Lupeol Alkanoates in Brazilian Propolis

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High temperature high resolution gas chromatography coupled to mass spectrometry (HT-HRGC-MS) isa powerful analytical tool. In this work we applied this technique to the study of crude extracts of propolis collected near the city of Uberlândia – Minas Gerais State. Eucalyptus trees and native plants from "cerrado" (savannah) were the material sources disposable for the *Apis mellifera* bees. A lot of known propolis constituents were identified, however, several high molecular weight compounds including lupeol alkanoates were identified for first time in propolis.

Introduction

Propolis (CAS No. 9009-62-5) is a complex mixture that honeybees collect from plants and use in construction, protection (*e.g.* to prevent the decomposition of dead insects) and adaptation of their nests. In Brazil as in other tropical countries, propolis shows significant differences in their chemical composition, because of the high plant biodiversity.

Recently, high temperature high resolution gas chromatography (HT-HRGC) is now employed as a standard technique in many GC laboratories. The term usually denotes temperature programmable GC operation with final column temperatures of 370 °C or higher. Today, non-polar and medium polar high temperature capillary columns can be conveniently operated at temperatures up to 420 °C. The extension of the working range from 370 °C to 420 °C, may appear of little practical significance. However, expressed in mass units of the compounds, which can be analyzed, the working range can be extended by more than 400 daltons. Apart from a few specialized GC groups, most potential users do not clearly appreciate the scope of high temperature work as a routine technique (Aquino Neto *et al*., 1994; Pereira and Aquino Neto, 1999). It should be stressed that the use of HT-HRGC conditions for conventional HRGC range samples, also improves the analysis through lower bleeding, faster analysis and longer column life.

As the natural product propolis contains mainly compounds of high molecular weight, the present work applies for the first time the HT-HRGC technique to analyze this mixture.

Experimental

Sample material

Propolis sample was supplied by "Apiarios Santa Rita" and was collected near Uberlândia (Minas Gerais State, Brazil) from hives located in the "cerrado" (savannah) where the dominant vegetation was native plants and *Eucalyptus* trees. The sampling was realized in July of 2000 (drought season).

Standards

Lupeol was obtained from professor Angelo da Cunha Pinto (Instituto de Química – Universidade Federal do Rio de Janeiro).

Fractionation of extracts

Propolis (3 g) was extracted sequentially tree times each with 20 ml of dichloromethane, 20 ml of acetone and 20 ml of methanol. All extractions were performed using ultrasonic agitation for 30 min at room temperature. The combined extracts for each solvent were concentrated under vacuum, and the resulting crude extracts were analyzed by HT-HRGC.

Crude extracts were weighed after solvent removal under vacuum and drying in vacuum desiccators with P_2O_5, and gave values of 1.28 g (42.7%, dichloromethane); 0.55 g (18.3%, acetone) and 0.88 g (29.3%, methanol), respectively.

Derivatization

The acetone and methanol crude extract was converted to trimethylsilyl esters prior to HT-HRGC and HT-HRGC-MS analyses by reaction with BSTFA (Sigma, St. Louis, USA) at 60 °C during 30 min.

Gas chromatography

Gas chromatography was performed on fused silica capillary columns (15 m × 0.25 mm i.d.; J&W, Folson, CA, USA) coated with 0.2 μm of DB-5HT (5%-phenyl-95%-methylpolysiloxane).

An on-column injector (Carlo Erba, Rodano – Italy) was mounted on a Hewlett-Packard (Palo Alto, USA) model 5890-II gas chromatograph. The column temperature was maintained at 40 °C during injection, then programmed at 10 °C/min to 390 °C and held for 10 min. The flame ionization detector (FID) and the on-column injector were operated at 400 °C and room temperature, respectively. Hydrogen was used as carrier gas at a linear velocity of 50 cm/s and the sample volume injected was 0.5 μl. GC data were acquired and processed with a HP 3396-II integrator.

Mass spectrometry

HTHRGC-MS analyses were carried out on a HP 5972A spectrometer (Hewlett Packard, Palo Alto, USA), under electron impact ionization (70 eV). The GC operating conditions were as described above. The on-column injector and the transfer line temperatures were set to 40 °C and 390 °C, respectively, and the ion source temperature to 300 °C (MS scan range was 40 to 700 a.m.u.). Helium was used as carrier gas at a linear velocity of 38 cm/s.

