High-temperature gas chromatography–mass spectrometry with glass capillary columns for the screening of natural products

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Abstract

High-temperature high resolution gas chromatography (HT-HRGC) and HT-HRGC coupled to mass spectrometry (HT-HRGC–MS) are powerful but relatively unexplored tools for the analysis of crude extracts and fractions of natural products. To illustrate the scope of the technique the direct characterization of several compounds, present in crude extracts of leaves and stems of *Croton hemiargyreus* Muell. Arg. var. *hemiargyreus* was undertaken, without derivatization or clean-up procedures. Both practical aspects and limitations of HT-HRGC and HT-HRGC–MS were evaluated resulting in a simple, straightforward and extremely powerful technique for the analysis of complex mixtures. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Croton hemiargyreus; Plant materials; Gas chromatography

1. Introduction

High-temperature high resolution gas chromatography (HT-HRGC) is now utilized 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, 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 370 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 u. Apart from a few specialized GC groups, most potential users do not clearly appreciate the scope of high-temperature work as a routine technique ([1,2] and references therein). 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.

The use of HT-HRGC and HT-HRGC coupled to

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mass spectrometry (HT-HRGC–MS) in the analysis of crude extracts and fractions of natural products permitted the direct characterization of several compounds, without derivatization and, in some cases, also without clean-up procedures. Thus, typical samples for HPLC became amenable to HT-HRGC. Despite the enormous analytical potential of HT-HRGC for natural product chemistry, only a dismal number of papers have been published so far [3–13].

The main objective of this work is to show that HT-HRGC and HT-HRGC–MS are valid and potent analytical methods for natural products research. The possibility of the analysis of crude extracts can be extremely useful for the systematic study of medicinal plants and other sources of biologically active compounds. They are fast screening methods, which could guide subsequent phytochemical, or specific, target-oriented work. To illustrate these features, leaves and stems of *Croton hemiargyreus* Muell. Arg. var. *hemiargyreus* were selected, because the genus *Croton* (Euphorbiaceae) is widely distributed in Brazil: in the State of Rio de Janeiro alone 39 species have been identified and some have been used in folk medicine in a large number of applications, including their use as anticancer drugs/agents [14,15].

2. Experimental

2.1. Materials

The leaves and stems of *Croton hemiargyreus* Muell. Arg. var. *hemiargyreus* were collected near Nova Friburgo (Rio de Janeiro, Brazil). The species was identified by Professor Arline Souza de Oliveira (UFRJ – Universidade Federal do Rio de Janeiro). Voucher samples have been deposited in the herbarium of UFRJ (number R182775).

Triglycerides were obtained from Sigma (St. Louis, MO, USA).

2.2. Fractionation of extracts

Two samples (5.2419 and 5.3655 g) of the powdered plant (leaves and stems) were extracted sequentially five times each with 50 ml of dichloromethane, 50 ml of acetone, 50 ml of methanol and,

finally, 50 ml of carbon disulfide. 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 0.2825 and 0.2671 g (dichloromethane); 0.0336 and 0.0331 g (acetone); 0.3553 and 0.3988 g (methanol) and 0.0176 and 0.0164 g (carbon disulfide), respectively.

2.3. Derivatization

The methanol crude extract was converted to trimethylsilyl esters prior to HT-HRGC and HT-HRGC–MS analyses by reaction with bis-(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma) at 60 °C during 30 min.

2.4. Gas chromatography

2.4.1. Columns

Gas chromatography was performed on borosilicate capillary columns (25 m×0.25 mm I.D.; Duran-50, Vidrolex, Brazil) that were coated in our laboratory (according to a literature procedure [16]) with 0.2 µm of PS-086 (5% phenyl–95% methylpolysiloxane; Petrarch Systems, USA). During the analysis by HRGC–MS, the capillary column was connected to a 2 m piece of a treated empty capillary tube (interface GC–MS) prepared from 0.25 mm I.D. high-temperature fused-silica (HTFS; J&W, Folsom, CA, USA). The treatment involved purging with hydrogen at 180 °C for 15 min and deactivation by flushing with HMDS–DPTMDS (1:1), sealing the capillary, and heating at 400 °C for 12 h. The tubing was then rinsed with 1 ml each of hexane, methanol and diethyl ether.

Column performance was checked prior to use by the Grob test [17,18], the columns showed an inertness similar to the commercially coated capillary columns.

