Rapid Screening of Polar Compounds in Brazilian Propolis by High-Temperature High-Resolution Gas Chromatography−Mass Spectrometry

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Methanol extracts of propolis from six different places, five in Rio de Janeiro state and one in São Paulo state, both in the Southeast of Brazil, were investigated using high-temperature high-resolution gas chromatography (HT-HRGC) and HT-HRGC−mass spectometry. The main purpose of the study was to establish the applicability of HT-HRGC as an analytical method for systematic studies of polar propolis fractions. Several compounds, including carbohydrates, phenolic acid derivatives, and high molecular weight compounds (e.g., wax esters of long chain fatty alcohols) could be readily characterized in the crude extracts by HT-HRGC−MS. HT-HRGC and HT-HRGC−MS were shown to be quick and informative tools for rapid analysis of crude polar extracts without cleanup.

Keywords: *Propolis; high-temperature gas chromatography; polar compounds; carbohydrates; high molecular weight compounds*

INTRODUCTION

Propolis is a resinous beehive product consisting of a mixture of wax, sugars, and plant parts (branch, flowers, pollen, and buds) collected by the honeybee from different plants depending on the geographic location. Bees use propolis as a sealer for their hive and to prevent the decomposition of carcasses of insect invaders which enter the hive and are killed by the soldier bees (Banskota et al., 1998; Pereira et al., 1998a).

Propolis has been used as folk medicine at least since 300 B.C. (Banskota et al., 1998). In recent years interest in this material has increased not only as a potential source in folk medicine but mainly as a prospect raw material in the pharmaceutical industry. It presents several biological activities; e.g., anticancer, antioxidant, antiinflammatory, antibiotic, and antifungal properties have been reported for propolis and its constituents (Bankova et al., 1987; Banskota et al., 1998; García-Viguera et al., 1993; Pereira et al., 1998a). Because of the wide range of activity, propolis is now being used as a health food supplement. Reports have been published about successful clinical use of propolis to aid the healing of wounds, ulcers, and tuberculosis, in treatment of fungal infections, eczems, and mouth infections, etc. (Marcucci, 1995; Burdock, 1998). The only side effect reported so far appears to be the allergic reactions caused in sensitive individuals (García-Viguera et al., 1992). Previous studies showed that flavonoids, aromatic acids, and phenolic derivatives appear to be the principal components responsible for the therapeutic effects of European propolis, and the anticancer activity in Brazilian propolis seems to be primarily due to phenolic derivatives (Banskota et al., 1998; Ghisalberti et al., 1979; Marcucci, 1995). The occurrence and concentration of the different compounds depend, among other things, on the type of plant and time of collection (Banskota et al., 1998).

The high biodiversity characteristic of tropical countries such as Brazil increases the possible variability of constituents of propolis (Nothenberg, 1997); in Brazil this fact is more expressive because of the continental size. The major biologically active constituents of propolis from European and North American sources are flavones, flavanones, and flavonols (García-Viguera et al., 1993). Besides the flavonoids, alcohols, aldehydes, aliphatic and aromatic acids, aliphatic and aromatic esters, chalcones, terpenoids, steroids, sugars, and amino acids were also identified in propolis (Nothemberg, 1997; Greenaway et al., 1991). In Brazilian propolis the main active compounds already characterized are derivatives of aromatic acids (e.g., cinnamic acids) and diterpenoids (Bankova et al., 1996).

In 1997 the estimated world consumption of propolis was about 700−800 tons/year, with raw material prices ranging from, in U.S. dollars, 120.00/kg to 180.00/kg, depending on the nature and concentration of active principles (Nothenberg, 1997).

