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Three series of high molecular weight alkanoates found in Amazonian plants

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Abstract

Electron impact mass spectra were measured by high temperature high resolution gas chromatography–mass spectrometry (HT-HRGC–MS) for three homologous series of high molecular weight compounds present in the Amazonian plants Marupá (*Simaruba amara*) and Brazil nut (*Bertholettia excelsa*). Based on their mass spectra, the compounds were identified as three wax ester series of α-tocopherol (vitamin E), β-tocopherol and phytol (2,6,10,14-tetramethylhexadec-14-en-16-ol). The interpretations are supported by high resolution mass spectrometry and GC retention indices of authentic standards.
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1. Introduction

The epicuticular waxes cover the surfaces of the aerial organs of plants and are usually composed of a mixture of long chain aliphatic compounds such as *n*-alkanes, esters, aldehydes, ketones, alcohols and acids (Tava et al., 1996). High molecular weight (HMW) compounds, such as wax esters, are also part of the saponifiable matter of vegetable oils (internal plant lipids) with concentrations ranging from 0 to 3% (Reiter et al., 1999). Waxes are important for repelling water and controlling the gas balance between a plant and its environment; other possible functions may be related to the protection of epidermal cells against mechanical damage and inhibition of fungal and insect attack (Tava et al., 1996).

High temperature high resolution gas chromatography (HT-HRGC) is an established technique for the separation of complex mixtures containing HMW compounds. It has been used to characterize HMW components in different matrices (e.g. petroleum, environmental, natural products, etc.) (Pereira and Aquino Neto, 1999; Elias et al., 1997, 1998, 1999). The analysis of natural products (e.g., from higher plants) by HT-HRGC coupled to mass spectrometry (HT-HRGC–MS) is a promising technique for the characterization of HMW compounds which is quite often neglected by current researchers (Elias et al., 1998, 1999).

Pentacyclic triterpenes and mainly sterols esterified with fatty acids have been studied in numerous plant species as well as in individual tissues and organelles (e.g., microsomal and mitochondrial fractions) (Grunwald, 1970; Wojciechowki et al., 1972; Garcia and Mudd, 1978; Takaoka et al., 1987). Despite this, their precise role in plants remains unclear. For example, changes have been observed in the abundance of steryl esters at all vital stages of the plant life cycle, and it is therefore likely that esters play an equally important role in plant metabolism (Dyas et al., 1991).

In this work, three series of high molecular weight compounds, α-tocopherol, β-tocopherol and phytol

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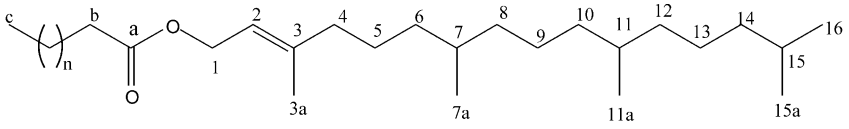
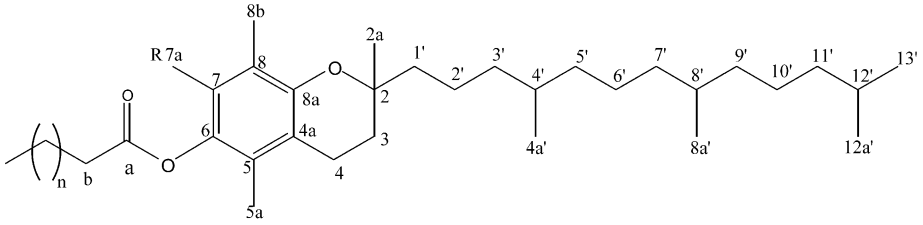


1 n = 9 **8** n = 17
2 n = 11 **9** n = 18
3 n = 12 **10** n = 19
4 n = 13 **11** n = 20
5 n = 14 **12** n = 21
6 n = 15 **13** n = 22
7 n = 16



14 R = H, n = 9 **20** R = CH_3, n = 11
15 R = H, n = 11 **21** R = CH_3, n = 12
16 R = H, n = 12 **22** R = CH_3, n = 13
17 R = H, n = 13 **23** R = CH_3, n = 15
18 R = H, n = 15 **24** R = CH_3, n = 17
19 R = CH_3, n = 9

esterified to fatty acids (**1**–**24**), were characterized for the first time by routine HT-HRGC and HT-HRGC–MS analyses of samples from Amazonian plants. Amyryl alkanoates were also found in the extracts of these Amazonian plants. Their mass spectra had been described earlier in smoke particulate matter (Elias et al., 1997, 1998, 1999), because these HMW components were stripped into smoke when such plant biomass was burned.