2.4.2. Chromatographic conditions

An on-column injector (Carlo Erba, Rodano, Italy) was mounted on a Hewlett-Packard (Palo Alto, CA,

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 detection (FID) system and the on-column injector were operated at 400 and 40 °C, 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.

2.5. Mass spectrometry conditions

HT-HRGC–MS analyses were carried out on a HP 5972A spectrometer (Hewlett-Packard), 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 and 390 °C, respectively, and the ion source temperature to 300 °C (MS scan range was 40 to 700 u). Helium was used as carrier gas at a linear velocity of 38 cm/s.

3. Results and discussion

Faster analyses (<40 min) with good resolution (Fig. 1) was obtained by the use of glass capillary columns coated with PS-086 stationary phase and permitted the direct characterization of more than 40 compounds per fraction (Tables 1 and 2). Many of these compounds are polyfunctional and of a reasonably high molecular mass, traditionally considered to be amenable to HPLC analyses. Therefore HT-HRGC should be seen as a extremely useful tool for the routine analysis in natural products research.

Previous reports [19,20] on the applications of HT-HRGC in the analysis of high-molecular mass (M_r) and highly polar compounds, showed that the usual HRGC procedure used in natural products studies needed to be slightly changed in order to identify the M_r and highly polar compounds present in crude extracts. In addition to the new features of high-temperature capillary columns, an even more critical requirement is the injection technique. An injector that introduces samples with little loss of the M_r or thermally labile compounds is mandatory. The traditional cold on-column technique proved especially useful, particularly for M_r compounds and thermally labile compounds, because the sample is deposited directly into the column, giving the highest reproducibility, lowest discrimination and minor sample decomposition. Cold on-column has advantages when compared with programmable temperature vaporizing (PTV) injectors. Even though results obtained for standards were comparable, for both techniques when real samples (e.g. crude petroleum) were analyzed, the chromatograms for the PTV injector suffered from several drawbacks, even after many attempts to optimize performance [1].

Previously studies have shown that HT-HRGC represents an attractive and fast method for the direct analysis of biologically active (including some thermally labile) compounds (e.g. isoquinoline alkaloids) in crude plant extracts [15]. It is obvious that, due to the complexity of natural product samples (Fig. 1), precise quantification by this method is very difficult. However, an estimate of the mass distribution for natural product compound classes can be performed. This is extremely important for comparative purposes of extracts and as a screening technique.

Fig. 2 shows a percentual quantitative estimate based on flame FID peak areas and considering all response factors equal to 1, for a few classes of substances present in *Croton hemiargyreus* that are amenable to HT-HRGC screening.

The characterization of the constituents and their relevance for each class of substances will be discussed below.