Results and Discussion

Substance identification was based on mass spectra interpretation, retention time, the compar-

Table I. Compounds characterized in acetone and methanol extracts.

Retention time [min]	Compound	Acetone extract	Methanol extract
3.89	Lactic acid	1.2	trace
4.43	Hydracrylic acid	0.1	trace
4.93	L-Valine		trace
5.35	Glycerol	0.8	7.9
5.38	Phosphate		0.7
5.59	Succinic acid	trace	trace
5.78	2,3-Dihydroxypropanoic acid	trace	trace
5.82	Fumaric acid		trace
5.99	1,2,4-Butanetriol		trace
6.39	2,4-Dihydroxybutanoic acid		trace
6.45	Hydrocinnamic acid	0.2	trace
6.55	3,4-Dihydroxybutanoic acid		trace
6.59	2 (3H)-Dihydro-furanone, 3,4-dihydroxy		trace
6.91	Erythritol	0.7	trace
7.08	Malic acid	6.0	1.7
7.29	Erythrose		12.0
7.59	Trihydroxybutyric acid (isomer)		trace
7.74	Trihydroxybutyric acid (isomer)		trace
7.79	3,4-Dimethoxy-phenethylamine	0.2	
9.11	3,4-Dimethoxybenzoic acid	0.5	
12.11	Palmitic acid	2.2	0.6
13.00	Caffeic acid		trace
13.61	Oleic acid	2.4	0.6
18.44	Tetracosanoic acid	9.0	3.7
19.79	Hexacosanoic acid	2.1	1.8
21.07	Octacosanoic acid	1.7	1.3

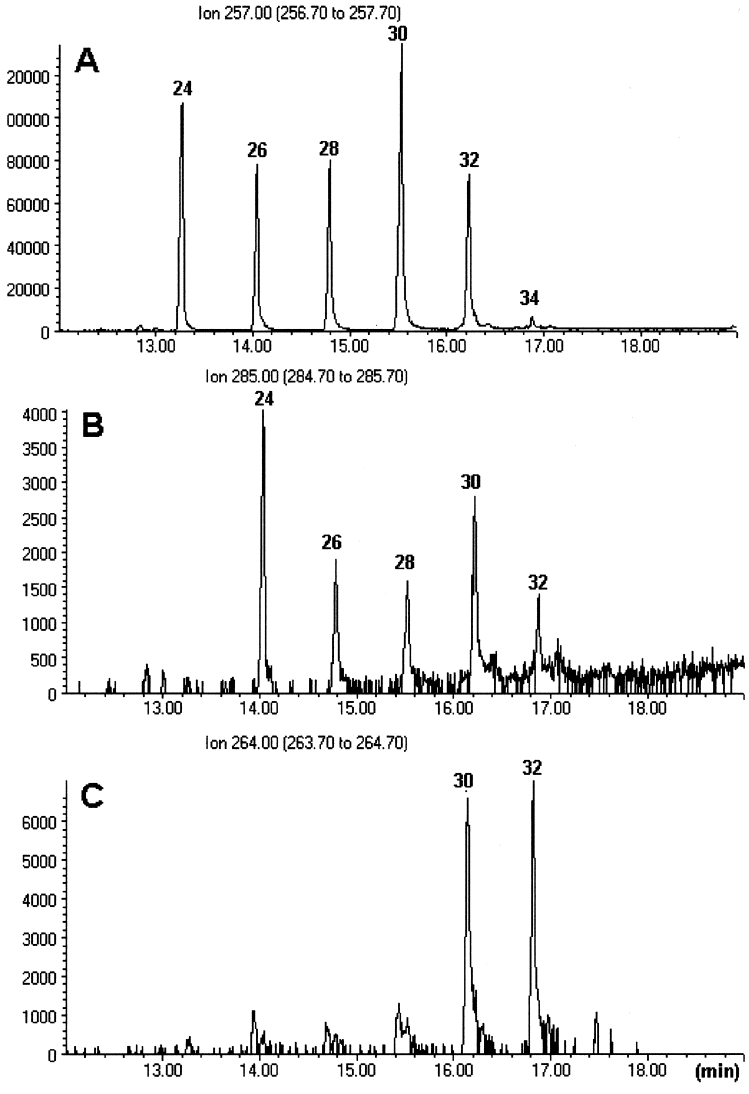

Fig. 1. HT-HRGC mass fragmentograms of the esters of long chain fatty alcohols found in propolis: A) *m/z* 257 (palmitic acid); B) *m/z* 285 (stearic acid) and C) *m/z* 264 (oleic acid). The numbers at the peaks refer to the number of carbons of the chain fatty alcohols.

ison with the mass spectra library Wiley 275 and comparison with authentic standard. In studies on crude propolis, the accurate quantification is very difficult due to the complexity of these samples. An estimate of the concentrations was therefore performed using a response factor of 1 (one) for the mass spectrometry detector in total ion chromatogram for all compounds.