Vitamin E, as it occurs naturally, consists of eight compounds which belong to two series of methyl substituted chromanols, with either a saturated (the tocopherols) or unsaturated (the tocotrienols) side chain in the 2-position. Each of these compounds has different vitamin E activity and antioxidant properties (Van Niekerk, 1988). α-Tocopherol, ($C_{29}H_{50}O_2$) was initially described as a factor in vegetable oils capable of reestablishing the fertility of sterile mice fed exclusively with cow milk (Rosenberg, 1945). Wheat and rice germ oils are rich sources of vitamin E, which acts as a lipid antioxidant compound, protecting membranes from molecular oxygen damage (Rosenberg, 1945), and as a chemopreventive agent (inhibitor of carcinogen formation) (Pezzuto, 1993).

Another common natural product found in higher plants is phytol (2,6,10,14-tetramethylhexadec-14-en-16-ol, $C_{20}H_{40}O$), which commonly occurs as part of the chlorophyll molecule. Phytol is also a cancer preventive agent (Duke, 1992).

The long chain fatty acid esters of these natural products (**1**–**24**) were found in the smoke from burning Amazonian plants (Elias et al., 1997, 1998, 1999). Due to the importance and novelty of these inferred natural products, their structural elucidation in lipids extracted from the actual plants was carried out. Here we describe their characterization using electron impact (EI) ionization MS coupled to HTGC.

2. Results and discussion

Most previous studies concerning fatty acid esters, e.g., with pentacyclic triterpenoids or steroids, have proceeded by alkaline hydrolysis of the intact molecules, followed by separate analyses of the constituent triterpenols or sterols and the acyl moieties. This methodology, however, loses potentially useful information on the nature of the intact esters. The direct high temperature GC–MS method was used in previous studies of HMW compounds in the smoke of Amazonian plants (Elias et al., 1997, 1998, 1999) and showed the presence of several different compound classes [e.g., long chain wax esters, triterpenyl (α-amyryl, β-amyryl and taraxasteryl) fatty acid esters (TFAE) and triglycerides] in emissions from biomass burning. During a thorough study of the mass fragmentation of the minor compounds, three additional HMW compound series could be identified: wax esters of phytol (**1**–**13**), in the dichloromethane (CH_2Cl_2) extract of smoke from burning of Marupá (*Simaruba amara*), and wax esters of α- and β-tocopherol (**14**–**24**), in the (CH_2Cl_2) extract of smoke from burning of Brazil nut (*Bertholettia excelsa*). In this work these compounds were also characterized in

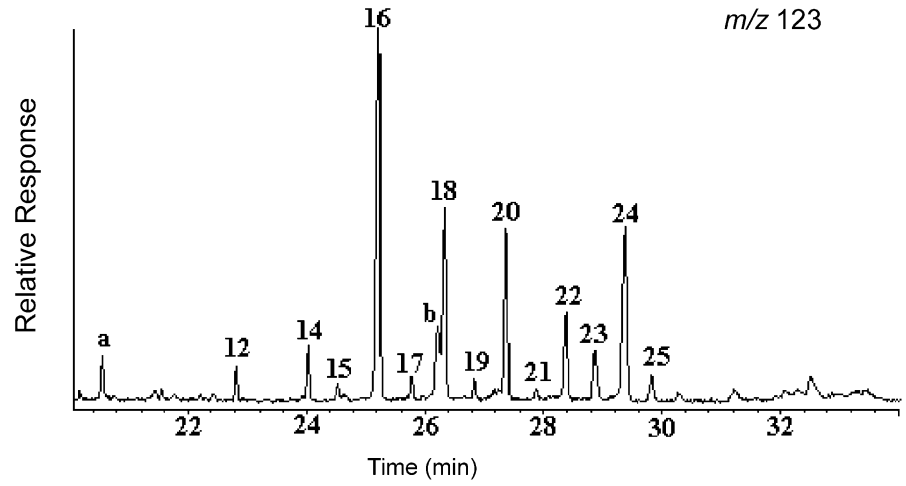


Fig. 1. Representative HT-HRGC–MS fragmentogram (*m*/*z* 123) showing the homologous series of phytyl alkanoates in Marupá (*Simaruba amara*) leaves. Numbers refer to carbon chain length of the esterified fatty acids; a and b are squalene and an unknown compound, respectively.

