Systematic Study of High Molecular Weight Compounds in Amazonian Plants by High Temperature Gas Chromatography-Mass Spectrometry

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High Temperature Gas Chromatography/MS, *Simaruba amara, Bertholletia excelsa*

The fractions of hexane and dichloromethane extraction from marupá (*Simaruba amara)* and (*Bertholletia excelsa*) leaves were analyzed by HT-HRGC (high temperature high resolution gas chromatography) and HT-HRGC coupled to mass spectrometry (HT-HRGC-MS). Several compounds can be characterized including unusual high molecular weight compounds.

Introduction

Isolation and purification of substances from plant extracts through classical phytochemistry involve methods that are both costly and time-consuming. Also significant amounts of extract are required and detection of minor compounds is not frequently possible (Patittucci *et al.*, 1995).

In the GC community, it is now becoming common knowledge, that high resolution gas chromatography (HRGC) can be used to analyze compounds traditionally known as intractable by GC, as a result of being polar or "thermolabile". Today, high resolution gas chromatography coupled to mass spectrometry (HRGC-MS), is an important and consolidated method for the systematic analysis of natural products (Patittucci *et al.*, 1995; Godoy *et al.*, 1987). In the study of medicinal plants, however, use of HRGC-MS has been generally restricted to the analysis of low-polarity compounds of low molecular weight.

The method described in this work involves direct analysis of crude or pre-fractionated apolar extracts by high-temperature high-resolution gas chromatography coupled to mass spectrometry (HT-HRGC-MS).

In this work, we report the application of HT-HRGC to analyze extracts from two common species of trees of the Amazonian forest (Brazil). The plants studied in this work were *Simaruba amara* (Marupá) and *Bertholletia excelsa* (Brazil nut).

Experimental

Materials

The leaves from Marupá (*Simaruba amara)* and Brazil nut (*Bertholletia excelsa*) were collected (INPA forestry reserve) near the city of Manaus in Amazonia State, Brazil, in July 1998.

Fractionation of extracts

The samples were minced, placed on 100 ml jars, and extracted sequentially five times each with 15 ml of hexane, 15 ml of dichloromethane, 15 ml of acetone and, finally, 15 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, the final volume was approximately 2 ml, and the resulting crude extracts (before drying) were analyzed by HT-HRGC.

The extracts obtained were concentrated under N_2. The dry residues were fractionated by Thin Layer Chromatography (TLC) using a mixture of hexane: diethyl ether (9:1, v/v), the TLC elution regions corresponding to hydrocarbons, esters, ketones (and aldehydes), alcohols and origin were scraped off, eluted with CH_2Cl_2 and concentrated by rotatory evaporation followed by nitrogen blowdown, and transferred to 2 ml vials.

All extracts and factions were kept in a refrigerator until analyzed.

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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 (20 m × 0.25 mm i.d.; Duran-50 glass, Vidrolex, Brazil) coated with 0.1 μm of PS-090 (20%-phenyl-80%-methylpolysiloxane; Ohio Valley Specialty Chemical, Co., USA). The columns were prepared according to a literature procedure (Blum and Englinton, 1989). Column performance was checked prior to use by the Grob test (Grob Jr. *et al.*, 1978; Grob *et al.*, 1981). Sample volumes were 0.5 μl, with the injector at room temperature and the detector at 400 °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, 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

Previous reports (Pereira *et al.*, 1998a; Pereira *et al.*, 1998b) on the applications of HT-HRGC in the analysis of high molecular weight (HMW) and highly polar compounds, showed that the usual HRGC procedure used in natural products research studies needed to be slightly changed in order to deal with these substances. Basically, the main change is the injection technique. The cold on-column technique proved essential, particularly for HMW compounds. Because the sample is deposited directly into the column, giving the highest reproducibility, lowest discrimination and minor sample decomposition.

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

More that 50 compounds were characterized in the samples analyzed (n-alkanes, triglycerides, terpenes, etc.).

Hydrocarbons

In both samples the usual odd carbon number predominance distribution of n-alkanes was observed, with 15 up to 32 carbons for Marupá and C_{21} up to C_{31} for Brazil nut. Squalene and neophytadiene were also detected in these two samples.

