Application of High Temperature High Resolution Gas Chromatography to Crude Extracts of Propolis

Alberto dos Santos Pereira*, Angelo C. Pinto, Jari Nobrega Cardoso, Francisco Radler de Aquino Neto

Instituto de Química, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Cidade Universitária, Centro de tecnologia, Bloco A, Rio de Janeiro, RJ – Brazil 21949–900

Mônica Freiman de Souza Ramos, Gisela M. Dellamora-Ortiz, Elisabete Pereira dos Santos

Faculdade de Farmacia, Universidade Federal do Rio de Janeiro, Ilha do Fundão, CCS, Bloco K, 2° Andar, Rio de Janeiro, RJ – Brazil 21941-590

Ms received: November 10, 1997; accepted: May 21, 1998

Key Words: *High temperature gas chromatography; flavonoids; propolis; high molecular weight compounds*

Summary

The underivatized acetone and hexane fractions from propolis samples (predominant flora *Citrus* spp. and *Vernonia polyanthes*) 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 were characterized in 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 extracts without need for prior derivatization and purification.

1 Introduction

Propolis (bee glue) is a complex mixture, formed from resinous and balmy material, collected by bees from parts of plants (branch, flowers, pollen, and buds) and modified in the beehive by addition of salivate secretions and beeswax [1–3]. Bees use it as a sealer for their hives and, more importantly, to prevent the decomposition of creatures which have been killed by bees after an invasion of the hive [4].

Propolis shows a number of 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, treatment of mycotic infections and eczema, in stomatology, *etc.* [4, 5].

These valuable properties of propolis created an 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 [4, 6]. The proportion of these compounds varies and depends, among many other variables, on the place and time of collection [7].

The flavonoids, aromatic acids and phenolic derivatives are believed to be the principal components responsible for the therapeutic effects of propolis [8–11]. Flavonoids and other phenolic compounds are widespread in plants and are also important as active ingredients of many photogenic preparations in cosmetics and medicine [12]. Estimated world consumption is about 700–800 tons/year, with raw material prices ranging from US dollars 120.00 to 180.00/kg, depending on the nature and concentration of active principles [13].

Characterization of flavonoids is usually accomplished by classical phytochemical techniques, comprising a step of isolation before identification by 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 thereupon decide as to the interest for unambiguous identification of the isolated unknown substances [14]. Unfortunately, analysis by HRGC of several classes of bioactive compounds is made 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 which do not elute when analyzed on ordinary HRGC columns [15]. As such, HT-HRGC may be an excellent alternative to classical analytical phytochemistry and a potent tool for the study of medicinal plants. HT-HRGC has been already reported in the analysis of high molecular weight compounds as, for example: porphyrins [16], cyclodextrin derivatives [17–18], HMW *n*-alkanes[19], triglycerides[20], oligossaccharides [21], lipids [22], in widely different matrices, including, recently, environmental samples [23–25].

2 Experimental

2.1 Material

Propolis was collected from bee hives at Sapucaia, RJ, Brazil with predominant local flora of *Citrus* spp. and *Vernonia polyanthes*.

2.2 Fractionation of Extracts

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

2.3 Chromatographic Analysis

HT-HRGC analyses were performed on a HP 5890-II gas chromatograph with flame ionization detector (FID, Hewlett Packard,

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Palo Alto, USA), using a cold on-column injector (Carlo Erba, Milano, Italy). The column used was a fused silica capillary (10 m × 0.3 mm i.d.) coated with a 0.1 µm film of Silaren-30 (30% diphenylpolysiloxane, 40% sildiphenylene ether, 30% dimethyl polysiloxane; BGB Analytik AG, Rothenfluh, Switzerland). Sample volumes were 0.2 µL, with the injector at room temperature and the detector at 400 °C.

The column temperature was programmed as follows:

- analysis of crude hexane extracts: 40 °C (0.5 min), 10 °/min to 380 °C (20 min).
- analysis of crude acetone extracts: 40 °C (0.5 min), 30 °/min to 100 °C and 10 °/min to 370 °C (10 min).

Hydrogen was used as carrier gas, at the linear velocity of 50 cm/s. The data were acquired and processed on a HP 3396-II 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).

2.4 Mass Spectrometric Analysis

HT-HRGC-MS analysis was performed on a HP 5987A mass spectrometer (Hewlett Packard, Palo Alto, USA), under electron impact ionization (70 eV). MS scan range was 40 to 700 amu. 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.

