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Regiospecific analysis of neutral ether lipids by liquid chromatography/electrospray ionization/single quadrupole mass spectrometry: validation with synthetic compounds

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A reversed-phase high-performance liquid chromatography (HPLC) method with on-line electrospray ionization/collision-induced dissociation/mass spectrometry (ESI/CID/MS) is presented for the regiospecific analysis of synthetic reference compounds of neutral ether lipids. The reference compounds were characterized by chromatographic retention times, full mass spectra, and fragmentation patterns as an aid to clarify the regiospecificity of ether lipids from natural sources. The results clearly show that single quadrupole mass spectroscopic analysis may elucidate the regiospecific structure of neutral ether lipids. Ether lipid reference compounds were characterized by five to six major ions in the positive ion mode. The 1-*O*-alkyl-*sn*-glycerols were analyzed as the diacetoyl derivative, and showed the $[M - \text{acetoyl}]^+$ ion as an important diagnostic ion. The diagnostic ions of directly analyzed 1-*O*-alkyl-2-acyl-*sn*-glycerols and 1-*O*-alkyl-3-acyl-*sn*-glycerols were the $[M - \text{alkyl}]^+$, $[M + H - H_2O]^+$ and $[M + H]^+$ ions. Regiospecific characterization of the fatty acid position was evident from the relative ion intensities, as the *sn*-2 species had relatively high $[M + H]^+$ ion intensities compared with $[M + H - H_2O]^+$, whereas the reverse situation characterized the *sn*-3 species. Furthermore, corresponding *sn*-2 and *sn*-3 species were separated by the chromatographic system. However, loss of water was promoted as fatty acid unsaturation was raised, which may complicate interpretation of the mass spectra. The diagnostic ions of directly analyzed 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols were the $[M - \text{alkyl}]^+$, $[M - \text{sn-2-acyl}]^+$ and $[M - \text{sn-3-acyl}]^+$ ions. Regiospecific characterization of the fatty acid identity and position was evident from the relative ion intensities, as fragmentation of the *sn*-2 fatty acids was preferred to the *sn*-3 fatty acids; however, loss of fatty acids was also promoted by higher degrees of unsaturation. Therefore, both structural and positional effects of the fatty acids affect the spectra of the neutral ether lipids. Fragmentation patterns and optimal capillary exit voltages are suggested for each neutral ether lipid class. The present study demonstrates that reversed-phase HPLC and positive ion ESI/CID/MS provide direct and unambiguous information about the configuration and identity of molecular species in neutral 1-*O*-alkyl-*sn*-glycerol classes. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: alkyldiacylglycerols; electrospray ionization; fragmentation pattern; liquid chromatography/mass spectrometry; regiospecific

INTRODUCTION

The use of cod and shark liver oils, in alternative medicine and for dietary supplementation of ω-3 polyunsaturated fatty acids, is popular in Scandinavia. The shark liver oils have, *qua* their high content of ether lipids, especially 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols (DAGEs), been associated with the ability to reduce radiation damage, suppress tumor growth, increase hemopoesis, and accelerate wound healing.[1] Commonly consumed food of animal origin also contains substantial

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amounts of ether lipids, as ether phospholipids.[2] Diets rich in ether lipids modulate both lipid class and alkyl-group composition in endogenous ether lipids,[3,4] which may have biological impact, as endogenous ether lipids are precursors of, for instance, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycerophosphocholine).[5,6] Platelet-activating factor is an ether phospholipid with diverse and potent physiological and pathological effects in a variety of cells and tissues.[6,7]

Functional properties are closely related to the structural composition, emphasizing the need for rapid, reliable and specific analytical techniques to investigate all transformation products of ether lipids, *inter alia* those of dietary sources. Furthermore, the elucidation of structure by, for instance, mass spectrometry(MS), and evaluation of metabolism and physical chemistry of these structures are mandatory. Previously, high-performance liquid chromatography (HPLC) methods, employing chiral stationary phases, have been developed for the separation of enantiomeric pairs of alkyl-*rac*-glycerols and alkylacyl-*rac*-glycerols.[8,9] However, it is generally accepted that ether lipids from natural sources specifically show the alkyl function in the *sn*-1 position, yielding 1-*O*-alkyl-*sn*-glycerols (GEs).[10,11] In contrast to the neutral ether lipids, the various ether phospholipid classes have been extensively studied for their mass spectroscopic behavior, employing soft ionization techniques such as the electrospray ionization (ESI) in the positive and negative ion modes under various analytical conditions.[12–15]

This report presents an analysis combining reversed-phase HPLC with on-line ESI MS (LC/ESI/MS) for the regiospecific analysis and characterization of synthetic reference neutral ether lipid compounds, i.e. GE, 1-*O*-alkyl-2-acyl-*sn*-glycerols (2-MAGEs) and 1-*O*-alkyl-3-acyl-*sn*-glycerols (3-MAGEs), and DAGEs, by collision-induced dissociation (CID) using a single quadrupole mass spectrometer.

EXPERIMENTAL

Materials

Palmitic acid (16 : 0), palmitoleic acid (16 : 1*n*-7), oleic acid (18 : 1*n*-9), linoleic acid (18 : 2*n*-6), 4-dimethylaminopyridine, and *N*,*N*′-dicyclohexylcarbodiimide were obtained from Sigma–Aldrich (St Louis, MO). 1-*O*-Octadecyl-*sn*-glycerol was obtained from Fluka (Ronkonkoma, NY). Natural mixtures of GEs were obtained from Sigma–Aldrich. All solvents used were analytical or HPLC grade.

