

Pentacyclic Triterpenoid Alkanoates in Propolis

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Z. Naturforsch. **54c**, 1115–1118 (1999); received June 1/July 23, 1999

Propolis, High Temperature Gas Chromatography/MS, Triterpenyl Alkanoates,
High Molecular Weight Esters, Bee Wax

A series of high molecular mass pentacyclic triterpenoid wax esters (TTFAE) were characterized for the first time in hexane extracts of Brazilian propolis (Carangola, Minas Gerais State). Identification was by HT-HRGC (High Temperature High Resolution Gas Chromatography) and HT-HRGC coupled to mass spectrometry (HT-HRGC-MS).

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 salivary secretions and beeswax (Vanhaelen and Vanhaelen-Fastré, 1979; Ghisalberti, 1979 and Bankova *et al.*, 1987). Bees use it as a sealer for their hives and, more importantly, to prevent the decomposition of insects killed by the bees after attempted invasion of the hive (Marcucci, 1995).

The interesting 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 (Greenaway *et al.*, 1991 and Marcucci, 1995). The distribution and relative proportion of these compounds vary and depend, among many other variables, on the place and time of collection (Bankova *et al.*, 1992).

In this regard, the analysis of propolis samples by High Temperature High Resolution Gas Chromatography (HT-HRGC) and HT-HRGC coupled to Mass Spectrometry (HT-HRGC-MS) may permit the characterization of higher molecular weight compounds not previously reported.

HT-HRGC is now an established technique for separation of complex mixtures containing higher

molecular weight compounds in different matrices, such as petroleum (Aquino Neto *et al.*, 1994), environmental samples (Elias *et al.*, 1998) and natural products (Pereira *et al.*, 1998a; Pereira *et al.*, 1998b; Pereira and Aquino Neto, 1999). The heavier compounds in these samples however may be missed since they elute at much higher temperatures than the upper limit of conventional capillary columns (Pereira and Aquino Neto, 1999).

In this work, we report for the first time the characterization of two series of triterpenyl fatty acid esters in propolis.

Experimental

Materials

A commercial (30%, w/v) ethanolic solution of propolis, collected (March, 1998) in the city of Carangola, Minas Gerais state, localized in the Brazilian Atlantic forest, was used in this work. In addition to the endemic species, the region has farms of the *Citrus* spp. and *Pinus* spp.

Fractionation of extracts

The propolis solution (30 ml) was diluted with distilled water (30 ml). Hexane (pesticide grade, 50 ml) was added, and the mixture was stirred in an orbital shaker at 120 rpm for 24 h. The layers were separated and the aqueous phase submitted to the hexane extraction procedure two more



times. The organic layers were combined and the solvent removed under reduced pressure at room temperature. The solid residue obtained, 1.2 g (4% w/v), was kept in a refrigerator until analyzed.

Chromatographic analysis

HT-HRGC analyses were performed on a HP 5890-II gas chromatograph (Hewlett Packard, Palo Alto, USA), with flame ionization detector and using a cold on-column injector (Carlo Erba, Milano, Italy) for sample introduction. Gas chromatography was performed on borosilicate capillary columns (18 m × 0.30 mm i.d.; Duran-50 glass, Vidrolex, Brazil) coated with 0.1 μ m 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 μ l, with the injector at room temperature and the detector at 380 °C. Column temperature was programmed as follows: 40 °C, 10 °C/min to 390 °C (10 min). Hydrogen was used as carrier gas, at a linear velocity of 50 cm/s. Data were acquired and processed on a HP 3396 integrator.

Mass spectrometric analysis

HT-HRGC-MS analyses were 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 analysis by HRGC-MS, the end of the glass capillary column was connected to a 2 m piece of high temperature fused silica (HTFS, 0.25 mm i.d., J&W, USA) which served as interface. The HTFS was previously 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,