2.5 Compound Characterization

The compounds were characterized by mass spectra interpretation and comparison with library searches. Library searches were of relatively limited help in the case of the HMW compounds, because many of these compounds have not been analyzed previously by GC-MS.

3 Results and Discussion

Crude extracts of propolis are complex samples, containing several acids and phenolic compounds forming, in many instances, mixtures which are 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 [26]. The utilization of short columns (≤10 m) and thin films of the stationary phase (≤0.1 µm), favor the more rapid elution (at lower temperatures) of compounds with high molecular mass and/or high boiling point, but limit even more the sample capacity and resolving power of the capillary column. Despite this fact, the acetone crude extract of propolis could be analyzed by HT-HRGC, with no clean-up, affording an informative chromatogram (see below).

Analysis of the hexane fraction of propolis by HT-HRGC (**Figure 1**) showed the presence of several high molecular weight compounds possessing values of Kovats retention indices, exceeding 4200 (**Table 1**). Analysis of this same fraction by HT-HRGC-MS allowed characterization of several acids and phenolic compounds (**Table 2**) and some of the major compound classes will be presented below.

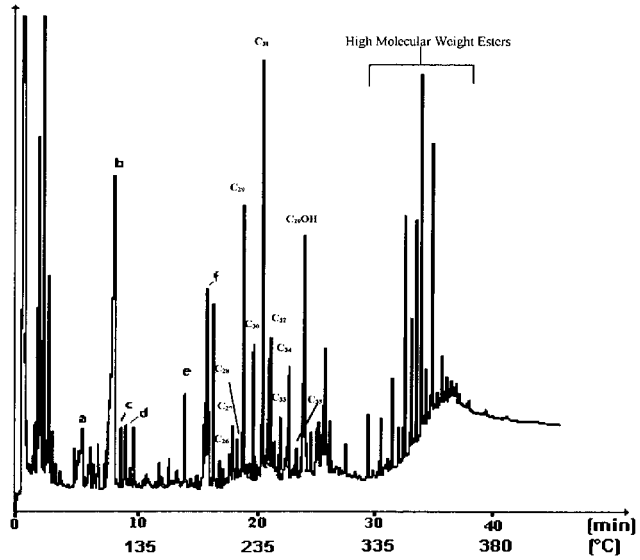

Figure 1. Chromatogram of the hexane crude extract of propolis. a) benzoic acid; b) hydrocinnamic acid; c) vanillin; d) cinnamic acid; e) hexadecanoic acid, and f) benzyl cinnamate; $C_{29}OH$ (1-nonacosanol); $C_{26}-C_{35}$ (hexacosane – pentatriacontane). HT-HRGC conditions: see experimental.

Table 1. Compounds characterized in hexane or acetone crude extracts, retention times (t_R) and Kovats retention indices (*I*).

Compound	Molecular formula	t_R(min)	*I**
Benzoic acid	$C_7H_6O_2$	5.3	973
Hydrocinnamic acid	$C_9H_{10}O_2$	7.7	1329
Vanillin	$C_8H_8O_3$	8.7	1472
Cinnamic acid	$C_9H_8O_2$	9.0	1515
Hexadecanoic acid	$C_{16}H_{32}O_2$	14.0	2329
Benzyl cinnamate	$C_{16}H_{14}O_2$	16.3	2624
Naringenin 3′,4′-dimethoxy	$C_{17}H_{16}O_6$	21.9	3500
1-Nonacosanol	$C_{29}H_{60}O$	22.8	3588
Betuleol	$C_{17}H_{14}O_7$	24.7	3800
Kaempferid	$C_{16}H_{12}O_6$	25.2	3892
Tetracosyl hexadecanoate	$C_{40}H_{80}O_2$	30.7	4255
Pentacosyl hexadecanoate	$C_{41}H_{82}O_2$	31.0	4289
Heptacosyl hexadecanoate	$C_{43}H_{86}O_2$	31.9	4389
Octacosyl hexadecanoate	$C_{44}H_{88}O_2$	32.3	4433
Nonacosyl hexadecanoate	$C_{45}H_{90}O_2$	32.7	4478
Triacontyl hexadecanoate	$C_{46}H_{92}O_2$	33.0	4511
Dotriacontyl hexadecanoate	$C_{48}H_{96}O_2$	33.8	4600
Tetratriacontyl hexadecanoate	$C_{50}H_{100}O_2$	35.5	4750

* Calculated according to E. Kovats [30], using as reference compounds a mixture of even carbon number saturated hydrocarbons (Polywax 655).