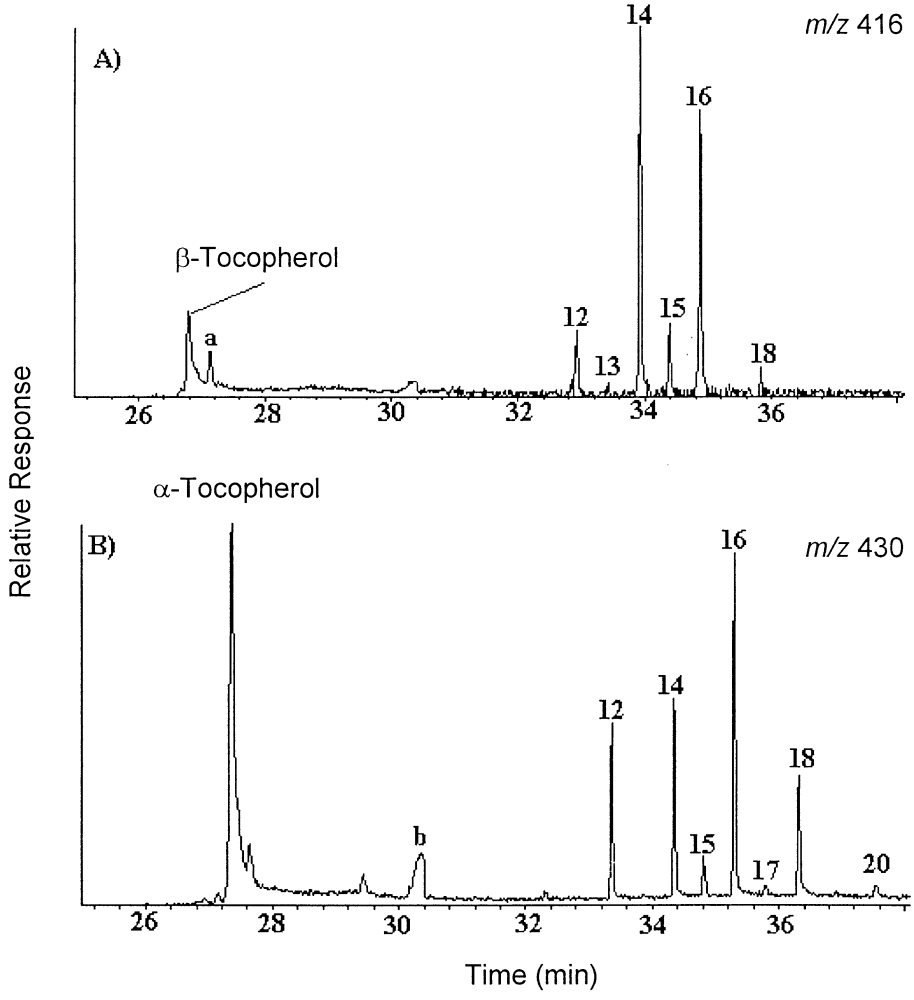


Fig. 2. Representative HT-HRGC–MS fragmentograms: (A) *m*/*z* 416 showing the homologous series of β-tocopheryl alkanoates; and (B) *m*/*z* 430 showing the homologous series of α-tocopheryl alkanoates in Brazil nut (*Bertholettia excelsa*) leaves. Numbers refer to carbon chain length of the esterified fatty acids; a and b are unknown compounds.