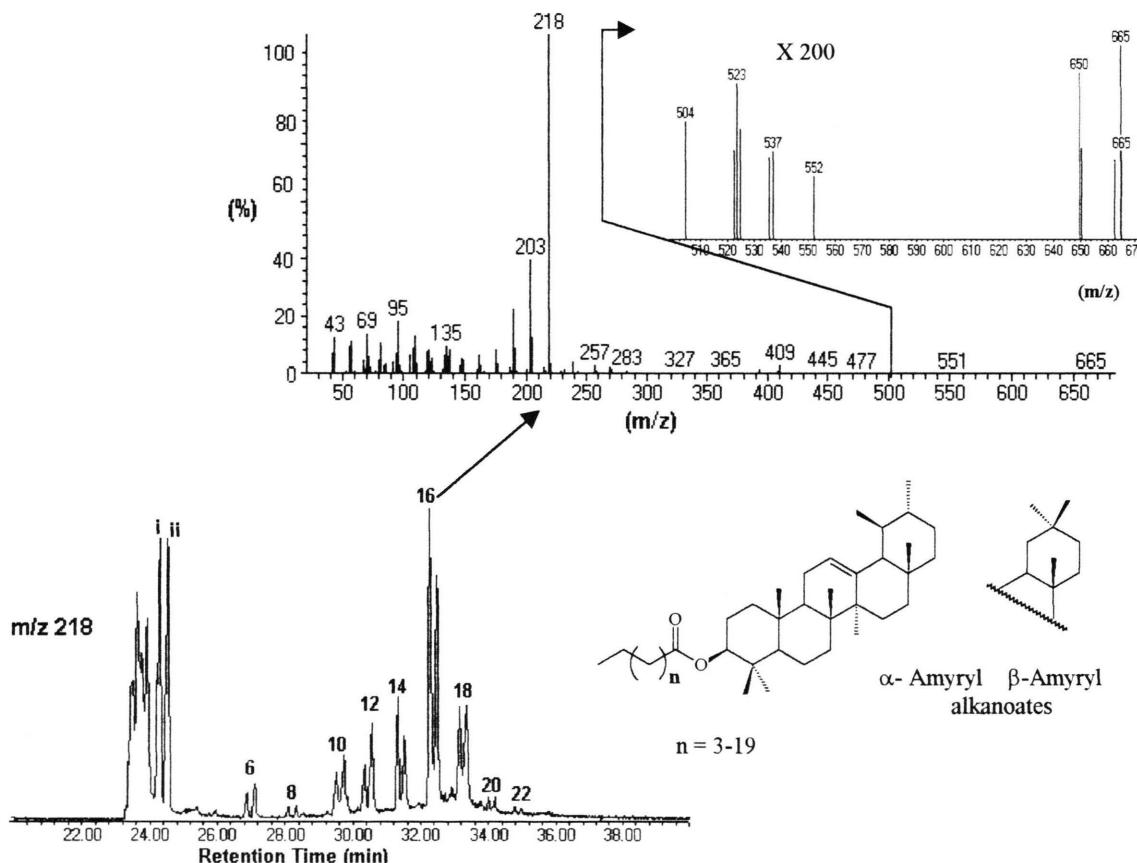


Fig. 1. Representative HT-HRGC mass fragmentogram (m/z 218) of a hexane propolis extract showing the homologous series of amyril alkanoates and a representative mass spectrum. i and ii are β -amyrin and α -amyrin, respectively. Numbers refer to carbon chain lengths of the esterified fatty acids.

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.

Results and Discussion

HT-HRGC is a relatively new technique for routine analyses, apparently because commercial high temperature capillary columns are now becoming available. Traditional concepts against analysis of labile or higher molecular weight substances by GC, are also an obstacle to the rapid acceptance of HT-HRGC by potential users. HT-HRGC offers however the possibility of analyzing a great variety of high molecular weight samples (Pereira *et al.*, 1998a).

The use of HTHRGC and HTHRGC-MS in the analysis of propolis samples 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. Although wax esters (C_{34} to C_{50}) have been extensively described in the literature, long chain wax esters (LCWE) were reported only for a few cases of higher plant waxes and phytoplankton lipids, despite their likely wider occurrence (Kolattukudy, 1976; Elias *et al.*, 1998).