3.1 Hydrocarbons

Alkanes between 26 and 35 carbon numbers are present in the crude hexane extract (Figure 1). These compounds comprise more that 30% of the total area in the FID chromatogram.

3.2 Acids

Several free aromatic and aliphatic acids could be detected without derivatization in the hexane crude extract. A list of all the

Table 2. Flavonoids and related substances present in hexane and acetone crude extracts of propolis. Mass spectra fragmentation: *m/z* (relative intensity,%).

Compound	Molecular formula	M^+	M-15	M-17	M-18	M-43	M-55	other ions
Hydrocinnamic acid	$C_9H_{10}O_2$	150 (39)	—	—	—	—	—	105 (17); 104 (48); 103 (12); 91 (100); and 77 (12)
Vanillin	$C_8H_8O_3$	152 (91)	137 (3)	135 (2)	—	109 (15)	—	151 (100).
Cinnamic acid	$C_9H_8O_2$	148 (61)	—	131 (15)	130 (6)	105 (6)	93 (4)	147 (100); 135 (57); 105 (6); 103 (42); 91 (39); and 77 (42)
Benzyl cinnamate	$C_{16}H_{14}O_2$	238 (10)	—	—	—	—	—	193 (30); 192 (45); 131 (85); 115 (15); 103 (45); 91 (100); 77 (36); and 65 (15)
Naringenin 3′,4′-di-methoxy	$C_{17}H_{16}O_6$	316 (35)	301 (12)	—	—	273 (8)	261 (1)	243 (82); 225 (25); 189 (28); 165 (33); 164 (94); 147 (67); 105 (61); and 91 (100)
Betuleol	$C_{17}H_{14}O_7$	330 (70)	315 (12)	313 (9)	312 (36)	287 (100)	275 (3)	269 (6); 232 (9); 165 (15); 135 (24); 105 (21); 91 (29); 77 (24); and 69 (30)
Kaempferid	$C_{16}H_{12}O_6$	300 (100)	285 (21)	283 (1)	282 (3)	257 (15)	—	229 (19); 150 (12); 135 (18); 105 (15); 91 (18); 77 (18); and 69 (18).

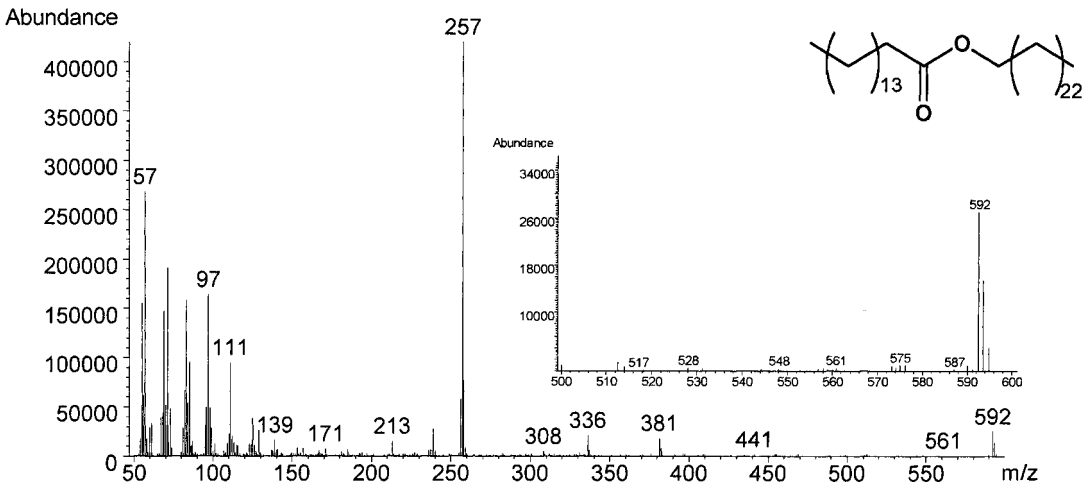

Figure 2. Mass spectra of tetracosyl hexadecanoate, representative of the homologous series of palmitic acid esters.

acids characterized is shown in Table 1. The mass spectral data of a few components is presented in Table 2.

3.3 Other Compounds

Vanillin, benzyl cinnamate, and 1-nonacosanol were characterized in the hexane extract.