High-resolution gas chromatography (HRGC) and HRGC coupled to mass spectrometry (HRGC−MS) have become traditional methods to obtain the distribution profiles and identities of compounds in complex fractions

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Table 1. Composition of Methanolic Extracts of Propolis by HT-HRGC−MS[a]

TR (min)	compound	A	B	C	D	E	F
5.24	glycerol (3 TMS)	2.1					
6.49	2,3-dihydroxypropanoic acid (3 TMS)	3.1	<0.5	1.3		<0.5	<0.5
6.98	phosphate (3 TMS)	<0.5	2.3	3.1		<0.5	2.3
7.62	butanedioic acid (2 TMS)		<0.5	1.4			
9.00	benzenepropanoic acid (1 TMS)	1.8	0.8				
10.50	malic acid (3 TMS)		1.1	3.3		0.9	
11.24	erythritol (4 TMS)		<0.5		<0.5	<0.5	1.0
11.63	trihydroxybutyric acid isomer (4 TMS)					<0.5	
11.88	hydroxybenzoic acid (2 TMS)	<0.5					
11.88	trihydroxybutyric acid isomer (4 TMS)					<0.5	
13.87	hydroxymethoxybenzoic acid isomer (2 TMS)			<0.5			
13.99	hydroxymethoxybenzoic acid isomer (2 TMS)			<0.5			
14.04	dihydroxymethoxybenzoic acid isomer (3 TMS)	2.2					
14.05	hydroxymethoxybenzoic acid isomer (2 TMS)			<0.5			
14.84	dihydroxymethoxybenzoic acid isomer (3 TMS)			0.9			
14.92	dihydroxymethoxybenzoic acid isomer (3 TMS)			0.5			
15.36	inositol (6 TMS)	1.2		2.3			2.6
16.23	*p*-cinnamic acid (2 TMS)	6.7	3.5				<0.5
16.82	trihydroxymethoxybenzoic acid isomer (4 TMS)		<0.5			<0.5	
17.34	hexadecanoic acid (1 TMS)		1.2		4.4		
17.40	vanilethanediol (3 TMS)						1.6
17.60	trihydroxymethoxybenzoic acid isomer (4 TMS)			2.3			
17.63	ferulic acid (1 TMS)				0.8		
17.72	trihydroxymethoxybenzoic acid isomer (4 TMS)			4.9			
17.84	isoferulic acid (1 TMS)				1.3		
18.57	caffeic acid isomer (1 TMS)			<0.5			
18.70	caffeic acid isomer (1 TMS)	2.4	1.5	<0.5			1.2
19.73	octadecanoic acid (1 TMS)				<0.5		
25.09	scalene				<0.5		
25.50	tetracosanoic acid (1 TMS)					2.3	
26.03	hexacosanol (1 TMS)					1.0	
27.14	hexacosanoic acid (1 TMS)					<0.5	
27.67	cholest-5-en-3-ol (1 TMS)				<0.5	1.0	
28.06	octacosanol (1 TMS)					<0.5	
28.70	octacosanoic acid (1 TMS)					<0.5	
29.07	stigmast-5-en-3-ol (1 TMS)				<0.5		
29.52	triacontanol (1 TMS)					<0.5	
30.15	triacontanoic acid (1 TMS)					<0.5	
30.96	dotriacontanol (1 TMS)					<0.5	
31.53	dotriacontanoic acid (1 TMS)					<0.5	
32.86	tetratriacontanoic acid (1 TMS)					<0.5	
34.86	hexadecanoic acid tetracosyl ester					<0.5	
36.03	hexadecanoic acid hexacosyl ester					<0.5	
37.14	hexadecanoic acid octacosyl ester					<0.5	
38.23	hexadecanoic acid triacontyl ester					<0.5	
39.24	hexadecanoic acid dotriacontyl ester					<0.5	

[a] Relative concentrations (%) were determined from the TIC. For HRGC conditions, see the Materials and Methods.

and subsequently decide on the convenience and best strategy for isolation and unambiguous identification of unknown substances (Patitucci et al., 1995; Pereira and Aquino Neto, 1999). 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 (HT-HRGC−MS) are established techniques for separation of complex mixtures and identification of high molecular weight compounds, many of which do not elute when analyzed on ordinary HRGC columns (Pereira and Aquino Neto, 1999). HT-HRGC may be an excellent alternative to classical analytical phytochemistry and a potent tool for the rapid evaluation of the composition of crude natural products and medicinal plants (Pereira et al., 1998b).