The identification of several classes 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. For example, the fragmentation of trimethylsilyl (TMS) derivatives of carbohydrates shows a base peak ion at m/z 204 or m/z 217, and are more prominent in the mass spectra of six-membered ring and five-membered ring isomers, respectively [21]. In the methanol extract more than 20 carbohydrates and glycosides could be seen. In view of the similarity and complexity of the mass spectra of this class of natural products, at the present screening level only the predominance of six-membered ring monosaccharides was firmly determined.
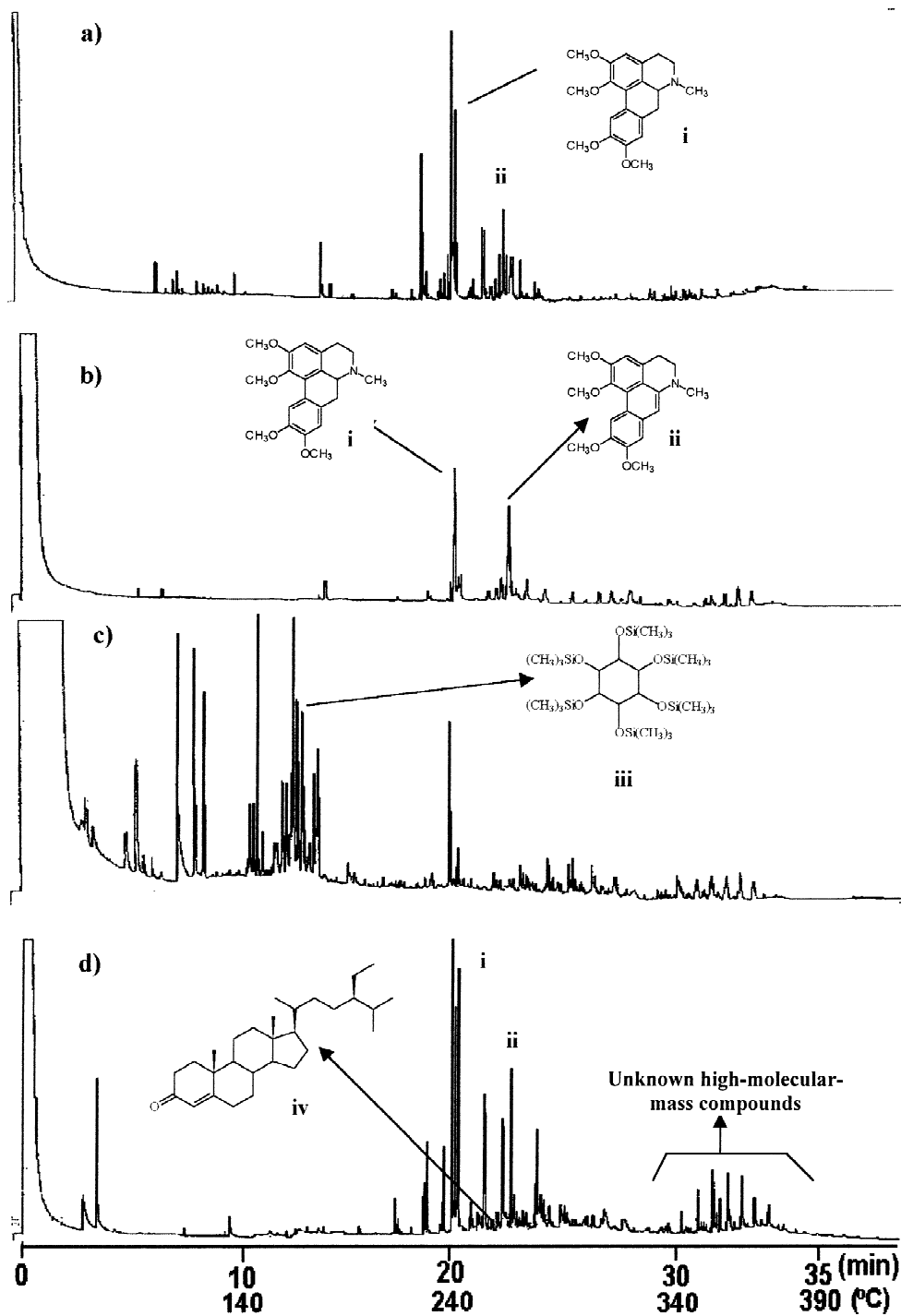
However, the use of HT-HRGC–MS permitted the

Fig. 1. High-temperature high-resolution gas chromatograms: (a) dichloromethane crude extract, (b) acetone crude extract, (c) methanol crude extract (derivatized with BSTFA) and (d) carbon disulfide crude extract. For HT-HRGC conditions, see Experimental. (i) Glaucine, (ii) dehydroglaucine, (iii) inositol (hexakis-trimethylsilyl) and (iv) stigmast-4-en-3-one.

Table 1
Substances characterized in crude extracts of *Croton hemiargyreus* Muell. Arg. var. *hemiargyreus*