3.4 High Molecular Weight Compounds

The high molecular weight compounds detected in the hexane fraction of propolis, comprise a homologous series with fragment ions at *m/z* 257 (base peak, **Figure 2**), characterized as fatty acid esters (FAE) of long chain fatty alcohols with more than 590 Daltons.

Figure 2 shows as an example the mass spectrum of the tetracosyl hexadecanoate as representative of this FAE series (see Table 1). The interpretation of the mass spectra of these compounds clearly indicates a FAE structure. This is based on the molecular ions, fragmentation patterns and retention indices and the fact that several FAE compounds have been reported previously in propolis [4]. Also, hexanoic acid is the only free fatty acid clearly seen in the extract ((**e**) Figure 1).

3.5 Flavonoids

Analysis of the acetone crude extract by HT-HRGC-MS (**Figure 3**), confirmed the presence of several flavonoids. The mass spectral characteristics of these compounds are presented in Table 2.

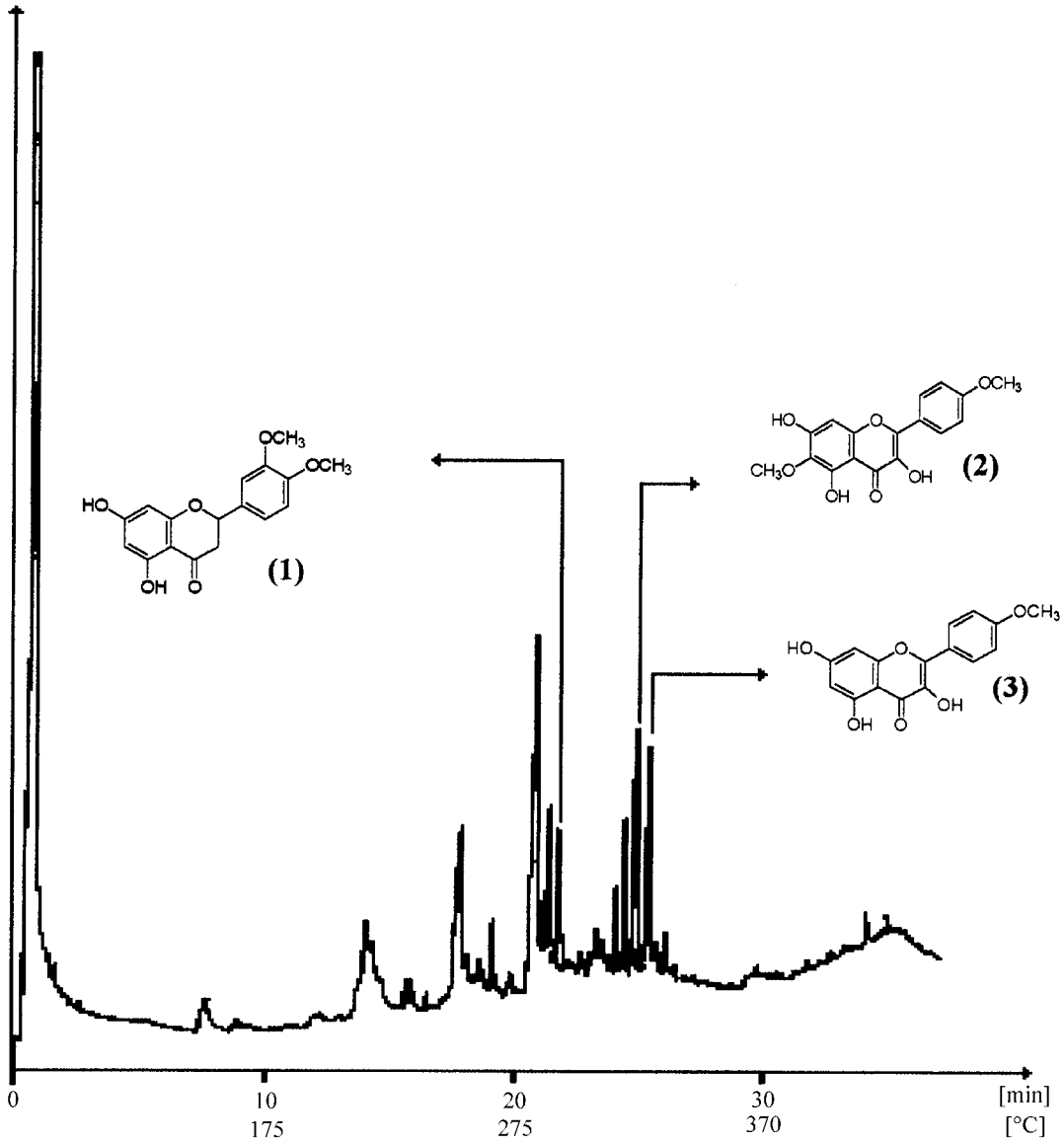

Figure 3. Chromatogram of the acetone crude extract of propolis. 1) naringenin 3′,4′-di-methoxy; 2) betuleol and 3) kaempferid. HT-HRGC conditions: See experimental.