Terpenes

The terpenoids constitute the largest class of natural products found in abundance in higher plants (Thomson, 1993). Compounds such as triterpenes can have an elaborate structure which requires a more detailed analysis for elucidation. However, even such structures could be rapidly detected through monitoring of characteristic fragments and molecular ions. The Δ^{12}-oleananes and Δ^{12}-ursenes are the most common naturally occurring pentacyclic triterpenes. Also known as the α and β amyrins series, they have a characteristic double bond at C_{12}-C_{13}. This feature has proved to be readily recognizable by mass spectrometry, since the molecular ion undergoes the equivalent of a Retro-Diels-Alder fragmentation to generate a very characteristic peak (*m/z* 218).

In the samples analyzed several terpenoids were characterized. In the Marupá samples the main terpenes characterized were sesquiterpenes (e.g. copaene, humulene, amorphane, caryophyllene)

and in Brazil nut samples the main terpenoids were the triterpenoids α- and β-amyrine.

Wax esters

Several series of high molecular weight compounds characterized in the two hexane extracts analyzed, were the wax acid esters of long chain fatty alcohols (LCWE). The hexadecanoic acid esters of long chain fatty alcohols series was previously characterized by HT-HRGC in hexane crude extracts of propolis (Pereira *et al.*, 1998a; Pereira *et al.*, 1999a). The base peak is formed by a double rearrangement fragmentation at the ester group (see Fig. 1). In this case, the fragment $[C_{16}H_{31}O_2H_2]^+$ (*m/z* 257, base peak) is formed by transfer of two hydrogen atoms to the acid moiety, to give rise to a protonated ionic species with a mass equal to the acid + 1, this is a variation of the McLafferty rearrangement (Gülz *et al.*, 1994; Reiter *et al.*, 1999).

The main wax acid esters of long chain fatty alcohols series characterized in this two plants were of the hexedecanoic, octadecanoic and eicosanoic acid esters. In Marupá samples besides these three LCWE series, more three LCWE series were characterized in low concentration; of the dodecanoic, tetradecanoic and tetraeicosanoic acids.

Although wax esters have been described extensively in the literature, LCWE are reported as such for only a few cases of higher plant waxes and phytoplankton lipids, despite their likely widespread occurrence (Kolattukudy, 1969). A possible reason could be that LCWE are not eluted on the conventional (low temperature) high resolution gas chromatography used to analyze lipid mixtures during common phytochemistry work. Homologous series of fatty acids esterified with fatty alcohols are the ester moiety of epicuticular waxes of many Angiosperm species.

Triterpenyl fatty acid esters

In crude hexane extracts of the Brazil nut, two series of triterpenyl fatty acid esters (TTFAE) similar to the ones found in the crude hexane extract of propolis were characterized by mass spectral data (Pereira *et al.*, 1999b). They occur as homologous series of α- and β-amyrin esters with the acyl carbon chain extending from 14 to 18 carbon atoms.

Despite their relatively complex structures, the mass spectra of TTFAE are relatively simple. Basically, they are composed of the molecular ion $[M]^{\bullet+}$, $[M - CH_3]^{+\cdot}$ and $[M - \text{fatty acid}]^{\bullet+}$ 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 amyryl fatty acid esters was reported previously (Elias *et al.*, 1997).