Chromatographic and mass spectrometric procedures

Preparative thin-layer chromatography (TLC)

Various transformation products were purified by preparative TLC on plates prepared in the laboratory ($200 \times 200 \times 0.25\ mm^3$) with silica gel G containing 5% boric acid. The plates were activated for 2 h at 110 °C before lipids were applied and resolved in *n*-hexane/isopropyl ether/acetic acid (50 : 50 : 4, by volume). Lipids were visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol.[16] Migration of a component is given as the relative retention factor (Rf). Lipids were recovered from the TLC plates by scraping off the gel, extraction with chloroform/methanol (2 : 1, by volume), washing with 1% ammonium hydroxide, drying with sodium sulfate, evaporating under nitrogen, and dissolution in chloroform/methanol (2 : 1, by volume).

Gas–liquid chromatography (GLC)

GLC analysis of GE was carried out with the acetylated derivative, i.e. 1-*O*-alkyl-2,3-diacetoyl-*sn*-glycerol (diacetoyl-GE). The acetylation was performed with acetic anhydride/pyridine (1 : 1, by volume) at 80 °C for 30 min, as described by Myher *et al*.[17] The GLC system consisted of a polar capillary column (SP 2380, 15 m × 0.32 mm i.d., Supelco, Mississauga, ON) installed into a gas chromatograph (Hewlett-Packard, Palo Alto, CA; Model 5880) equipped with a flame ionization detector (FID). Hydrogen was used as carrier gas at 3 psi. Injections were made at 100 °C and after 30 s the oven temperature was raised by 20 °C min^{-1} to 180 °C and then to 240 °C at 5 °C min^{-1}.

HPLC

Reversed-phase HPLC of the prepared ether lipid compounds were performed on an HP ODS Hypersil column (5 μm; 200 mm × 2.1 mm i.d.; Hewlett-Packard), installed into a Hewlett-Packard Model 1090 liquid chromatograph. The elution gradient was slightly modified from that reported by Kim *et al*.[18] Reference compounds were eluted isocratically with 100% solvent A for 3 min, followed by a linear gradient to 100% solvent B in 25 min, and kept for 6 min before returning to 100% solvent A. The solvent flow was 0.4 ml min^{-1}. The solvent compositions of A and B depended on the lipid class analyzed; however, 30% ammonium hydroxide (0.5%, by volume) was added to all solvents. Analysis of diacetoyl-GE: solvent A, methanol/water (88 : 12, by volume) and solvent B, methanol/*n*-hexane (88 : 12, by volume). Analysis of 2- and 3-MAGE: solvent A, methanol/water (95 : 5, by volume) and solvent B, methanol/*n*-hexane (75 : 25, by volume). Analysis of DAGE: solvent A, methanol/*n*-hexane (88 : 12, by volume) and solvent B, methanol/*n*-hexane (75 : 25, by volume).

LC/ESI/MS

Reversed-phase LC/ESI/MS was performed by admitting the entire effluent into a Hewlett-Packard Model 5988B single quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray interface (Hewlett-Packard Model 59987A) as described by Ravandi *et al*.[19] Nitrogen gas was used as both nebulizing gas (60 psi) and drying gas (60 psi, 270 °C). Positive ESI spectra were taken in mass range of 100–1100 amu unless stated otherwise. Capillary, endplate, and cylinder voltages were −4 kV, −3.5 kV, and −5 kV respectively in the positive ion mode. The capillary exit voltage (CapEx), which determines the extent of fragmentation by means of a CID process, was varied between +90 and +250 V. The HPLC column effluent contained ammonium hydroxide to enhance the ESI of the selected compounds *via* adduct ions like $[M + NH_4]^+$ $(M + 18)$ and $[M + Na]^+$ $(M + 23)$. The identification of the various reference compounds was performed on the basis of retention time (RT), averaged full spectra, and the fragmentation patterns.

Synthesis of ether lipids

Synthesis of 2- and 3-MAGE

The esterifications of 1-*O*-octadecyl-*sn*-glycerol with either palmitic, palmitoleic, oleic or linoleic acid were performed by the carbodiimide method.[20] Fatty acid (75 μmol), 1-*O*-octadecyl-*sn*-glycerol (100 μmol) and 4-dimethylaminopyridine (10 μmol) were suspended in dry *n*-hexane (5 ml). This suspension was added to a suspension of *N*,*N*′-dicyclohexylcarbodiimide (100 μmol) in dry *n*-hexane (5 ml) and shaken vigorously for 17 h at room temperature. After filtration, the solvent was evaporated under nitrogen, and the residue was subjected to preparative TLC. The major products, 2- and 3-MAGE (Rf 0.32 and 0.41 respectively), were clearly resolved from one another, as well as reactants and by-products: GE, free fatty acid (FFA) and DAGE (Rf 0.05, 0.66 and 0.90 respectively). A DAGE with identical fatty acids in the *sn*-2 and *sn*-3 position was formed as a by-product in this synthesis.

Synthesis of DAGE

The esterifications of prepared 2- and 3-MAGE with either palmitic, oleic or linoleic acid were performed by the carbodiimide method as described above. Excess fatty acid was added to the prepared and purified 1-*O*-octadecyl-2(3)-acyl-*sn*-glycerol to obtain higher yields. The major product, DAGE, was purified by preparative TLC.

Hydrolysis with pancreatic lipase

As pancreatic lipase hydrolyzes the primary, but not the secondary, esters of ether glycerolipids,[21] the reference DAGE was hydrolyzed by digestion with pre-extracted pancreatic lipase[22] in order to verify the regio-configuration of the synthetic compounds. The digestion was performed in the presence of gum arabic for 30 min and the digestion products were extracted with diethyl ether. The major product, 2-MAGE (Rf 0.32), was recovered by preparative TLC and analyzed by LC/ESI/MS. Only very small amounts of 3-MAGE and GE were observed, which suggests a low degree of isomerization of 2-MAGE during the hydrolysis and purification.