In the present work HT-HRGC and HT-HRGC−MS analyses of the highly polar and high molecular weight compounds that were present in samples of six different propolis collected in the states of Rio de Janeiro and São Paulo, Brazil, are reported.

MATERIALS AND METHODS

Materials. The propolis samples were collected from beehives in the following cities in southeast Brazil: A in Sapucaia, B and C in Nova Friburgo, D in Fregresia, and E in Visconde de Mauá, all in the state of Rio de Janeiro; F in Santo Antonio do Pinhal, in the state of São Paulo.

These regions have a high biodiversity because they correspond to cities localized in the Brazilian Atlantic forest (Wilson and Peter, 1997). The dominant vegetation in each area of collection was as follows: sample A, *Citrus* spp. and *Vernonia polyanthes;* samples B and C, *Citrus* spp.; sample D, *Citrus* spp. and *Pinus* spp.; sample E, *Pinus* spp.; sample F, the flora was undetermined.

Fractionation of Extracts. Powdered propolis was initially extracted with hexane (1:25, w/v), the extraction residue being further extracted with acetone at room temperature. The extraction residue was finally extracted with methanol, all at room temperature. An ultrasonic bath, Bransonic 72 (Branson), was used for all extractions.

Derivatization. The propolis fractions were dried at room temperature, using a desiccator with P_2O_5 under vacuum. A 100 μL sample of bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added to 10 mg of the dried residue samples. The samples were left for 16 h at 60 °C to complete the derivatization. BSTFA containing 1% trimethylsilyl (TMS) was obtained from Sigma (St. Louis, MO).

Chromatographic Analyses. HT-HRGC analyses were performed on an HP 5890-II gas chromatograph with a flame ionization detector (FID; Hewlett-Packard, Palo Alto, CA), using a cold on-column injector (Carlo Erba, Milano, Italy). A glass column (22 m, 0.2 mm) coated with PS-086 (15% phenyl and 85% methyl polysiloxane) was used, prepared in our laboratory, according to a literature procedure (Blum, 1985). Sample volumes of 0.5 μL were injected. The chromatograph was programmed as follows: The injector was at room temperature and the detector at 400 °C. Oven programming was 40 °C, 8 °C/min to 390 °C, with a final hold of 10 min. Helium was used as carrier gas, at a linear velocity of 38 cm/s. The data were acquired and processed on an HP 3396-II integrator.

Mass Spectrometric Analysis. HT-HRGC−MS analyses were performed on an HP 5972 MSD (Hewlett-Packard, Palo Alto, CA), under electron impact ionization (70 eV). The interface was at 320 °C, and the MS scan range was 40−700 Da. The column temperature program and injection mode were the same as for HT-HRGC.

Compound Characterization. The compounds were characterized by mass spectral interpretation and comparison with library searches. Library searches were of relatively limited help in the case of the high molecular weight compounds, because many such structures had not been previously analyzed by MS.

RESULTS AND DISCUSSION

Previous reports (Pereira et al., 1998a, 1999) on the applications of HT-HRGC to the analysis of high molecular weight and highly polar compounds showed that the usual HRGC procedure used in natural products research needed to be changed to identify the high molecular weight and highly polar compounds present in crude extracts. The main change was the technique of injection. Cold on-column injection proved essential, particularly for high molecular weight compounds, because the sample is deposited directly into the column, giving the highest reproducibility, lowest discrimination, and smallest sample decomposition.

Due to the complexity in composition of propolis, accurate quantitation is very difficult, because of the differences in detector response. In this work approximate concentrations were calculated using a correction factor equal to one (total ion chromatogram (TIC) response).

Previous studies of low polarity fractions of Brazilian propolis have characterized more that 60 compounds by HT-HRGC. Several flavonoids in acetone fractions were characterized without derivatization (Pereira et al., 1998a, 1999), but for substances extracted with methanol, derivatization with BSTFA was necessary. The compounds characterized are shown in Table 1.