Twenty-six polar compounds (see Table I); were characterized in polar fractions (acetone and methanol), most of these compounds are commonly found in different propolis samples (Marcucci, 1995).

The characterization of the constituents in the dichloromethane extract will be discussed below.

Hydrocarbons

A usual distribution of *n*-alkanes with 25 up to 31 carbons, with odd carbon number predominance and maxima at C_{29} was characterized. Neophytadiene (2,6,10-trimethyl-14-ethylene-14-pentadecene) was also detected.

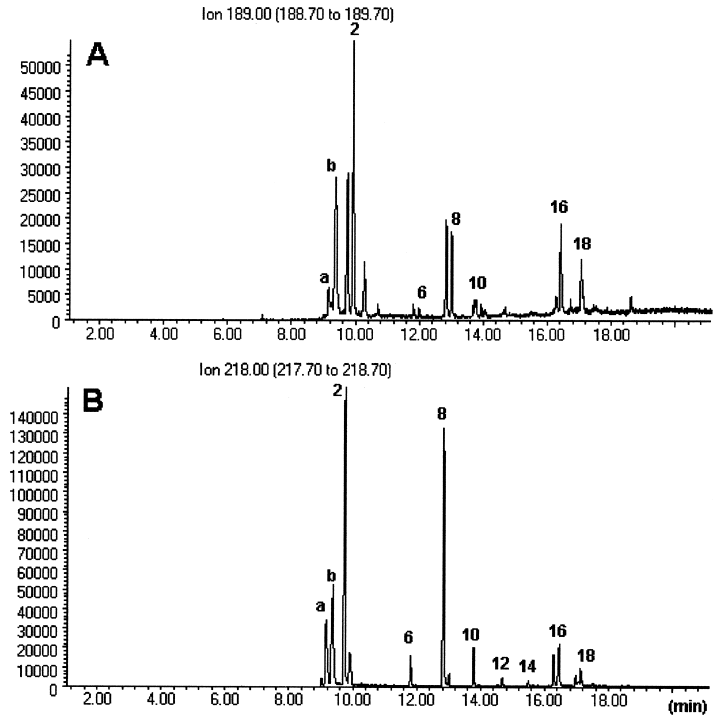

Fig. 2. HT-HRGC mass fragmentograms of the esters of triterpenyl alkanoates: A) *m/z* 189 (lupeol alkanoates) and B) *m/z* 218 (β-amyrin alkanoates). The numbers into the peaks refer to the number of carbons of the chain fatty alcohols, the **a** is the peak of β-amyrin and **b** is the peak of lupeol.

Wax esters

Three long chain alcohol series esterified (Fig. 1) with the palmitic acid (base peak *m/z* 257), stearic acid (base peak *m/z* 285) and oleic acid (base peak 264) were characterized. The oleic esters of long chain fatty alcohols series (Fig. 1C) showed the common fragmentation of the aliphatic esters, however in fragmentation of the palmitic and stearic esters series the base peak is formed by a double rearrangement fragmentation of the ester group, in these case, the fragments ($C_{16}H_{31}O_2H_2^+$, *m/z* 257 and $C_{18}H_{35}O_2H_2^+$, *m/z* 285) is formed by transfer of two hydrogen atoms to the acid moiety, to give rise to protonated ionic species with a mass equal to the acid + 1, this is a variation of McLafferty rearrangement (Gülz *et al.*, 1994; Reiter *et al.*, 1999).

Triterpenes and triterpenyl alkanoates

Four pentacyclic triterpenes were characterized in the dichloromethane crude extract: α-amyrin, β-amyrin, lupeol and lupenone. These compounds can have an elaborate structure thus requiring a more detailed analysis for elucidation. Distributions of such structures, however, can be rapidly screened through monitoring of characteristic fragments and molecular ions. The combination of two or more key fragmentograms can determine, in general with good reliability, the basic skeletons. Therefore, in several cases, total characterization can be obtained by correlation with the vast collection of mass spectra of triterpenoids available in the literature (*e.g.*, Budzikiewicz *et al.*, 1964).