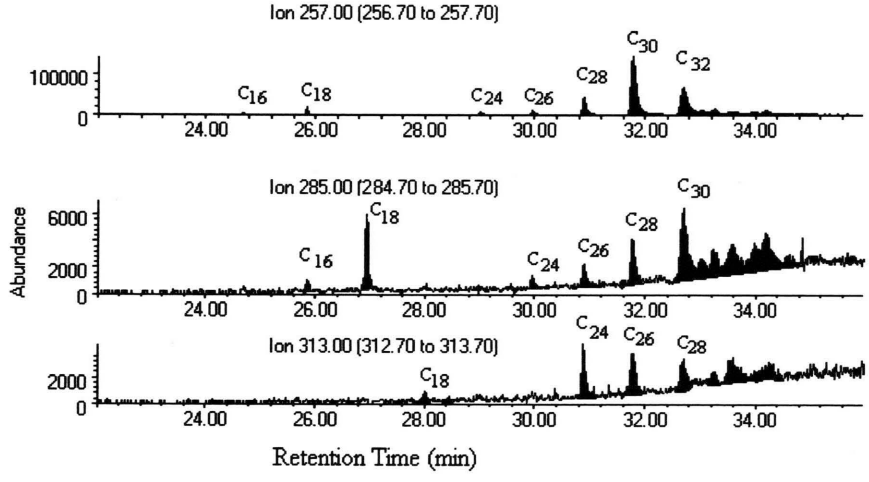

Fig. 1. Three representative HT-HRGC mass fragmentograns [(*m/z* 257) of the hexadecanoic acid, (*m/z* 285) of the octadecanoic acid and (*m/z* 313) of the eicoscanoic acid] esters of long chain fatty alcohols found in Marupá. The numbers refer to the number of carbons of the chain fatty alcohols.

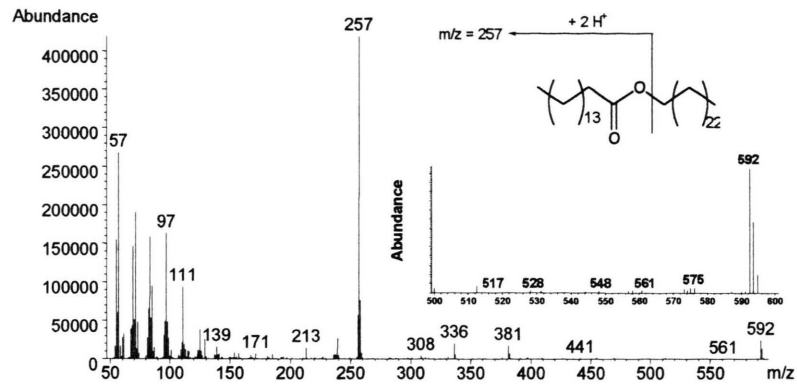

Fig. 2. Mass spectra of tetraicosylhexadecanoate, representative of the homologous series of hexadecanoic acid esters of fatty alcohols.

Triglycerides

Only the Marupá sample showed four triglycerides (in low concentration). The compounds were glyceryl-tripalmitate ($C_{51}H_{98}O_6$, MW 806), glyceryl-oleate-dipalmitate ($C_{53}H_{100}O_6$, MW 832), glyceryl-trioleate ($C_{57}H_{104}O_6$, MW 884) and glyceryl-oleate-palmitate-stearate ($C_{57}H_{104}O_6$, MW 884). The mass spectra of these compounds match with data for authentic standards. No ions above 700 Daltons (e.g., the $M^{\bullet+}$ ion) could be obtained due to the technical constraint of the HP 5972 MSD instrument which limits the MS data acquisition to a maximum of 700 Daltons.

Other high molecular weight compounds

Several other HMW compounds were observed mainly in Marupá samples but it was not possible to characterize them only with mass spectral data because of their low concentration and lack of mass spectral information about high molecular weight compounds. A few ethers have been reported, but occurring in other matrixes, e.g.: cholesteryl hexadecyl ether from bovine cardiac muscle (Funasaki and Gilbertson, 1968) and C_8 and C_9 alkyl steryl ethers identified in diatomaceous oozes (Boon and Leeuw, 1979). A interesting D. Sc. thesis, reported the identification of the wax acid esters of cinnamic acid derivatives identified in soils (Ries-Kautt, 1986). Their published mass spectra do not match with the ones observed for the Marupá and Brazil nut HMW compounds.

Origin of the high molecular weight compounds

Formation and composition of wax in higher plants depends, to some extent, on the environmental conditions under which plants are grown. It is in fact, amply documented that both morphology and chemistry of surface lipids can be significantly influenced by water, soil and light (Biachi *et al.*, 1986).

Other high molecular weight compounds are formed by depolymerization of the polyesters that surround the external plant structure. There are two models of these polyesters (Cutin and Suberin), these structures are not only a shield to protect the plant from its environment, but also play an essential role in the life of plant, controlling the diffusion of molecules (Kolattukudy, 1980).

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