faster development of this field of knowledge. Additionally, this diverse chemical composition, also promotes variation of the biological and pharmacological properties of propolis exacerbating the need for fast, simple and efficient analytical techniques to support pharmacological studies of this important natural product. Such a technique would also be useful in quality control of commercial samples including detection of adulteration.

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