RESULTS

LC/ESI/MS analysis of GEs

Figure 1A shows the total positive ion current profile of the acetylated reference compound 1-*O*-octadecyl-*sn*-glycerol at RT 16.6 min. Figure 1B shows the full mass spectrum averaged over the entire peak of 1-*O*-octadecyl-2,3-diacetoyl-*sn*-glycerol (CapEx +120 V, *m*/*z* 100–700). Five ions are observed that together represent 1-*O*-octadecyl-2,3-diacetoyl-*sn*-glycerol (MW 428) as indicated by $[M - alkyl]^+$ (*m*/*z* 159), $[M - acetoyl]^+$ (*m*/*z* 369), $[M + H]^+$ (*m*/*z* 429), $[M + NH_4]^+$ (*m*/*z* 446), and $[M + Na]^+$ (*m*/*z* 451). The proposed fragmentation, i.e. loss of acetoyl and alkyl groups, is shown schematically in Fig. 1B. Almost identical spectra were obtained at CapEx +90 and +120 V.

Furthermore, three natural mixtures, each enriched in the three most commonly encountered and biologically significant alkyl chains, i.e. 16 : 0, 18 : 1*n*-9, and 18 : 0, were analyzed. Corresponding spectra to that shown in Fig. 1B were obtained, as only minor changes in the relative ion abundances of the five characteristic ions were introduced. Nevertheless, the reversed-phase HPLC RT values changed, as expected: 12.5 min (16 : 0), 13.0 min (18 : 1*n*-9), and 16.6 min (18 : 0).

The reference 1-*O*-octadecyl-*sn*-glycerol (Fig. 1) was preferred for the synthesis of other neutral ether lipids, because of its high chemical purity (estimated by GLC-FID as 99.5%), compared with the reference GEs isolated from natural sources (estimated as 67–73%).

LC/ESI/MS analysis of 2-MAGEs and 3-MAGEs

Figure 2A shows the total positive ion current profile of the synthetic reference compound 1-*O*-octadecyl-3-octadecadienoyl-*sn*-glycerol at RT 22.4 min. The peak at RT 5.0 min in Fig. 2A is due to an impurity from the carbodiimide reaction, which co-chromatographed with the 3-MAGE species during preparative TLC. Fig. 2B–E shows the full mass spectra averaged over the entire peak of 1-*O*-octadecyl-3-octadecadienoyl-*sn*-glycerol at CapEx +120 V, +150 V, +175 V, and +250 V respectively (*m*/*z* 200–800).

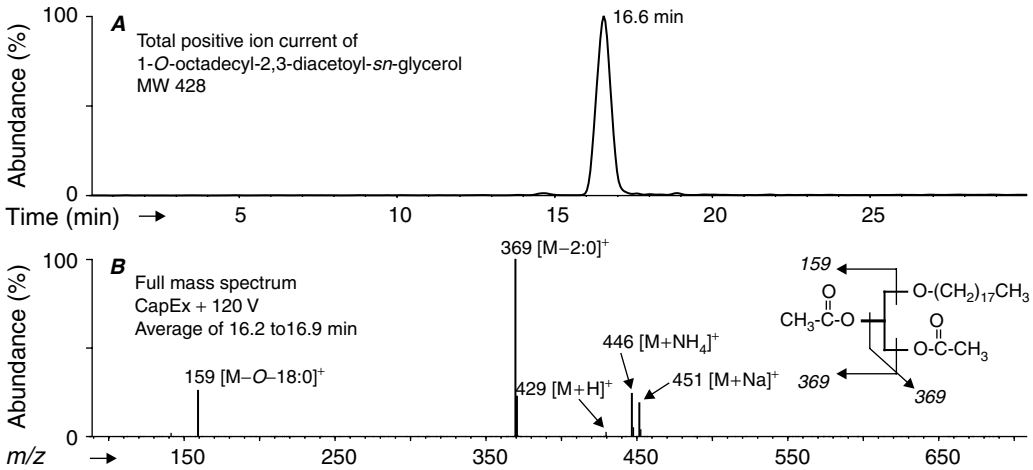

Figure 1. Reversed-phase LC/ESI/MS analysis of acetylated reference GEs. (A) Total positive ion current profile of 1-*O*-octadecyl-2,3-diacetoyl-*sn*-glycerol; (B) full mass spectrum averaged over the entire peak of 1-*O*-octadecyl-2,3-diacetoyl-*sn*-glycerol in (A) at CapEx +120 V. The proposed fragmentations are shown schematically in (B).