Classes	Substances characterized
Acids	Propanedioic (2 TMS)*[c], fumaric (2 TMS)[c], malic (3 TMS)[c], hexadecanoic[b], linoleic[b] and octadecanoic[b].
Alcohols	Inositol (6 TMS)[c], phytol[a,b], 1-tetracosanol[a], 1-hexacosanol[a], 1-octacosanol[a,d] and 1-triacontanol[d].
Aldehydes	1-tetracosanal[a], 1-hexacosanal[a] and 1-octacosanal[a].
Alkaloids	Glaucine[a,b,d] and dehydroglaucine[a,b,d].
Alkanes	Heneicosane[a,d], docosane[a,d], tricosane[a,b,d], tetracosane[a,d], pentacosane[a,b,d], hexacosane[a,d], heptacosane[a,b,d], octacosane[a,d], nonacosane[a,b,d], triacontane[a,d], henetriacontane[a,b,d] and tritriacotane[a,d].
Alkenes	Neophytadiene[b] and squalene[a,b].
Amides	Urea (2 TMS)[c].
Amines	Ethanolamine (2 TMS)[c], 2,2,6,6-tetramethyl-4-piperidinone[c].
Aminoacids	Alanine (2 TMS)[c], 2-methylalanine (2 TMS)[c], 2-aminobutyric acid (2 TMS)[c], valine (2 TMS)[c], leucine (2 TMS)[c], serine (2 TMS)[c], threonine (3 TMS)[c], pyroglutamic acid (2 TMS)[c], phenylalanine (2 TMS)[c], glutaminic acid (3 TMS)[c], asparagine (2 TMS)[c], and glutamic acid (3 TMS)[c].
Aromatic	3-Phenylundecane[a].
Esters	Methyl esters of α-hydroxybenzenepropanoic acid[d]; hexadecanoic acid[d]; octacosanoic acid[d]; triacontanoic acid[d]; dotriacontanoic acid[d] and tetracosyl-[a]; hexacosyl-[a] and octacosyl-[a] esters of hexadecanoic acid.
Ketones	6,10,14-Trimethyl-2-pentadecanone[a] and 2-tritriacontanone[d].
Steroids	Ergost-5-en-3-ol[a,b,d], stigmast-5,22-dien-3-ol[a,b,d], stigmast-5-en-3-ol[a,b,d], and stigmast-4-en-3-one[a,b,d].
Terpenes	*trans*-Caryophylene[a], α-humulene[a], germacrene d[a], bicyclogermacrene[a], spathulenol[a], calarene[a], D:C-friedooleanan-3-one[a], 3-keto-urs-12-ene[a], α-amyrine[a], β-amyrine[a], D:B-friedo-B′:A′-neogammacer-5-en-3-ol (simiarenol)[a] and D:A-friedooleanan-3-one[a].
Triglycerides	Dihexadecanoate-glycerol[b,d], glyceryl-2-myristate-1,3-distearate[b,d], glyceryl-1-oleate-2-stearate-3-palmitate[b,d]
Other compounds	Glycerol (3 TMS)[c], vitamin E[a,b,d], vitamin E succinate[b,d] and γ-tocopherol[d].

* Is the number of trimethysilyl units in the compound.
[a] Compounds characterized in dichloromethane crude extract.
[b] Compounds characterized in acetone crude extract.
[c] Compounds characterized in methanol crude extract (derivatized with BSTFA).
[d] Compounds characterized in carbon disulfide crude extract.

characterization of several classes of natural products, including several *n*-alkanes, alcohols, ketones, acids, amino acids and triglycerides (see Table 1)

3.1. Wax esters

Several compounds were characterized (see Table 1) including the long chain wax esters (LCWEs): tetracosyl hexadecanoate ($C_{40}H_{80}O_2$), hexacosyl hexadecanoate ($C_{42}H_{84}O_2$) and octacosyl hexadecanoate ($C_{44}H_{88}O_2$). Although wax esters have been described extensively in the literature, LCWEs are reported as such for only a few cases of higher plant waxes and phytoplankton lipids, despite their likely

Table 2

Main fragments (*m/z*) and their relative abundances (% in parenthesis) in the mass spectra of triterpenyl fatty acid esters