Two series of triterpenyl fatty acid esters (TTFAE) were found in the crude hexane extract of propolis and characterized by mass spectral data. Fig. 1 shows a homologous series of α - and β -amyrin esters with the acyl carbon chain extending from 6 to 22 carbon atoms. Fig. 2 presents a ho-

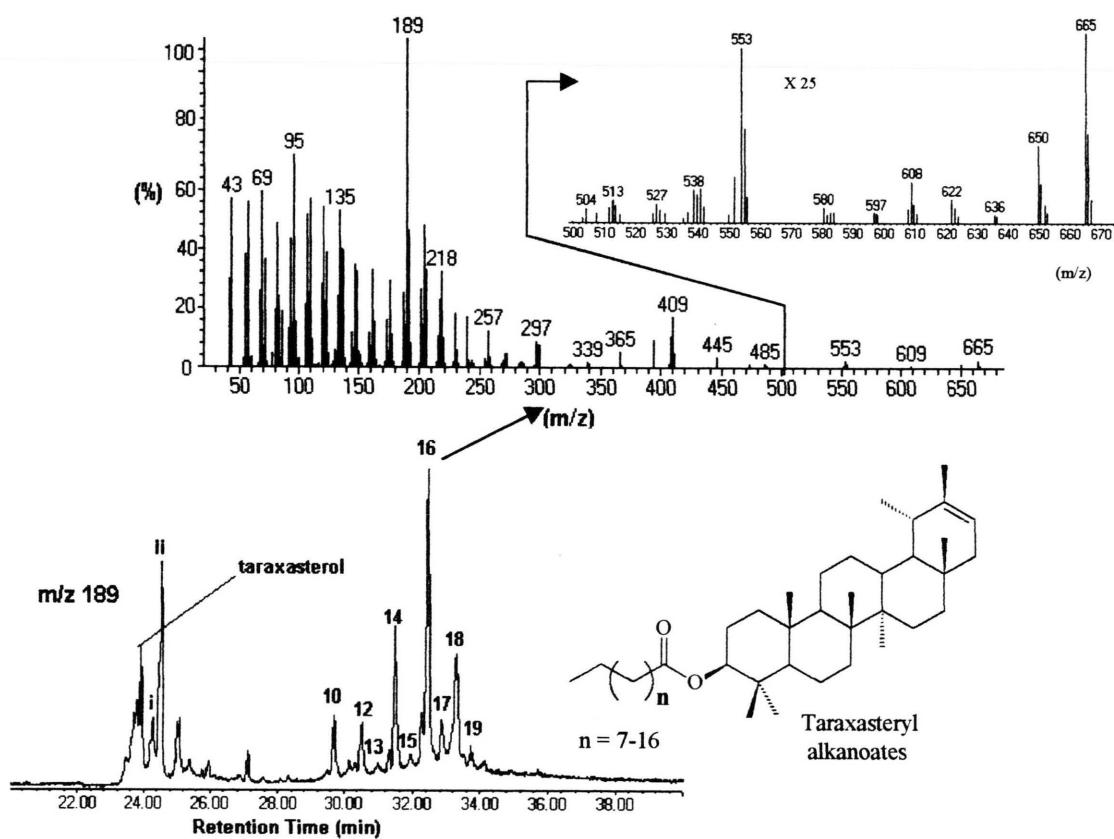


Fig. 2. Representative HT-HRGC mass fragmentogram (m/z 189) of a hexane propolis extract showing the homologous series of taraxasteryl alkanoates and a representative mass spectrum. i and ii are β -amyrin and α -amyrin, respectively. Numbers refer to carbon chain lengths of the esterified fatty acids.

mologous series of taraxasterol esters with the acyl carbon chain extending from 10 to 19 carbon atoms.

Despite their relatively complex structures, the mass spectra of TTFAE are relatively simple. Basically, they are composed of the molecular ion ($M\bullet^+$), ($M - CH_3\bullet^+$), and ($M -$ fatty acid) \bullet^+ ions besides the triterpenoid fragments. The dominant fragmentation is cleavage of the ester bond, either directly or by H-transfer via a McLafferty-type rearrangement, to yield the triterpenyl ions (m/z 408 and 409, respectively). A detailed interpretation of the mass spectra of amryl and taraxasteryl fatty acid esters were reported previously (Elias *et al.*, 1997).

HT-HRGC and HT-HRGC-MS have been used extensively as powerful analytical tools in natural product research. Today, the apolar and medium polar high temperature capillary columns can be conveniently operated at temperatures up to 420 °C. The extension of the working range from 320 °C to 420 °C may appear of little practical significance. However, in reality it is highly significant. Expressed in mass units of the compounds

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