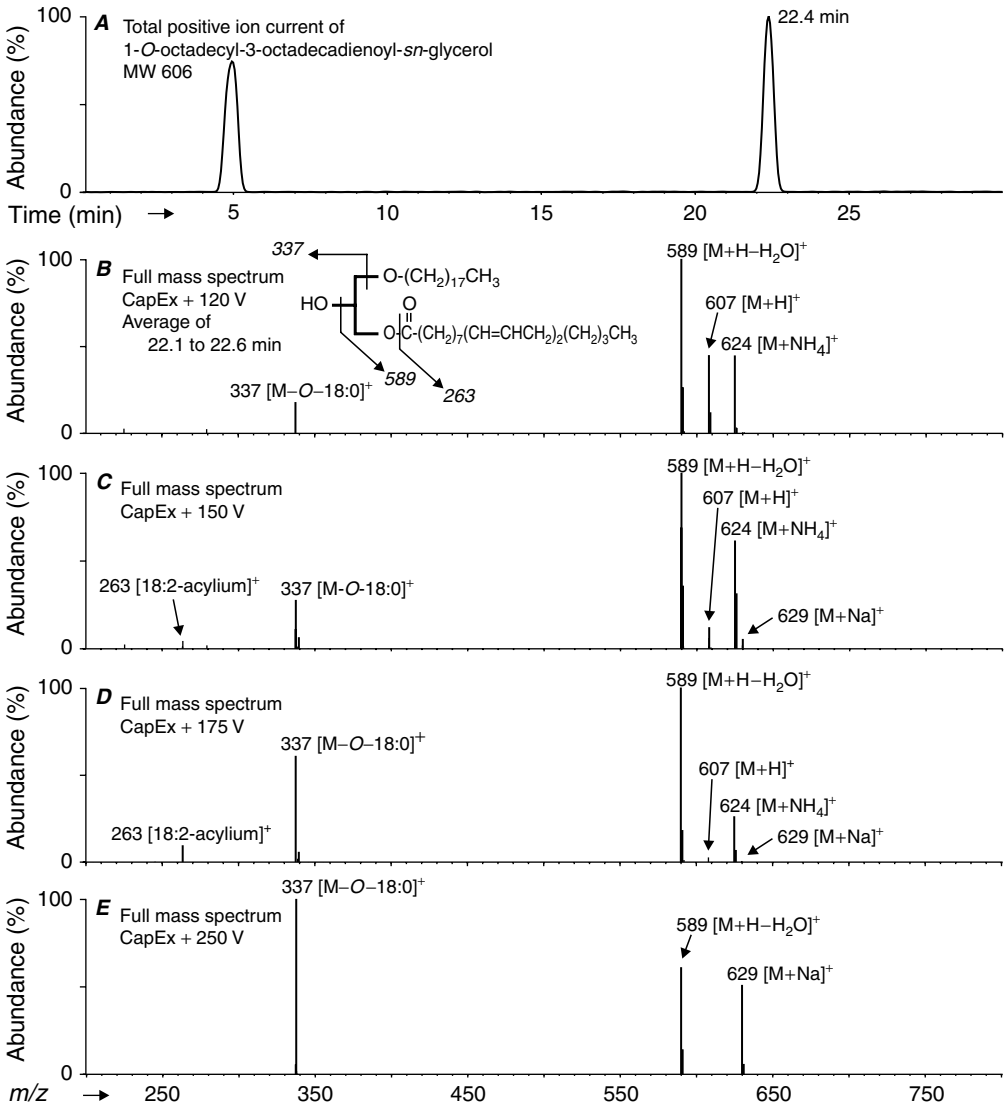

Figure 2. Reversed-phase LC/ESI/MS analysis of synthetic 3-MAGEs. (A) Total positive ion current profile of 1-*O*-octadecyl-3-octadecadienoyl-*sn*-glycerol; (B)–(E) full mass spectra averaged over the entire peak of 1-*O*-octadecyl-3-octadecadienoyl-*sn*-glycerol in (A) at CapEx +120 V, +150 V, +175 V, and +250 V respectively. The proposed fragmentations are shown schematically in (B). The 18 : 2*n*-6-acylium ion (*m*/*z* 263) corresponds to the $[CH_3(CH_2)_3(CH_2CH{=}CH)_2(CH_2)_7C{=}O]^+$ ion.

In total, six ions were observed, with varying intensities depending on the CapEx voltage, that together represent 1-*O*-octadecyl-3-octadecadienoyl-*sn*-glycerol (MW 606). The ions were [acylium ion of 18 : 2*n*-6]$^+$ (*m*/*z* 263), $[M - \text{alkyl}]^+$ (*m*/*z* 337), $[M + H - H_2O]^+$ (*m*/*z* 589), $[M + H]^+$ (*m*/*z* 607), $[M + NH_4]^+$ (*m*/*z* 624), and $[M + Na]^+$ (*m*/*z* 629). Ions representing loss of fatty acids were never observed for the 3-MAGE species. The proposed fragmentations, i.e. loss of water and alkyl groups, in addition to the formation of the acylium ion of 18 : 2*n*-6, are shown schematically in Fig. 2B.

LC/ESI/MS analysis of other synthetic 3-MAGE reference compounds, containing *sn*-3 16 : 1*n*-7, 16 : 0, and 18 : 1*n*-9 fatty acids, revealed the same types of characteristic ion at very similar relative ion intensities as shown for the *sn*-3 18 : 2*n*-6 isomer (Fig. 2) in the CapEx voltage range examined: +120 to +250 V (spectra not shown). Thus, the individual synthetic 3-MAGEs showed unique mass ions, characteristic spectra for their class, and similar relative ion intensities, indicating that the fatty acid species had only a minor effect on the fragmentation pattern. Furthermore, the synthetic 3-MAGE reference compounds showed different RT values, depending on the *sn*-3 fatty acid; 21.5 min, 22.4 min, 24.2 min, and 24.4 min for the 16 : 1*n*-7, 18 : 2*n*-6, 16 : 0, and 18 : 1*n*-6 species respectively.