Compound	Retention time (min)	$M^{.+}$	Base peak (100%)	M–Fatty acid	Other significant fragments
Simiarenol esters					
Simiarenyl dodecanoate	29.98	608	259	408 (4.1)	134 (70), 274 (93)
Simiarenyl tetradecanoate	30.96	636	259	408 (5.3)	134 (72), 274 (90)
Simiarenyl hexadecanoate	31.91	664	259	408 (6.2)	134 (73), 274 (83)
Simiarenyl octadecanoate	32.79	692	259	408 (3.1)	134 (68), 274 (91)
Simiarenyl eicosanoate	33.64	720*	259	408 (3.0)	134 (67), 274 (89)
Simiarenyl docosanoate	34.50	748*	259	408 (2.8)	134 (65), 274 (75)
Simiarenyl tetracosanoate	35.23	776*	259	408 (2.9)	134 (78), 274 (82)
Simiarenyl hexacosanoate	36.11	804*	259	408 (2.7)	134 (75), 274 (79)
Amyrin esters					
β-Amyryl octanoate	28.03	552 (0.4)	218	409 (1.2)	203 (33), 189 (17)
β-Amyryl decanoate	29.19	580 (0.5)	218	409 (1.3)	203 (32), 189 (15)
β-Amyryl dodecanoate	30.25	608 (1.0)	218	409 (0.8)	203 (30), 189 (15)
α-Amyryl dodecanoate	30.47	608 (1.1)	218	409 (0.8)	203 (17), 189 (22)
β-Amyryl tetradecanoate	31.25	636 (0.7)	218	409 (1.4)	203 (30), 189 (19)
α-Amyryl tetradecanoate	31.45	636 (0.6)	218	409 (1.2)	203 (15), 189 (19)
β-Amyryl hexadecanoate	32.18	664 (0.4)	218	409 (0.7)	203 (29), 189 (18)
α-Amyryl hexadecanoate	32.39	664 (0.5)	218	409 (0.6)	203 (20), 189 (25)
β-Amyryl octadecanoate	33.16	692 (0.6)	218	409 (1.2)	203 (32), 189 (22)
α-Amyryl octadecanoate	33.30	692 (0.2)	218	409 (1.4)	203 (17), 189 (25)
β-Amyryl eicosanoate	33.95	720*	218	409 (1.3)	203 (33), 189 (18)
α-Amyryl eicosanoate	34.16	720*	218	409 (0.8)	203 (18), 189 (26)
β-Amyryl docosanoate	34.73	748*	218	409 (0.7)	203 (33), 189 (17)
α-Amyryl docosanoate	34.94	748*	218	409 (1.0)	203 (18), 189 (25)
β-Amyryl tetracosanoate	35.55	776*	218	409 (0.9)	203 (32), 189 (17)
α-Amyryl tetracosanoate	35.78	776*	218	409 (1.1)	203 (14), 189 (17)
β-Amyryl pentacosanoate	35.98	790*	218	409 (1.0)	203 (33), 189 (17)
α-Amyryl pentacosanoate	36.22	790*	218	409 (1.1)	203 (15), 189 (18)
β-Amyryl hexacosanoate	36.48	804*	218	409 (0.8)	203 (33), 189 (15)
α-Amyryl hexacosanoate	36.75	804*	218	409 (0.8)	203 (14), 189 (22)
β-Amyryl octacosanoate	37.56	832*	218	409 (0.7)	203 (23), 189 (12)
α-Amyryl octacosanoate	37.90	832*	218	409 (0.6)	203 (18), 189 (25)
β-Amyryl triacontanoate	38.88	860*	218	409 (0.7)	203 (25), 189 (17)
α-Amyryl triacontanoate	39.31	860*	218	409 (0.7)	203 (10), 189 (19)

* The molecular ions of these compounds were not observed because the upper limit of the scan range was 700 u.

widespread occurrence [22,23]. A possible reason could be that LCWEs are not eluted on the conventional (low-temperature) high resolution gas chromatography commonly used to analyze lipid mixtures.

3.2. Triterpenes

Six triterpenes were characterized in the dichloromethane crude extract (see Table 1). 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 total characterization, in several cases, can be obtained by correlation with the vast collection of mass spectra of triterpenoids available in the literature [24–31].

3.3. Triterpenyl fatty acid esters

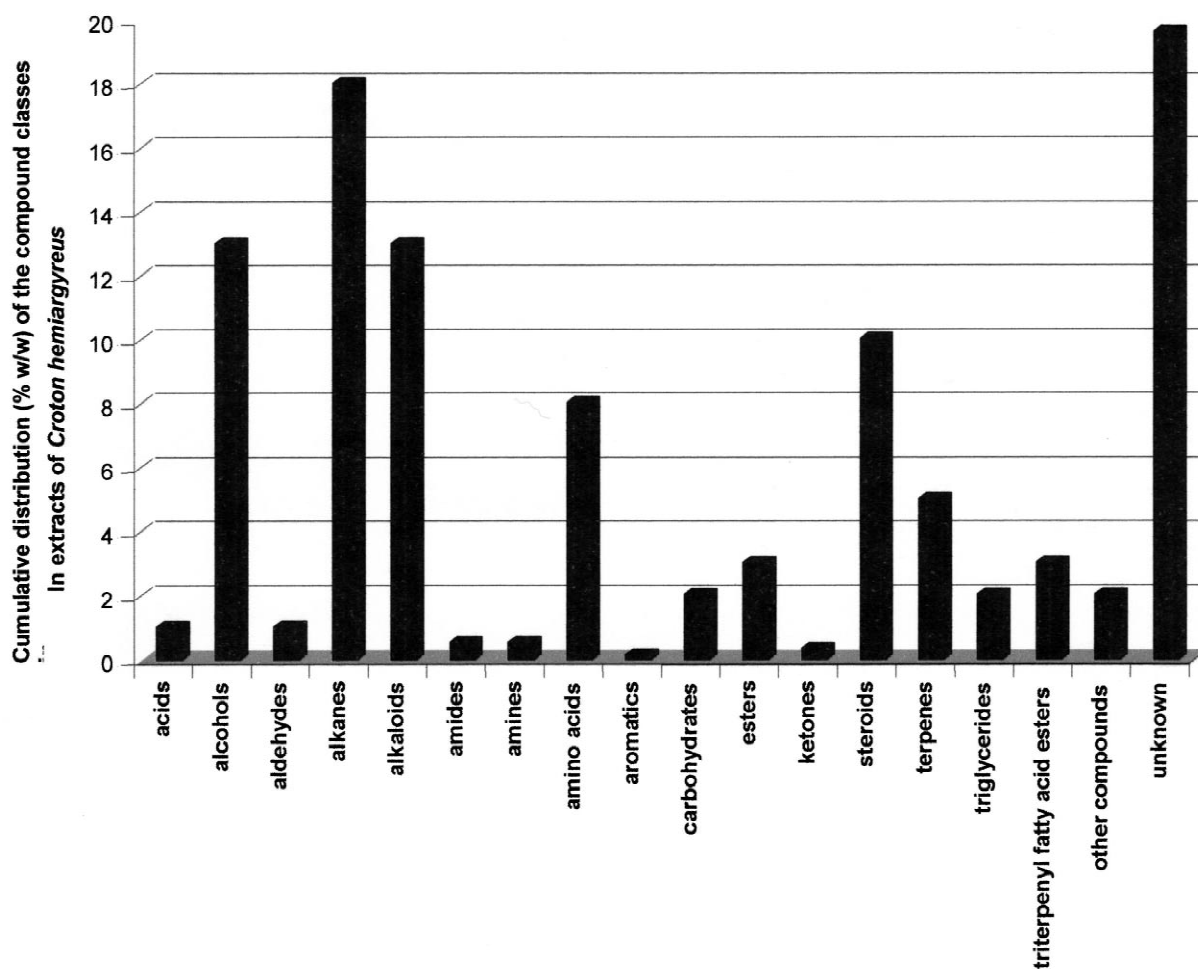A series of triterpenyl fatty acid esters (TFAEs)