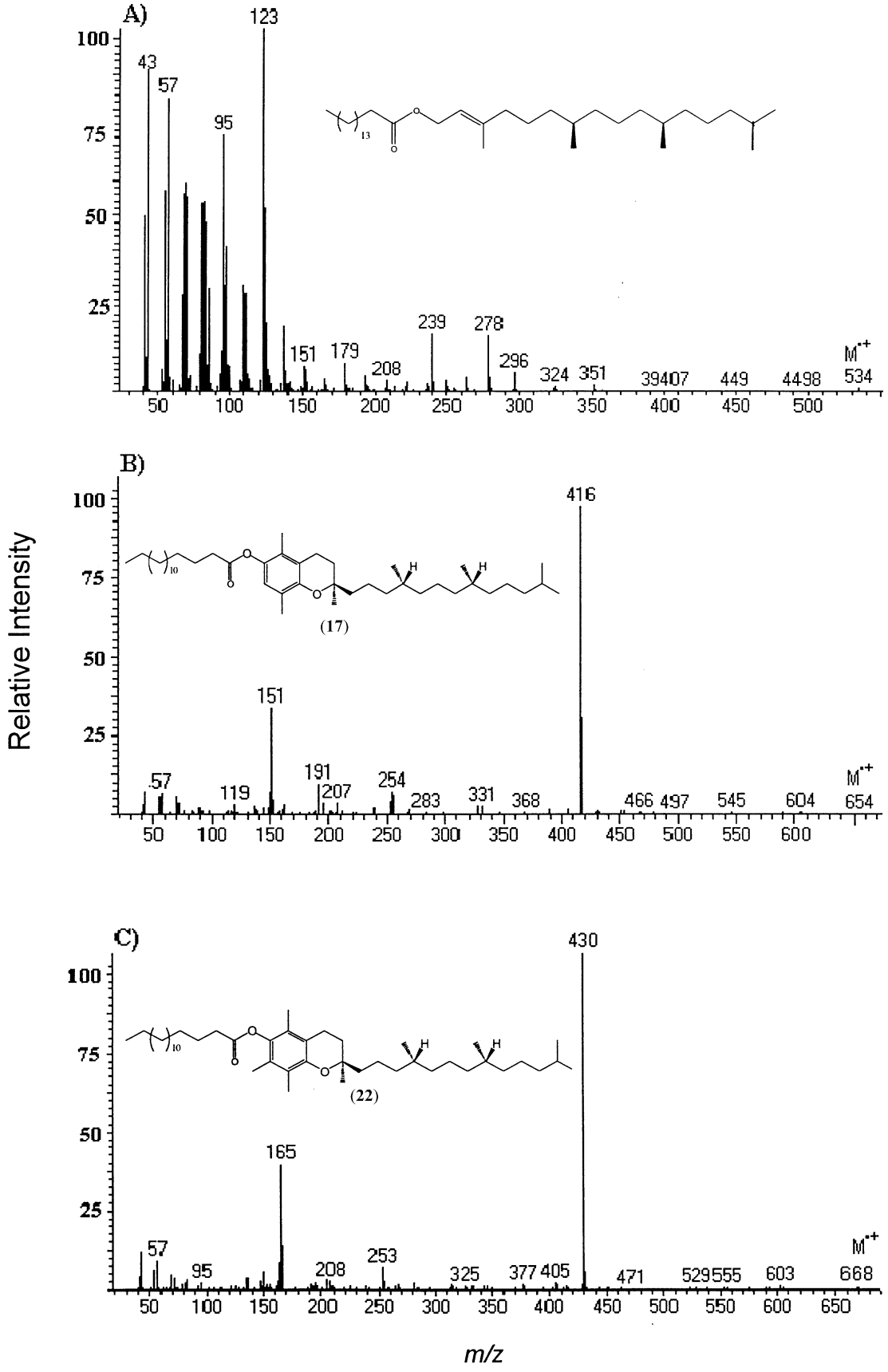


Fig. 3. Mass spectra of (A) phytyl-, (B) β-tocopheryl- and (C) α-tocopheryl hexadecanoates. Each is representative of the respective homologous series of phytol, α-tocopherol and β-tocopherol esterified to long-chain fatty acids. The structures for each compound are shown.

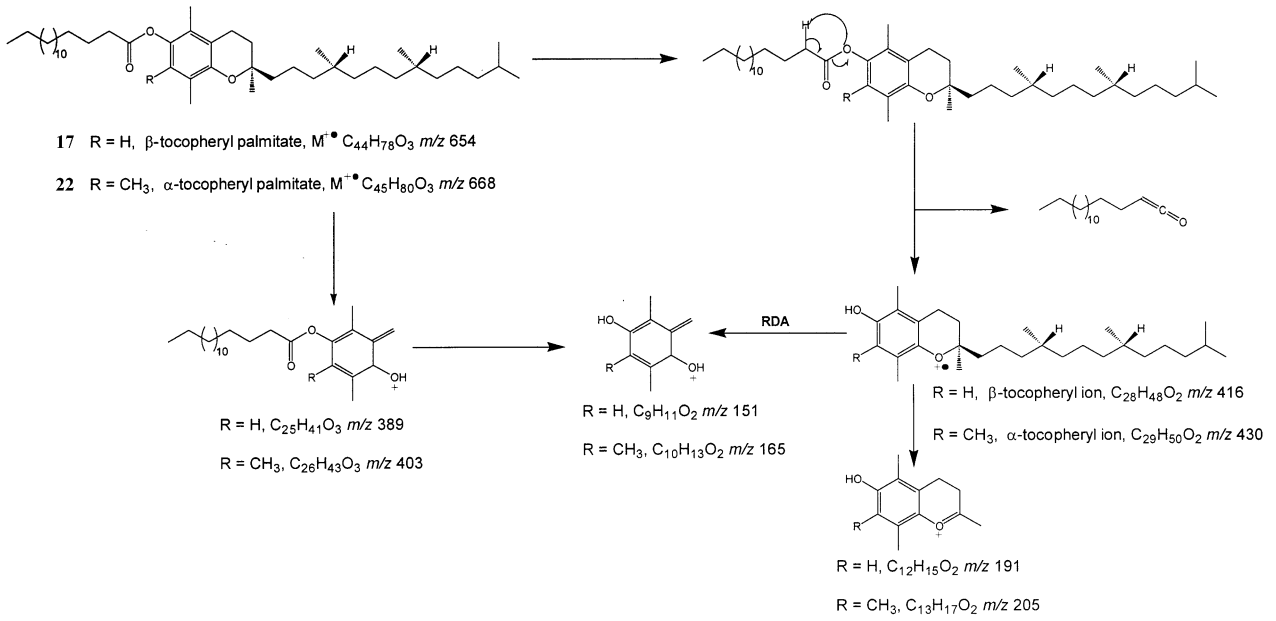


Fig. 4. Scheme summarizing the mass spectrometric fragmentation pattern of the α-tocopheryl (R = CH_3) and β-tocopheryl (R = H) fatty acid esters (RDA = Retro-Diels–Alder reaction).