Increasing the CapEx voltage changed the intensities of the fragment ions (Fig. 2B–E). This series of spectra illustrates very well the general effect of CapEx voltage on the fragmentation pattern of all the neutral ether lipids studied in this work. Loss of the alkyl group and formation of the acylium ion and the sodium molecular adduct ion were more pronounced at higher CapEx voltages. In contrast, the relative abundance of the protonated molecular ion and the ammonia molecular adduct ion decreased and disappeared at higher CapEx voltages. The ion formed due to loss of water, i.e. $[M + H - H_2O]^+$, had the highest relative abundance at low to intermediate CapEx voltages, and decreased only

slightly with increasing CapEx voltages. However, at high CapEx voltages the ion representing loss of the alkyl group was the base peak.

Figure 3A shows the total positive ion current profile of the synthetic reference compound 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol at RT 21.8 min. The other peaks in Fig. 3A are due to various impurities from the carbodiimide reaction, which co-chromatographed with the 2-MAGE species during preparative TLC. Figure 3B shows the reconstructed single-ion chromatogram, with the peak at RT 21.8 min representing the $[M + H - H_2O]^+$ ion (m/z 589) of 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol. Figure 3C shows the full mass spectrum averaged over the entire peak of 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol at CapEx +120 V (m/z 200–800). As in the spectra of the 3-MAGE species, the same six types of characteristic ion were observed (the acylium ion of 18 : 2*n*-6 was only detected in trace amounts at CapEx +120 V) to represent 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol (Fig. 3C). Ions representing loss of fatty acids were never observed for the 2-MAGE species. The proposed fragmentations, i.e. loss of water and alkyl groups, are shown schematically in Fig. 3B.

LC/ESI/MS analysis of other synthetic 2-MAGE reference compounds, containing *sn*-2 16 : 1*n*-7, 16 : 0, and 18 : 1*n*-9 fatty acids, revealed the same types of characteristic ion as shown for the *sn*-2 18 : 2*n*-6 isomer (Fig. 3) in the CapEx voltage range examined: +120 to +250 V (spectra not shown). The spectra of the 2-MAGE species showed similar relative ion intensities; nevertheless, the relative intensity of the $[M + H - H_2O]^+$ ion to, for instance, the protonated molecular ion increased with the number of double bonds in the fatty acid (Table 1; spectra not shown). Thus, the individual synthetic 2-MAGEs showed unique mass ions and characteristic spectra for their class, although the relative ion intensities of certain ions varied, indicating that the fatty acid species had an effect on the fragmentation pattern. The relative ion intensities changed with the CapEx voltage as described for the 3-MAGEs. Furthermore, the synthetic 2-MAGE reference compounds showed different RT values, depending on the *sn*-2 fatty acid; 21.1 min, 21.8 min, 23.8 min, and 24.1 min for the 16 : 1*n*-7, 18 : 2*n*-6, 16 : 0, and 18 : 1*n*-9 species respectively.

LC/ESI/MS analysis of DAGEs

Figure 4A shows the total positive ion current profile of the synthetic reference compound 1-*O*-octadecyl-2-hexadecanoyl-3-octadecadienoyl-*sn*-glycerol at RT 19.5 min. Figure 4B and C shows the full mass spectra averaged over the entire peak of 1-*O*-octadecyl-2-hexadecanoyl-3-octadecadienoyl-*sn*-glycerol at

Table 1. Calculated ratios of the $[M + H - H_2O]^+$ and $[M + H]^+$ ions in synthetic 2-MAGE species at CapEx voltage +120 V

Fatty acid *sn*-2	$[M + H - H_2O]^+/[M + H]^+$ ion ratio[a]
16 : 0	0.55
16 : 1*n*-7	0.70
18 : 1*n*-6	0.70
18 : 2*n*-6	1.05

[a] $[M + H]^+$, protonated molecular ion; $[M + H - H_2O]^+$, loss of H_2O from the protonated molecular ion.

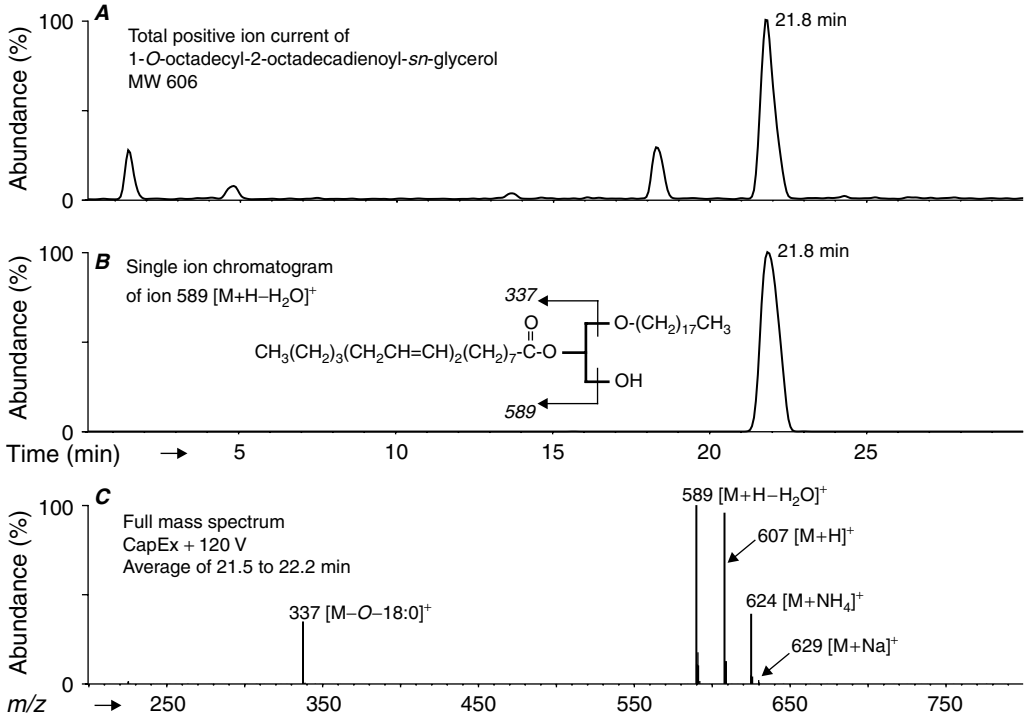

Figure 3. Reversed-phase LC/ESI/MS analysis of synthetic 2-MAGEs. (A) Total positive ion current profile of 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol; (B) reconstructed single-ion mass chromatogram of the m/z 589 $[M + H - H_2O]^+$ diagnostic ion of 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol; (C) full mass spectrum averaged over the entire peak of 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol in (A) at CapEx +120 V. The proposed fragmentations are shown schematically in (B).