Fig. 2. Approximate mass distribution for compound classes in *Croton hemiargyreus* Muell. Arg. var. *hemiargyreus*; based on FID peak areas and considering all response factors equal to 1.

was found in the dichloromethane crude extract. Fig. 3 shows an homologous series of α- and β-amyrins with the acyl carbon chain length extending from eight up to 30 carbon atoms. Fig. 4 presents an homologous series of simiarenol (D:B-friedo-B′:A′-neogammacer-5-en-3-ol) with the acyl carbon chain length extending from 12 up to 26 carbon atoms. The most important feature in the simiarenol esters mass spectra are peaks at m/z 274 (retro-Diels–Alder) and the base peak at m/z 259 (274 – CH$_3$), which characterize the Δ^5-unsaturated skeleton [24]. The semiarenol fatty acid esters now found represents a novel natural product series. The relative abundances of characteristic fragments in the mass spectra of the

TFAEs, are given in Table 2. Despite their relatively complex structures, the mass spectra of TFAEs are quite simple. Basically, they are composed of molecular ion ($M^{\cdot+}$), $(M-CH_3)^+$, $(M-\text{fatty acid})^{\cdot+}$, and the triterpenoid fragments. The detailed interpretation of the mass spectra of α- and β-amyrine fatty acid esters was reported previously [32].