Several constituents of the crude methanol extracts presented a mass spectrum containing ions typically related to the fragmentation of TMS derivatives of carbohydrates (base peak ion at *m*/*z* 204 or *m*/*z* 217). It was possible to recognize, for example, ring size: Consider the fragmentation involved in the formation of *m*/*z* 319; this ion retains four carbon atoms of the carbohydrate molecule and three $(CH_3)_3SiO$ groups, and is much more prominent in the mass spectra of the furanoses (and furanosides) than in the mass spectra of the pyranoses (and pyranosides). The ions *m*/*z* 204 and *m*/*z* 217 are more prominent in the mass spectra of six-membered-ring and five-membered-ring isomers, respectively (Dejongh et al., 1969).

Analyses of the mass spectral data show that all samples presented sugars, mainly monosaccharides. Sample A has a large number of sugar structures

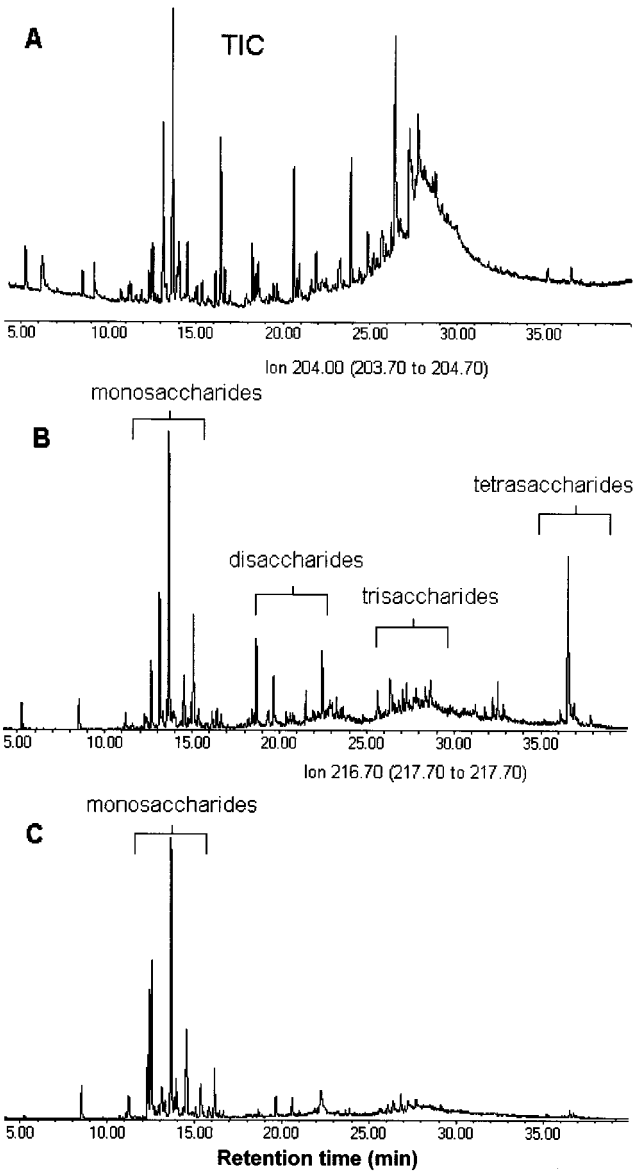

Figure 1. (A) TIC of the crude methanolic extract of propolis sample A (see the Materials and Methods). (B) Representative HT-HRGC mass fragmentogram (*m*/*z* 204) and (C) representative HT-HRGC mass fragmentogram (*m*/*z* 217) showing the distributions of saccharides (TMS derivatives) in the sample.

including tetrasaccharides (see Figure 1B). The presence in propolis of sugars containing more than one residue is reported for the first time in this paper.

The identification of mixtures of natural products by mass spectral analysis alone is rather difficult, because of the number of isomers and, in several cases, minor differences between their mass spectra (Bicalho et al., 2000). But more than 40 different compounds (excluding the carbohydrates) were characterized (e.g., phosphate, glycerol, cinammic acid, high molecular weight compounds, etc.; see Table 1).

The relative concentrations of all the compounds characterized in the samples are shown in Figure 2. Sample A contained the larger number of compounds, which could not be characterized using only their mass spectra. In three samples inositol was characterized (probably *myo*-inositol, the usual diastereoisomer found in natural products).