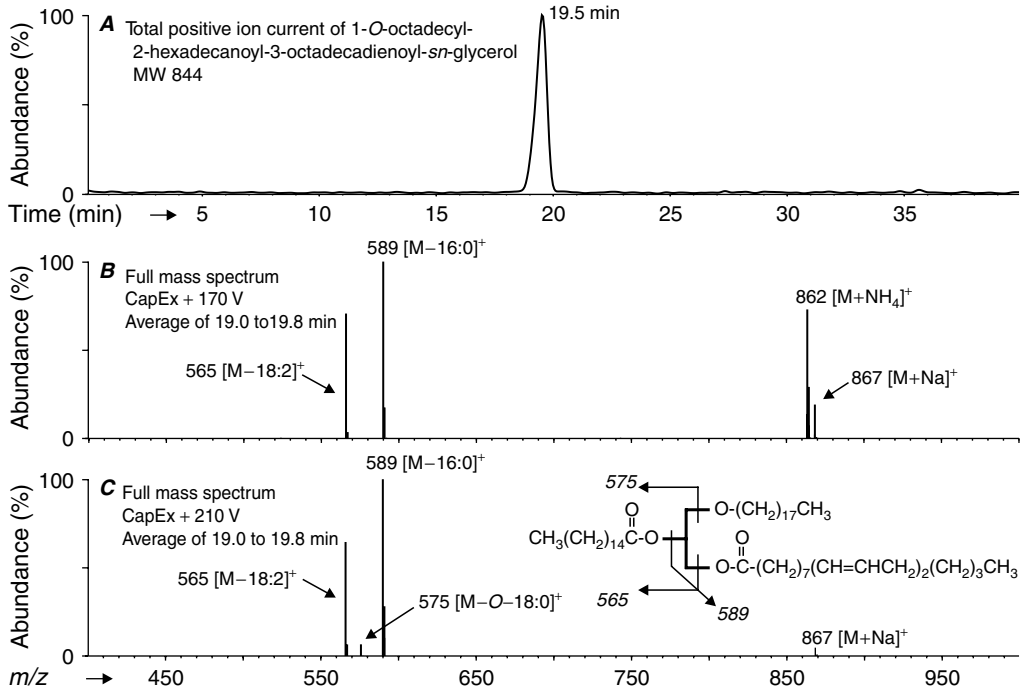

Figure 4. Reversed-phase LC/ESI/MS analysis of synthetic DAGEs. (A) Total positive ion current profile of 1-*O*-octadecyl-2-hexadecanoyl-3-octadecadienoyl-*sn*-glycerol; (B) and (C) full mass spectra averaged over the entire peak of 1-*O*-octadecyl-2-hexadecanoyl-3-octadecadienoyl-*sn*-glycerol in (A) at CapEx +170 V and +210 V respectively. The proposed fragmentations are shown schematically in (C).

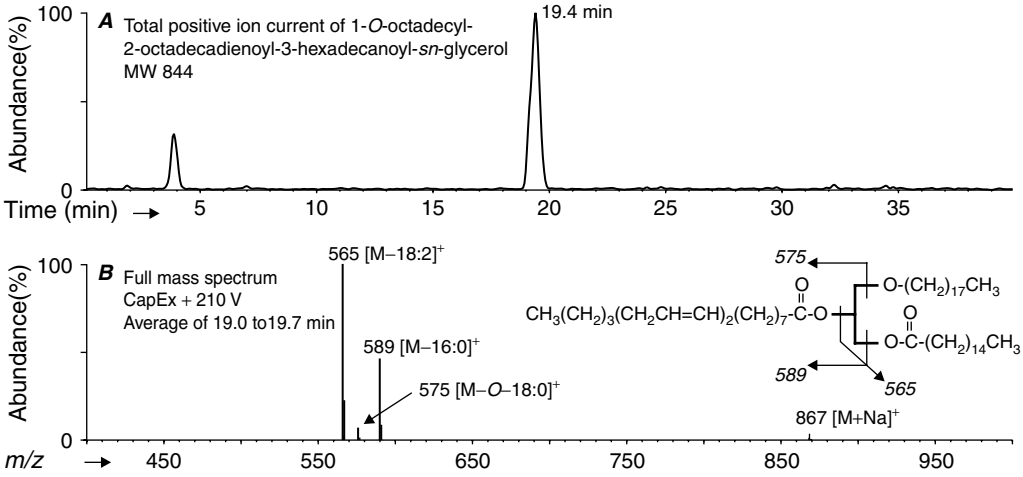

Figure 5. Reversed-phase LC/ESI/MS analysis of synthetic DAGEs. (A) Total positive ion current profile of 1-*O*-octadecyl-2-octadecadienoyl-3-hexadecanoyl-*sn*-glycerol; (B) full mass spectra averaged over the entire peak of 1-*O*-octadecyl-2-octadecadienoyl-3-hexadecanoyl-*sn*-glycerol in (A) at CapEx +210 V. The proposed fragmentations are shown schematically in (B).