3.4. Alkaloids

Two aporphine alkaloids were characterized: glaucine and dehydroglaucine. The mass spectra characteristic of the aporphine group consists of either the molecular ion or the ion formed by loss of
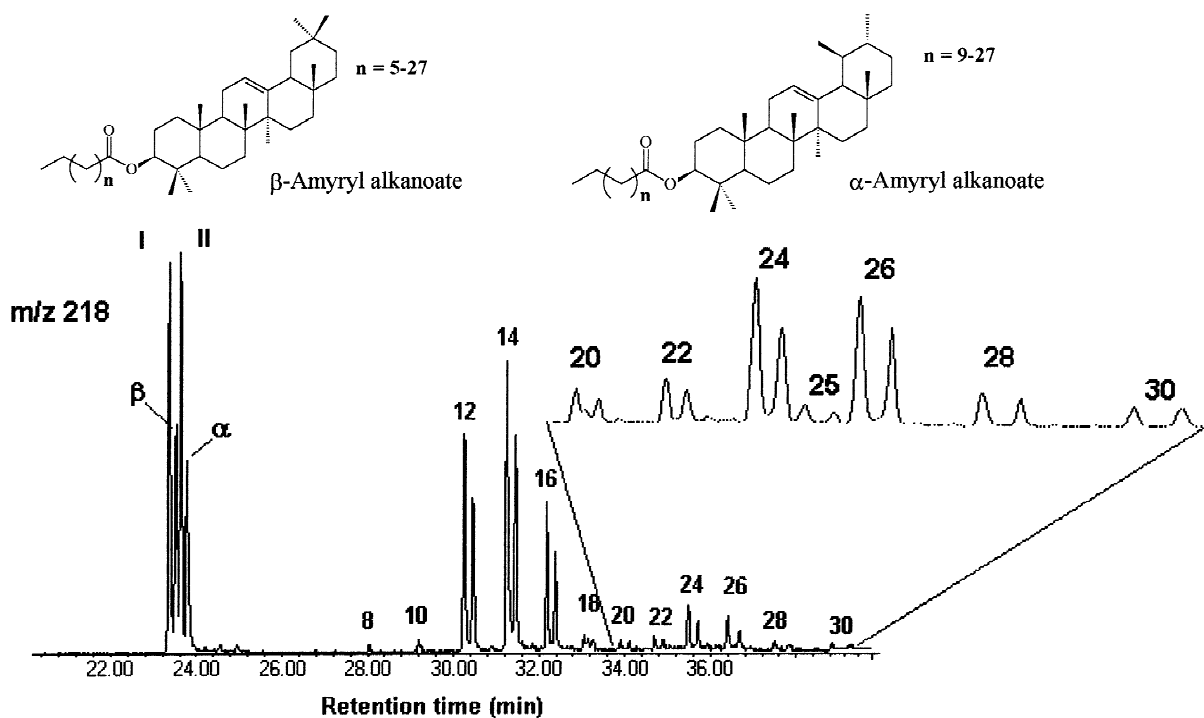
Fig. 3. Representative HT-HRGC mass fragmentogram (m/z 218) showing the homologous series of amyryl alkanoates; β and α are β-amyrin and α-amyrin, respectively. I is a triterpene not characterized and II is 3-keto-urs-12-ene.

a hydrogen atom therefrom as base peak. Other important ions are $(M-15)^+$ and $(M-31)^+$, formed by loss of methyl and methoxy groups from one of the aromatic rings [33,34].

3.5. Unknown compounds

Several unknown compounds are present in the crude extracts analyzed. Although partial chemical structures could be inferred (e.g. chemical class) the complete identification could not be accomplished due to unavailability of standards or published relevant mass spectra. Of special interest are the unknown high-molecular mass compounds (M_r above 800; Fig. 1d). They exemplify the new molecular mass "window" opened by the use of HT-HRGC. These compounds have been systematically overlooked due to the inability of HRGC or HPLC to analyze this molecular mass range.

3.6. Solvent extraction

Ideally, solvent extraction should quantitatively extract cellular lipids in an undegraded state. Membrane-associated lipids, such as glycolipids, require polar solvents (ethanol or methanol) to disrupt the bonds between the lipids and proteins [35]. The sequence of solvents used had the main objective to fractionate the extracts in a gradient of polarity: the apolar and lowest polar compounds extracted by dichloromethane and the polar compounds extracted by methanol.