Formation of the fragments **a** and **b** can be proposed to justify the presence of the peaks at *m/z* 165 in the mass spectrum of flavonoid **1** and *m/z* 135 in the mass spectra of flavonoids **2** and **3**, respectively (**Figure 4**). The peak at *m/z* 165 (33%) in the mass spectrum of **1** is consistent with the presence of a 3′,4′-dimethoxyphenyl group attached to C-2. The peak at *m/z* 135 in the mass spectra of **2** and **3** (24 and 18%, respectively), is consistent with the presence of a 3′-methoxyphenyl or 4′-methoxyphenyl moiety attached to carbon C-2.

The identification of this class 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. Usually only probable structures can be advanced using the mass spectral data together with biogenetic arguments. The presence of a 4′-methoxyphenyl moiety seems likely, based on the biogenetic formation of the flavonoids from shikimic acid [27]. For a discussion of the fragmentation characteristics of flavonoids, see Porter [28] and Takayama *et al.* [29].

The results of HT-HRGC and HT-HRGC-MS analysis of the crude extracts exhibited the advantage of the techniques: fast characterization of a great number of different classes of natural products, including highly functionalized compounds, such as flavonoids. As the chemical composition, as well as the source evaluation, of propolis depends on the place and time of collection, collecting bee species, source abundance, accessibility and attractive power, among other variables, a rapid and efficient screening method is mandatory to promote a faster development of this field of knowledge. By the same token, as this diverse chemical composition promotes variation of the biological and pharmacological properties of propolis, more efficient analytical

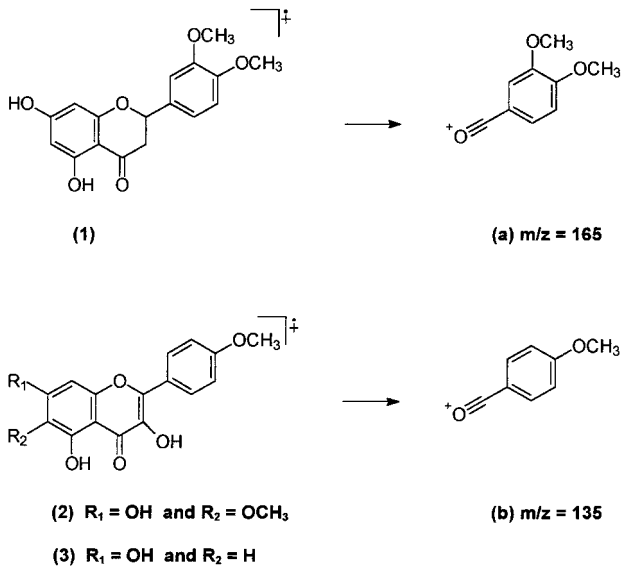

Figure 4. The mass spectrometric fragmentation of flavonoids with ions of *m/z* 135 and 165.

techniques are needed to support pharmacological studies of this important natural product. Specifically, in relation to propolis, fast characterization can be used in adulteration quality control.

4 Conclusions

The use of HT-HRGC and HT-HRGC-MS in the analysis of hexane and acetone crude extracts of propolis permitted the direct characterization of several compounds, without any clean-up, derivatization, or purification procedures. These included flavonoids and a homologous series of palmitic acid esters of long chain fatty alcohols. 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. The use of HT-HRGC-MS as an independent identification technique for flavonoids is somewhat hindered by the similarity between mass spectra of such highly functionalized compounds: to be conclusive in this respect, authentic standards are needed for coinjection. As a result, after the screening step, only a few unknown chromatographic peaks will remain dependent on classical analytical methodology (*e.g.* isolation) for reliable identification if necessary.

Acknowledgments

The authors wish to thank, FINEP, FAPERJ, CAPES, CNPq, and FUJB for financial support and/or fellowships.

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