CapEx +170 V and +210 V respectively (m/z 400–1000). In total, five characteristic ions were observed, with varying intensities depending on the CapEx, that together represent 1-*O*-octadecyl-2-hexadecanoyl-3-octadecadienoyl-*sn*-glycerol (MW 844). The ions were $[M - alkyl]^+$ (m/z 575), $[M - sn\text{-}2]^+$ (m/z 589), $[M - sn\text{-}3]^+$ (m/z 565), $[M + NH_4]^+$ (m/z 862), and $[M + Na]^+$ (m/z 867). Figure 5A shows the total positive ion current profile of the synthetic reference compound 1-*O*-octadecyl-2-octadecadienoyl-3-hexadecanoyl-*sn*-glycerol at RT 19.4 min. Figure 5B shows the full mass spectra averaged over the entire peak of 1-*O*-octadecyl-2-octadecadienoyl-3-hexadecanoyl-*sn*-glycerol at CapEx +210 V (m/z 400–1000). The two DAGE isomers showed the same characteristic ions. The proposed fragmentations, i.e. loss of acyl and alkyl groups, are shown schematically in Figs 4C and 5B.

LC/ESI/MS analysis of other synthetic DAGE reference compounds, containing different combinations of 16:0, 16:1*n*-7, 18:1*n*-9, and 18:2*n*-9 fatty acids, revealed the same types of characteristic ion at very similar relative ion intensities, as shown for the DAGE isomers in Figs 4 and 5, in the CapEx voltage range examined: +120 to

+250 V (spectra not shown). Increasing the CapEx voltage changed the intensities of the fragment ions. At +120 V only the two molecular adduct ions were detected, but, as the CapEx voltage was increased, the relative abundance of the prominent $[M + NH_4]^+$ ion decreased in contrast to the $[M + Na]^+$ ion, resulting in the $[M + Na]^+$ ion being the most prominent of the two molecular adduct ions at higher CapEx voltages (not all spectra shown). For the three fragment ions, the $[M - sn\text{-}2]^+$ ion was the base peak at CapEx voltages higher than +170 V, and thus the most prominent fragment ion, followed in intensity by the $[M - sn\text{-}3]^+$ ion and then the $[M - \text{alkyl}]^+$ ion (Fig. 4B and C).

Comparison of the full mass spectra of 1-*O*-octadecyl-2-hexadecanoyl-3-octadecadienoyl-*sn*-glycerol and 1-*O*-octadecyl-2-octadecadienoyl-3-hexadecanoyl-*sn*-glycerol (cf. Figs 4C and 5B) under identical analytic conditions (CapEx +170 and +210 V) revealed that the loss of *sn*-2 fatty acid was favored to the loss of *sn*-3 fatty acid and the alkyl group. Furthermore, the fatty acid species also influenced the fragmentation patterns, as the loss of 18:2*n*-6 was favored to the loss of 16:0, regardless of the positional effects (cf. Figs 4C and 5B). Thus, the individual synthetic DAGEs showed unique mass ions and characteristic spectra for their class, although the relative ion intensities of certain ions varied, indicating that the fatty acid species had an effect on the fragmentation pattern. Furthermore, the synthetic DAGE reference compounds showed different RT values, depending on the *sn*-2/*sn*-3 fatty acid contents: 17.1 min (18:2*n*-6/18:2*n*-6), 19.0 min (16:1*n*-7/16:0), 19.5 min (18:2*n*-6/16:0 and 16:0/18:2*n*-6), and 22.2 min (16:0/16:0, 18:1*n*-9/18:1*n*-9, and 18:1*n*-9/16:0).

Verification of structures of synthetic DAGE

Pancreatic lipase hydrolysis of 1-*O*-octadecyl-2-hexadecanoyl-3-octadecadienoyl-*sn*-glycerol followed by LC/ESI/MS analysis revealed one species of 2-MAGE only, which corresponded to that of 1-*O*-octadecyl-2-hexadecanoyl-*sn*-glycerol (RT 23.8 min, *m/z* 313, 565, 583, 600, and 605 at CapEx +120 V). Similar verification of 1-*O*-octadecyl-2-octadecadienoyl-3-hexadecanoyl-*sn*-glycerol revealed two species of 2-MAGEs, i.e. 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol (RT 21.7 min, *m/z* 337, 589, 607, 624, and 629 at CapEx +120 V) and 1-*O*-octadecyl-2-hexadecanoyl-*sn*-glycerol (RT 23.7 min, *m/z* 313, 565, 583, 600, and 605 at CapEx +120 V) in amounts of approximately 97% and 3% respectively, as judged by the total positive ion current areas.

DISCUSSION

No previous studies have been reported on mass spectroscopic analysis of neutral ether lipids using the soft ionization techniques presently employed in LC/MS. Therefore, reference compounds were prepared for the elucidation of ionization and fragmentation patterns using an electrospray interface. ESI/CID/MS was performed in the presence of ammonium to favor the formation of cationic species. Analysis of the synthetic reference compounds in the negative ion mode yielded mainly the $[M - 1]^-$ ions (data not shown); however, the sensitivity was not satisfactory in comparison with analysis in the positive ion mode. The structure of all synthetic compounds was established by a combination of TLC and LC/ESI/MS analysis and pancreatic lipase hydrolysis.

Proposed fragmentations

The ESI of the various synthetic reference neutral ether lipids produced a number of positive CID ions. These ions provided the basis for the proposed fragmentations of acetoyl, acyl, and alkyl esters, along with water and fatty acid acylium ions.