It was shown previously that CS_2 should be used as the best solvent for dissolution of high-molecular-mass lipids [1,36]. It has been seldomly used in applications as extraction solvent in classical phytochemistry. The aim of using CS_2 in this work was the extraction of high-molecular-mass compounds like alkanes and esters. However, the major peak in the extract was the alkaloid glaucine (see Fig. 1),
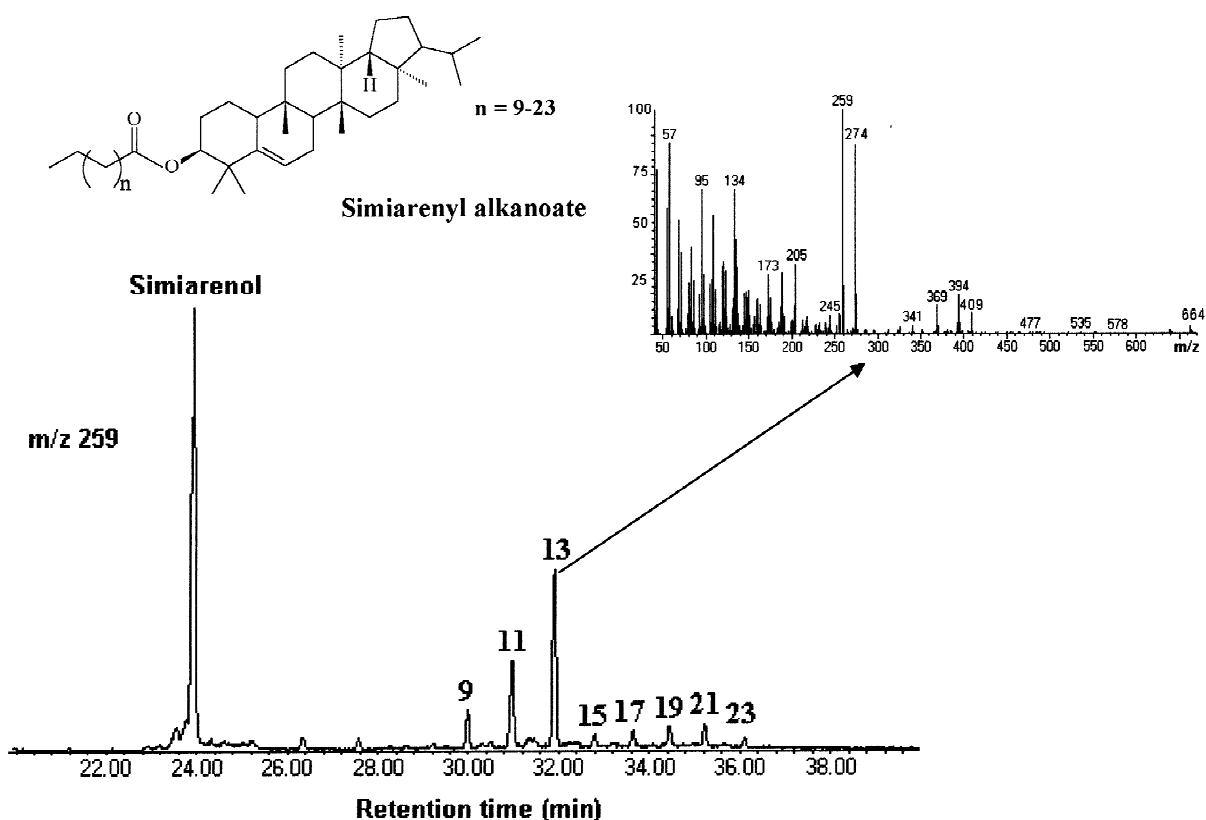
Fig. 4. Representative HT-HRGC mass fragmentogram (m/z 259) showing the homologous series of simiarenol (D:B-friedo-B′:A′-neogammacer-5-en-3-ol) esterified with fatty acids. The mass spectrum of simiarenyl octadecanoate is also shown.

which was not observed in the methanol crude extract (even by selective ion monitoring of characteristic ions).

The use of CS_2 as solvent increased in 10% the recovery of alkaloids. This result shows the importance of solvent selection in the study of the composition of higher plants, mainly in medicinal plant studies.

The presence of alkaloids in the carbon disulfide crude extract is not easily explained by the polarity of the solvent. The complex plant extracellular matrix, commonly referred to as the cell wall, is a heterogeneous macromolecular assembly consisting of cellulose microfibers embedded in a matrix of complex polysaccharides and glycoproteins. Possibly the presence of the same alkaloids in distinct crude extracts is due to the interaction of these compounds

with the macromolecular components of the plant extracellular matrix, which hinders the extraction of these compounds from the matrix with common solvents. The ability of CS_2 to penetrate in these matrices should be investigated further. Also, the usefulness of CS_2 to natural product (applied) chemistry has to be evaluated since there is little information concerning possible interference of CS_2 or its common contaminants with biological testing of natural product extracts and fractions.

4. Conclusions

HT-HRGC and HT-HRGC–MS were evaluated as powerful analytical tools in natural product research. A high level of information could be obtained

concerning the chemical composition of higher plants and other natural products, with the added possibility of direct analysis and characterization of compounds with molecular masses between 500 and 1000 in crude extracts.

Several classes of natural products, encompassing more than 100 compounds, including alkaloids, amino acids and high-molecular-mass compounds were characterized in this work in different crude extracts.

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 a result, only unknown chromatographic peaks should remain dependent on classical analytical methodology.

A new series of high-molecular-mass triterpenoid (simiarenol) wax esters was characterized in crude extracts of the leaves and stems of *Croton hemiargyreus* Muell. Arg. var. *hemiargyreus*. Amyrine fatty acid esters with acyl carbon chain lengths extending above 20 carbon atoms were also characterized, for the first time, in natural products.

Acknowledgements

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