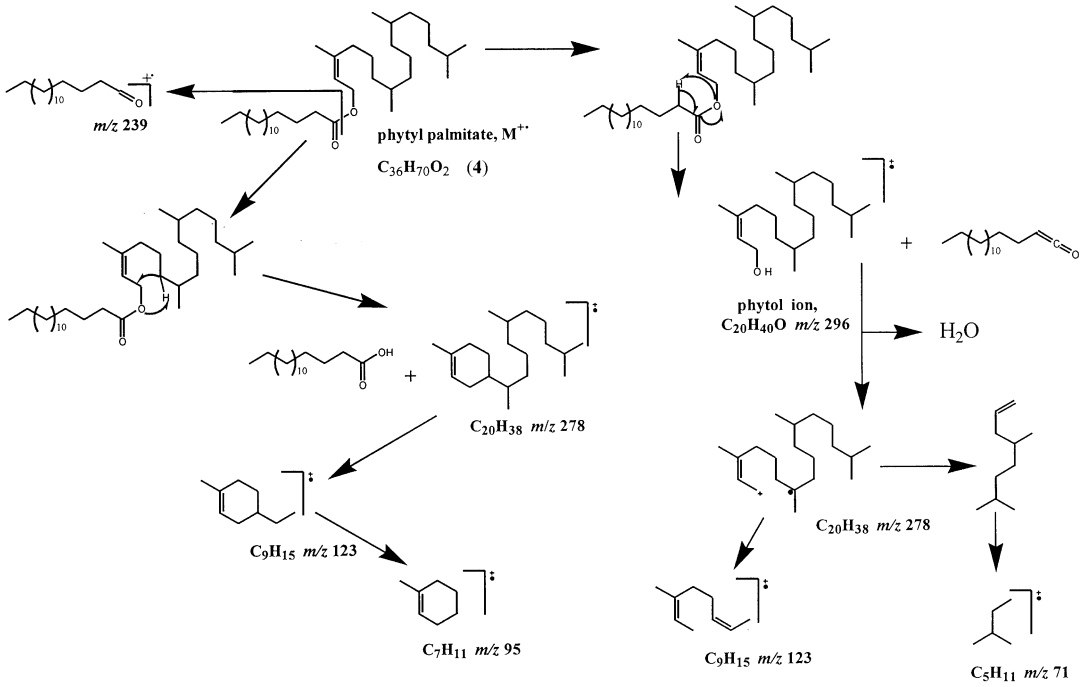


Fig. 5. Scheme summarizing the mass spectrometric fragmentation pattern of the phytyl fatty acid esters.

CH_2Cl_2 extracts of the epicuticular waxes of the plant leaves (Figs. 1 and 2). This demonstrates that these compounds were volatilized directly from the plants when they were burned, without chemical modification. These previous results showed the major HMW compounds in Brazil nut to be wax esters of α- and β-amyrin. Despite their relatively complex structures the mass spectra of the esterified triterpenols are quite simple, consisting of a molecular ion ($M^{\bullet+}$), M−CH_3, M−fatty acid, and triterpenoid fragments (Elias et al., 1997).

The mass spectrometric fragmentation patterns of these new series are also very simple (Fig. 3); however, the loss of the fatty acid moiety is distinctly different when compared with triterpenyl alkanoates (Elias et al., 1997).

In case of wax esters of the tocopherols (**14**–**24**) and phytol (**1**–**13**), the loss of fatty acid is by a retro-Diels–Alder reaction with hydrogen transfer (Figs. 4 and 5).

The mass spectra of the phytyl alkanoates **1**–**13** showed a similar fragmentation pattern (Fig. 3A) as that of neophytadiene (7,11,15-trimethyl-3-methylene-1-hexadecene). Two fragmentation routes are proposed in Fig. 5. The first route occurs through the loss of the acid as a ketene moiety, with the formation of the phytol ion (*m*/*z* 296). Elimination of H_2O yields a $C_{20}H_{38}$ ion at *m*/*z* 278 with subsequent fragmentation as in the case of phytadiene. The other fragmentation route cleaves the ester bond directly to retain the charge on the acyl fragment ($C_{16}H_{31}O$, *m*/z 239) or by H-transfer via a McLafferty-type rearrangement to yield the ion *m*/*z* 278. Other ions observed (Fig. 5) are at *m*/*z* 123 (C_9H_{15}, base peak), *m*/*z* (C_6H_9), *m*/*z* 69 (C_5H_9), and are characteristic of the isoprenoid side chain. However, additional typical fragments for saturated alkanes are also intense (i.e., *m*/*z* 43, 57, 71 and 85).