Two series of triterpenyl alkanoates were found in the dichloromethane crude extract. Figure 2 shows a homologous series of lupeol and β-amyrin with the acyl carbon chain length extending from two up to 18 carbon atoms. The β-amyrin alkanoates was previously found in a commercial sample of propolis collected in Carangola city, Minas Gerais State, Brazil (Pereira *et al.*, 1999) however the lupeol and its alkanoates are novel for propolis. Despite their relatively complex structures, the mass spectra of triterpenyl alkanoates are quite simple. Basically, they are composed of molecular ion ($M^{\bullet+}$), $(M-CH_3)^+$, $(M-\text{fatty acid})^{\bullet+}$, and the triterpenoid fragments. The detailed interpretation

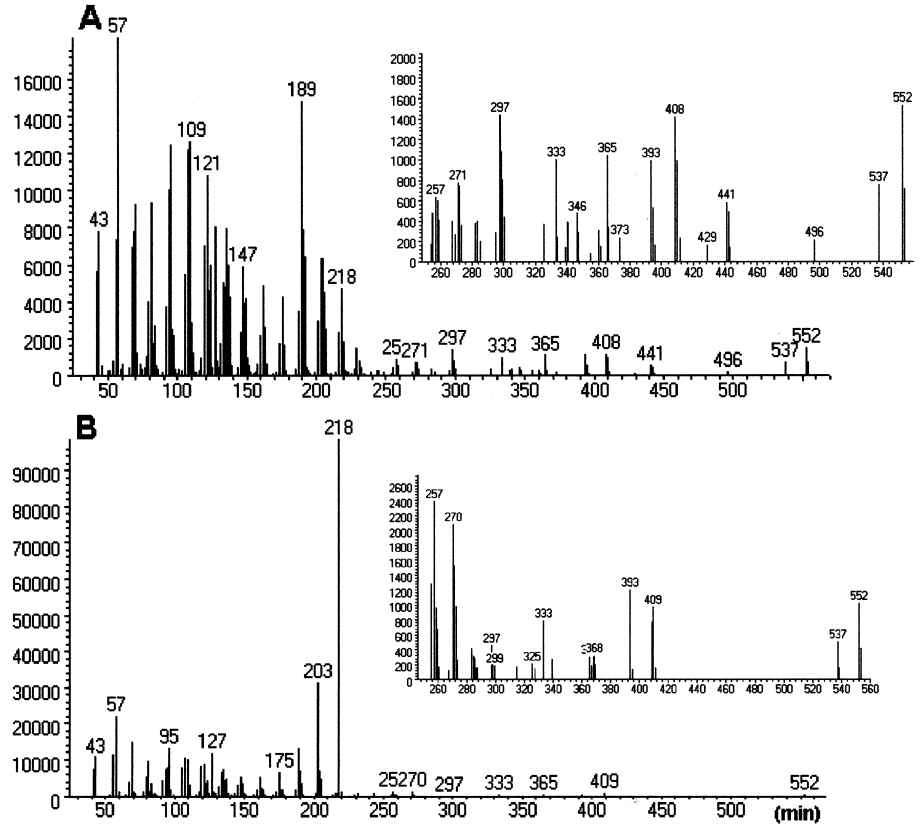

Fig. 3. Representative mass spectra of triterpenyl alkanoates: A) lupeol octanoate and B) β-amyrin octanoate.

of the mass spectra of β-amyrin fatty acid esters was reported previously (Elias *et al.*, 1997). Recently it was reported that lupeol and lupeol linoleate have a marked anti-inflammatory activity (Geetha and Varalakshmi, 2001). The amyrin alkanoates in epicuticular waxes of the red raspberry (*Rubus idaeus* L.) have been associated with resistance to aphid infestation (Shepherd *et al.*, 1999).

The 9,19-cyclolanosta-24-en-3-ol and its acetate were characterized in dichloromethane extract, and are also new in propolis.

HT-HRGC coupled to mass spectrometry is a valuable analytical too for propolis chemical research because it permits the simultaneous by detection of polar high molecular weight compounds as triterpenyl alkanoates (between 500 and 1000 daltons) in crude extracts.

Acknowledgments

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