Figure 2 presents the relative abundances of a few biologically active compounds in the samples. The

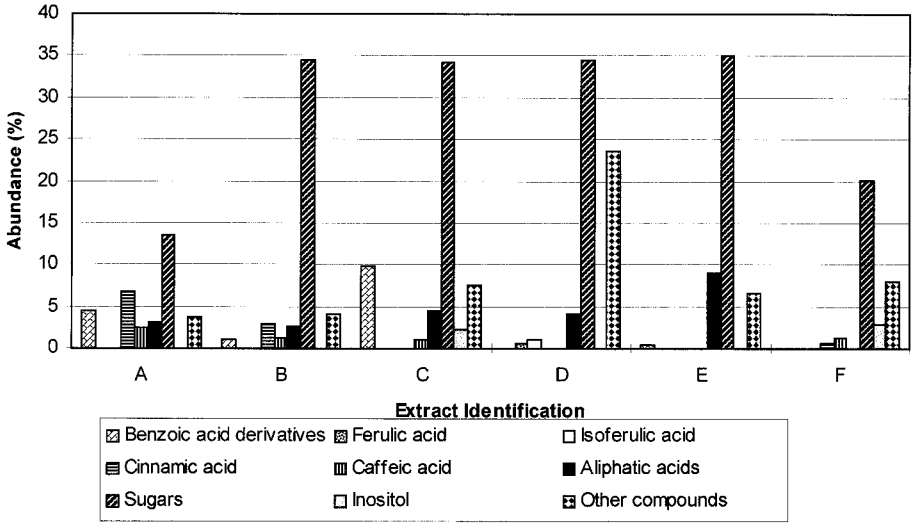

Figure 2. Histogram of the distributions of compounds or compound classes characterized in crude methanolic extracts of Brazilian propolis.

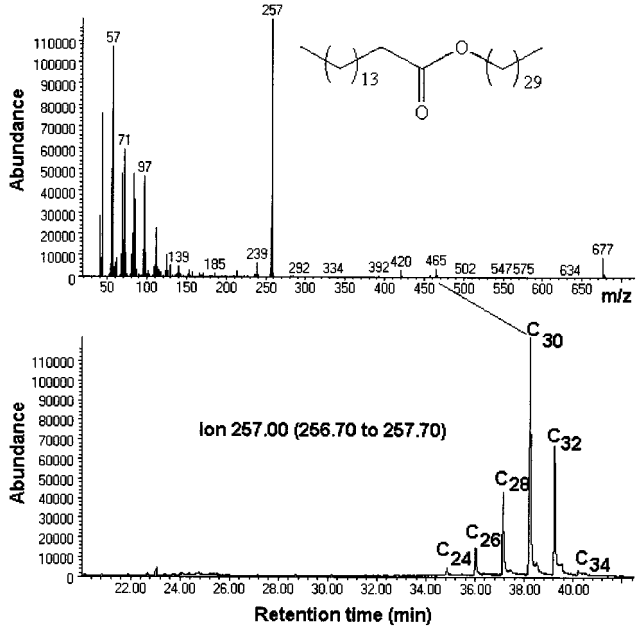

Figure 3. Representative HT-HRGC mass fragmentogram (*m*/*z* 257) of the hexadecanoic acid esters of long-chain fatty alcohols of a methanolic propolis extract (E). Also shown is the mass spectrum of octaicosylhexadecanoate (representative of the homologous series of palmitic acid esters found in propolis; C_n denotes the numbers of the carbon atoms of the alcohol chain).