A simpler fragmentation pattern is observed in tocopheryl alkanoates **14**–**24** (Figs. 3B,C and 4), with the base peak (tocopherol ion) being due to loss of the acid as a ketene moiety, resulting in the ions *m*/*z* 430 and 416 due to α- and β-tocopherol moieties, respectively. These ions undergo a retro-Diels–Alder reaction with hydrogen transfer to give an intense ion at *m*/*z* 165 and 151, respectively, and also an α-cleavage to give a weaker ion

Table 1
High resolution mass spectrometric data of the molecular ion and main fragments of the phytyl (**4**) and α-tocopheryl (**22**) hexadecanoates: experimental and theoretical values

Ion	Observed mass (Da)	Theoretical mass (Da)	Observed mass−theoretical mass (Da)
Phytyl hexadecanoate (**4**)			
$M^{\bullet}$ ($C_{36}H_{70}O_2$)	534.5378	534.5375	+ 0.0003
$C_{20}H_{40}O$	296.3557	296.5350	−0.1793
$C_{20}H_{38}$	278.3379	278.5202	−0.1823
$C_{17}H_{35}$	239.2828	239.2739	+ 0.0089
C_9H_{15}	123.1596	123.2175	−0.0579
C_7H_{11}	95.1238	95.1639	−0.0401
C_5H_{11}	71.0809	71.0860	−0.0051
α-Tocopheryl hexadecanoate (**22**)			
$M^{\bullet}$ ($C_{45}H_{80}O_3$)	668.6104	668.6107	−0.0003
$C_{29}H_{50}O_2$	430.3968	430.7120	−0.3152
$C_{22}H_{37}O$	317.2854	317.2844	+ 0.0010
$C_{17}H_{35}$	239.2678	239.2739	−0.0061
$C_{13}H_{18}O_2$	206.1350	206.1307	+ 0.0043
$C_{13}H_{15}O_2$	203.1170	203.1172	−0.0002
$C_{13}H_{12}O$	184.0890	184.0888	+ 0.0002
$C_{10}H_{13}O_2$	165.1217	165.2107	−0.0890

Table 2
Main fragments (*m*/*z*) and their relative abundances (%) (in parentheses) in the mass spectra of phytyl alkanoates

Compound	$M^{+\bullet}$	Base peak (100)	Other significant fragments
Phytyl dodecanoate **1**	478[a]	123	296 (5.5), 278 (8.6), 95 (91.4), 71 (60.6)
Phytyl tetradecanoate **2**	506 (1.0)	123	296 (5.7), 278 (12.7), 95 (82.4), 71 (64.3)
Phytyl pentadecanoate **3**	520 (0.3)	123	296 (5.0), 278 (8.1), 95 (81.4), 71 (56.6)
Phytyl hexadecanoate **4**	534 (1.1)	123	296 (5.4), 278 (15.7), 95 (71.2), 71 (56.7)
Phytyl heptadecanoate **5**	548[a]	123	296 (6.4), 278 (11.2), 95 (93.1), 71 (45.5)
Phytyl octadecanoate **6**	562 (0.6)	123	296 (5.6), 278 (18.5), 95 (78.2), 71 (57.6)
Phytyl nonadecanoate **7**	576[a]	69	296 (1.1), 278 (6.9), 123 (73.1), 95 (52.5)
Phytyl eicosanoate **8**	590 (0.6)	123	296 (7.9), 278 (16.7), 95 (73.7), 71 (63.5)
Phytyl heneicosanoate **9**	604[a]	43	296 (1.5), 278 (12.7), 123 (71.2), 95 (57.7)
Phytyl docosanoate **10**	618 (1.0)	123	296 (6.3), 278 (20.9), 95 (77.4), 71 (66.6)
Phytyl tricosanoate **11**	632 (1.5)	123	296 (9.3), 278 (22.2), 95 (80.0), 71 (73.5)
Phytyl tetracosanoate **12**	646 (0.3)	123	296 (6.8), 278 (20.4), 95 (72.4), 71 (69.0)
Phytyl pentacosanoate **13**	660[a]	57	296 (7.2), 278 (13.6), 123 (75.0), 95 (55.8)

[a] The molecular ions of these compounds were not observed because of their low intensity. Also the $M^{+\bullet}$ 618, 632, 646 and 660 occur as *m*/*z* 619, 633, 647 and 661 in the data due to the cumulative mass defect of H exceeding 0.7.

Table 3
Main fragments (*m*/*z*) and their relative abundances (%) (in parentheses) in the mass spectra of tocopheryl alkanoates

Compound	$M^{+\bullet}$	Base peak (100)	Other significant fragments
β-Tocopheryl dodecanoate **14**	598 (2.4)	416	191 (7.9), 151 (52.5), 57 (7.3)
α-Tocopheryl dodecanoate **19**	612 (1.7)	430	205 (4.1), 165 (23.1), 57 (9.7)
β-Tocopheryl tetradecanoate **15**	626 (1.8)	416	191 (5.1), 151 (29.7), 57 (5.1)
α-Tocopheryl tetradecanoate **20**	640 (1.2)	430	205 (3.1), 165 (32.4), 57 (4.7)
β-Tocopheryl pentadecanoate **16**	640 (2.6)	416	191 (13.7), 151 (39.5), 57 (15.5)
α-Tocopheryl pentadecanoate **21**	654 (0.3)	430	205 (1.1), 165 (12.3), 57 (3.6)
β-Tocopheryl hexadecanoate **17**	654 (1.0)	416	191 (9.2), 151 (37.4), 57 (7.6)
α-Tocopheryl hexadecanoate **22**	668 (0.8)	430	205 (3.5), 165 (35.6), 57 (8.4)
β-Tocopheryl octadecanoate **18**	668 (0.5)	416	191 (4.1), 151 (29.9), 57 (5.1)
α-Tocopheryl octadecanoate **23**	696 (1.1)	430	205 (1.4), 165 (31.5), 57 (4.9)
α-Tocopheryl eicosanoate **24**	710[a]	430	205 (1.0), 165 (33.2), 57 (5.7)

[a] The molecular ion of this compound was not observed because the upper limit of the scan range for the HP 5972 MSD is 700 Dalton. Also $M^{+\bullet}$ 668, 696 and 710 occur as *m*/*z* 669, 697 and 711 in the data due to the cumulative mass defect of H exceeding 0.7.

at *m*/*z* 205 and 191, respectively (α- and β-tocopherol). The retro-Diels–Alder reaction with or without hydrogen transfer also occurs (to a lesser extent) directly from the molecular ion to give ions *m*/*z* 403 and 389 (α- and β-tocopheryl hexadecanoates, respectively).

The fragmentation of the phytyl (**1**–**13**) and α- and β-tocopheryl (**14**–**24**) alkanoates were supported by direct analyses of synthetic phytyl (**4**) and α-tocopheryl (**22**) hexadecanoates by high resolution mass spectrometry (HRMS). The HRMS data for the characteristic fragments are given in Table 1. The highest mass difference (0.3152 Dalton) observed was for α-tocopherol ion ($C_{29}H_{50}O_2$, theoretical mass 430.7120) can be explained by contribution from the secondary fragmentation routes. A secondary ion can be formed by loss of the 4,8,12-trimethyltridecyl and methyl groups of the α-tocopheryl hexadecanoate (**22**) moiety with probable formation of a trimethyl-6-chromanoyl hexadecanoate ion ($C_{28}H_{46}O_3$, theoretical mass 430.3416). However, this type of fragmentation was observed in very low abundance for tocopheryl alkanoates **14**–**24**.

The relative abundances of the characteristic fragments in the mass spectra of the HMW phytyl and tocopheryl alkanoates are given in Tables 2 and 3, respectively. The tocopheryl alkanoates in Brazil nut (*B. excelsa*) have acyl carbon chain lengths extending from 12 to 20 for α- (**19**–**24**) and 12 to 18 for β-tocopherols (**14**–**18**), respectively. The phytyl alkanoates (**1**–**13**) in Marupá (*S. amara*) have acyl carbon chain lengths extending from 12 to 25. The dominant tocopheryl alkanoates are those of the α-tocopherol series (**19**–**24**) with a lesser amount of those of β-tocopherol (**14**–**18**).

Precise quantification is very difficult in crude extracts due to the complexity of these samples. An estimate of the concentrations was therefore made using a response factor of 1 for the mass spectrometric detection of all compounds. The total amount of phytyl alkanoates (**1**–**13**) was 0.3% of the CH_2Cl_2 extract from the leaves of Marupá (*Simaruba amara*), and the total amount of α-tocopheryl alkanoates (**19**–**24**) was 0.2% and β-tocopheryl alkanoates (**14**–**18**) was 0.05% of the CH_2Cl_2 extract from the leaves of Brazil nut (*B. excelsa*).

The HMW compounds present in epicuticular waxes as long chain wax esters have important functions in life support of plants. There is strong evidence supporting the hypothesis that long chain wax esters of plant waxes play an essential role in the epicuticular transport barrier which hinders the diffusion of water and solutes across the plant cuticle (Gülz et al., 1994). Recently it was reported that lupeol and lupeol linoleate have marked anti-inflammatory activities (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). However, there is no published data about the possible ecological functions of the phytyl or tocopheryl alkanoates.

3. Experimental

3.1. Materials

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

3.2. Fractionation of extracts

The samples (3 g) were minced, placed into 100 ml jars, and extracted sequentially five times each with hexane (15 ml), dichloromethane (15 ml), acetone (15 ml) and MeOH (15 ml), using ultrasonic agitation for 30 min at room temp. The combined extracts for each solvent were concentrated in vacuo to a final volume of approximately 2 ml and the resulting crude extracts (before drying) were analyzed by HT-HRGC.

The extracts were further concentrated under N_2 and weighed. The following yields were obtained for the solvent extracts in increasing polarity: hexane 1.11 and 0.05 g, dichloromethane 0.07 and 0.08 g, acetone 0.05 and 0.04 g, and MeOH 0.39 and 0.075 g, respectively, for Brazil nut and Marupá. The dry residues were fractionated by 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 fractions were kept in a refrigerator until analysis.