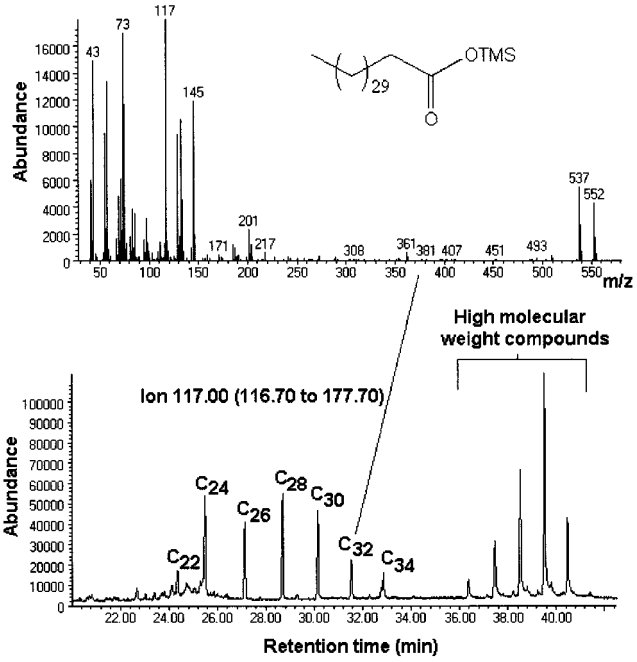

Figure 4. Representative HT-HRGC mass fragmentogram (*m*/*z* 117) of the *n*-acid esters of TMS of a methanolic propolis extract (E). Also shown is the mass spectrum of dotriacontanoic acid trimethylsilyl ester (representative of the homologous series of *n*-acids found in propolis; C_n denotes the number of carbon atoms of the acid chain). The high molecular weight compounds are uncharacterized compounds.

presence of caffeic acid derivatives known for their antitumoral properties could explain some of the possible therapeutic usefulness (Marcucci, 1995).

All methanolic extracts showed a series of high molecular weight hexadecanoate esters of long-chain fatty alcohols. This series was previously characterized in hexane crude extracts of propolis (Pereira et al., 1998a, 1999) and can be easily tracked by monitoring its base peak formed by a double rearrangement fragmentation at the ester group (see Figure 3). This material was probably added directly from the plant source, since it is part of the saponifiable matter of vegetable oils, where it occurs in concentrations sampling from 0 to 3% (Reiter et al., 1999).

Fatty acids and their metabolites are involved in the regulation of growth and morphogenesis in all living organisms. The diverse functions of these lipids include their structural role in membranes and their roles as a source of cellular energy, in regulation of brain function, in modulation of enzyme activity, etc. (Nes, 1994).

A series of wax acids were characterized with much longer carbon chains (C_{22}−C_{34}) (see Figure 4) than the usual fatty acids (C_{16}−C_{18}). Another series characterized was that of 1-alkanols (C_{26}−C_{32}). Since these relatively apolar compounds were only characterized in the methanolic extracts, there is probably a type of matrix effect that hinders their extraction by the previous, less polar solvents. This could be related to the fact that these fatty acids and their derivatives are commonly found in cellular membranes and in plant extracellular matrix, as a heterogeneous macromolecular assembly consisting

of cellulose microfibrils embedded in a matrix of complex polysaccharides and glycoproteins (Cutillas-Iturralde et al., 1998).

Another high molecular weight series was observed in the samples (see Figure 4), with molecular ions higher than 700 Da (the upper mass limit of the mass spectrometer used in this work). The analysis of the mass spectra of these compounds showed that these compounds were derivatized with BSTFA, but the information obtained by mass spectrometry was insufficient for characterization.

HT-HRGC is opening a new molecular weight "window" in the analysis of complex mixtures, ranging in mass from 400 to 1000 Da. And the possibility of analyzing crude extracts can be extremely useful for the systematic study of medicinal plants and other sources of biologically active compounds, as a quick screening method, which could guide subsequent phytochemical work.

As the chemical composition of propolis depends on the place and time of the collection, collecting bee species, quality/type and abundance of the source, etc., rapid and efficient analytical screening is mandatory to promote faster sample screening. Obviously, the differences in chemical composition also promote 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.

HT-HRGC and HT-HRGC−MS were demonstrated to be powerful analytical tools in propolis research. A high level of information can be obtained concerning the chemical composition, with the possibility of direct analysis and characterization of compounds with masses between 500 and 1000 Da in crude extracts. Polysaccharides up to four ring units and inositol were also characterized, for the first time, in propolis samples. HT-HRGC−MS would also be useful in quality control of commercial samples including detection of adulteration of propolis.

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