3.3. Instrumental analyses

General HRMS analyses were performed by direct inlet probe on a VG AUTOSPEC (VG Analytical, Manchester, UK); NMR spectra were acquired using a Brucker DPX 200 MHz instrument (Silberstreifen, Germany); prior to analysis the samples were carefully dried and dissolved in $CDCl_3$. FT-IR spectra were acquired using a Nicolet Magna, IR 760.

HT-HRGC–MS chromatography was performed with borosilicate capillary columns (20 m×0.25 mm i.d.; Duran-50, Vidrolex, Brazil) coated with 0.2 μm of PS-086 (15%-phenyl-80%-methylpolysiloxane, Petrarch Systems, Inc, USA). The columns were prepared in our laboratory, according to a published procedure (Blum, 1985) and connected to a 2 m piece of an empty capillary (interface GC–MS) prepared from 0.25 mm i.d. high temperature fused silica (J&W, USA). HT-HRGC–MS analyses were carried out on a HP-5890-II gas chromatograph coupled to a HP 5972 spectrometer (Hewlett Packard, Palo Alto, USA), under electron impact ionization (70 eV) and a MS scan range of 50–700 Da. The column temperature was programmed from 40 to 390 °C at 10 °C min^{-1} and held isothermal at 390 °C for 20 min. The on-column injector (Carlo Erba, Rodano, Italy) and the transfer line temperatures were set at 40 and 370 °C, respectively.

3.4. Synthesis of standards

3.4.1. Phytyl hexadecanoate (*4*)

Palmitoyl chloride (100 μl, 0.33 mmol), hexane (140 μl), phytol (140 μl, 0.40 mmol) and triethylamine (45 μl, 0.32 mmol) were heated (80 °C, 2 h) to afford phytyl hexadecanoate which was extracted with hexane (3×2 ml), concentrated by rotatory evaporation followed by nitrogen blowdown. The yellow oil obtained containing phytyl hexadecanoate **4** was purified by TLC, giving the product at 85% yield.

^{1}H NMR (200 MHz) δ 2.30 (b, *t*, $J=7.5$ Hz); δ 4.59 (1, *d*, $J=7.1$ Hz); δ 5.34 (2, *t*, $J=7.1$ Hz); δ 1.70 (3a, *s*) and δ 2.01 (4, *t*, $J=7.5$ Hz).

^{13}C NMR (50 MHz) δ 174.15 (a); δ 34.61 (b); δ 14.31 (c); δ 61.34 (1); δ 118.36 (2); δ 142.81 (3); δ 40.05 (4); δ 24.99 (5); δ 36.83 (6); δ 32.87 (7); δ 37.56 (8); δ 25.23 (9); δ 37.56 (10); δ 32.99 (11); δ 37.56 (12); δ 24.66 (13); δ 39.67 (14); δ 28.18 (15); δ 16.56 (3a); δ 19.94 (7a, 11a) and δ 22.90 (15a, 16).

IR cm^{-1}: 2925; 2854; 1738; 1464; 1378; 1273 and 1168.

3.4.2. α-Tocopheryl hexadecanoate (*22*)

α-Tocopherol (110 mg, 0.26 mmol), hexane (250 μl), palmitoyl chloride (85 μl, 0.28 mmol) and triethylamine (45 μl, 0.32 mmol) were heated (80 °C, 2 h) to afford α-tocopheryl hexadecanoate **22** which was extracted with hexane (3×2 ml), concentrated by rotatory evaporation followed by nitrogen blowdown. The white-yellow crystals obtained were purified by TLC, giving the product at 82% yield.

^{1}H NMR (200 MHz) δ 2.60 (a, *t*, $J=7.3$ Hz); δ 1.77 (b, *qt*, $J=7.3$ Hz); δ 2.10 (8b, *s*); δ 2.02 (7a, *s*); δ 1.97 (5a, *s*) and δ 0.88 (4a, 8a and 12a, d, $J=6.5$ Hz)

^{13}C NMR (50 MHz) δ 172.61 (a); δ 34.37 (b); δ 149.50 (8a); δ 140.68 (6); δ 126.87 (8); δ 125.09 (7); δ 123.17 (5); δ 117.53 (4a); δ 77.41 (2); δ 39.57 (11); δ 37.63 (3, 5, 7, 9); δ 37.48 (1); δ 32.99 (4, 8); δ 32.12 (3); δ 28.18 (12); δ 25.38 (10); δ 25.00 (6); δ 24.16 (2a); δ 22.92 (12a); δ 22.82 (13); δ 20.78 (4); δ 19.94 (4a); δ 19.85 (8a); δ 13.16 (7a); δ 12.31 (8b) and δ 12.02 (5a).

IR cm^{-1}: 2914; 2849; 2729; 1750; 1471; 1418; 1381; 1257; 1158; 1099 and 719.

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