The fragmentation mechanisms leading to loss of *sn*-2 and *sn*-3 fatty acids and acetoyl functions are most likely identical, as the esterified acetoyl group can be considered a 2:0 fatty acid. Ionization of triacylglycerols with resulting loss of fatty acids and generation of positive diacylglycerol-type ions has previously been shown to occur using different LC/MS equipment and conditions.[22–26] The acyl fragmentation obtained in the present study is similar to those reported in the aforementioned studies, and the ion detected represents the $[M + H - RCOOH]^+$ ion in ammonia chemical ionization.[27] In addition, Duffin *et al*.[25] have reported the detection of the acylium ions of fatty acids from monoacyl-, diacyl-, and triacyl-glycerols, i.e. the $[RC{\equiv}O]^+$ ion.

CID resulting in fragmentation of the alkyl group, i.e. the $[M - RO]^+$ ion, probably occurs by α-cleavage of the ether bond; however, this could not be verified by a literature survey. While studying the electrospray mass spectrometric identification of 1,2-diradyl-*sn*-glycerophospholipids, Kerwin *et al*.[12] observed a 'potential' loss of the alkyl group from the 1-*O*-alkyl-2-acyl-*sn*-glycerophosphoinositol.

In the mass spectra of 2- and 3-MAGE a loss of 17 amu was observed. Molecules with alcohol functional groups may produce an $[M - 17]^+$ fragment ion by 'hydroxide abstraction', as described by Westmore and Alauddin,[27] which is the loss of water from the protonated molecular ion, i.e. the $[M + H - H_2O]^+$ ion, or, more accurately, the $[M + NH_4 - H_2O - NH_3]^+$ ion in ammonia chemical ionization. Duffin *et al*.[25] have previously reported the loss of water (the $[M - 17]^+$ ion) from monoacyl- and diacylglycerols, and Taguchi *et al*.[15] and Khaselev and Murphy[14] more recently reported a similar fragmentation from diacylglycerols (daughter ions of diacyl phosphatidylcholine) and acyl lyso-glycerophosphocholine respectively.

Determination of the exact position of the double bonds in the fatty acids was not pursued, since Duffin *et al*.[25] have reported that such characterization cannot be performed by ESI/CID/MS owing to migration of the site of unsaturation during the collisions.

Analysis of GEs

The chromatographic and mass spectroscopic behavior of the acetylated reference compound 1-*O*-octadecyl-*sn*-glycerol are shown in Fig. 1. Of the five ions representing 1-*O*-octadecyl-2,3-diacetoyl-*sn*-glycerol (MW 428) at CapEx +120 V, the $[M - \text{acetoyl}]^+$ ion (*m/z* 369) was found to be the best diagnostic ion, as this abundant ion indicated the molecular weight of the *sn*-1 alkyl group. The $[M - \text{alkyl}]^+$ ion (*m/z* 159) was also an important ion, because it represents

2,3-diacetoyl-*sn*-glycerol, and thus confirms the ether lipid nature of the compound. A CapEx voltage at +120 V was chosen for regiospecific analysis of reference compounds as well as natural mixtures of GEs.

Analysis of 2-MAGEs and 3-MAGEs

The chromatographic and mass spectroscopic behavior of the synthetic references 1-*O*-octadecyl-3-octadecadienoyl-*sn*-glycerol and 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol are shown in Figs 2 and 3 respectively. Similar differences in the RT values, as those observed for the two MAGE isomers in Figs 2A and 3A (0.6 min), were also observed for the other synthetic reference compounds. Thus, the HPLC system resolved the MAGE regioisomers, with the 2-MAGE isomers having the lowest RT values.

Of the six ions representing the MAGEs, the best diagnostic ions were the $[M - alkyl]^+$, $[M + H - H_2O]^+$, and $[M + H]^+$ ions, since these abundant ions characterize the alkyl group and molecular weight, and thereby the acyl group of the MAGE. Comparison of the spectra of 3-MAGE and 2-MAGE species reveals that the main differences are the relative ion intensities of the $[M - alkyl]^+$, $[M + H - H_2O]^+$, and $[M + H]^+$ ions; e.g. the 2-MAGEs show higher intensities of the $[M - alkyl]^+$ and $[M + H]^+$ ions, whereas the 3-MAGEs show the $[M + H - H_2O]^+$ ion as a dominating base peak (Figs 2B and 3C). Obviously, the combination of the $[M - alkyl]^+$ and $[M + H - H_2O]^+$ ions and the $[M - alkyl]^+$ and $[M + H]^+$ ions form the best pairs of diagnostic ions for the characterization of the 3-MAGE and 2-MAGE species respectively. The interpretation of spectra of 2-MAGEs containing *sn*-2 polyunsaturated fatty acids may be complicated by the increased fragmentation of water, i.e. formation of the $[M + H - H_2O]^+$ ion, relative to the diagnostic ion pair, i.e. the $[M - alkyl]^+$ and $[M + H]^+$ ions. Accordingly, the two pairs of diagnostic ions have relatively high abundances at CapEx +175 V and +120 V for the 3-MAGE and 2-MAGE species respectively, indicating the optimal CapEx voltages for regiospecific analysis of reference compounds as well as of natural mixtures of MAGEs.

Hence, regiospecific characterization of 2-MAGE and 3-MAGE species may be performed both by the chromatographic and mass spectroscopic parts of the method presented.

Analysis of DAGEs

The chromatographic and mass spectroscopic behavior of the synthetic references 1-*O*-octadecyl-2-hexadecanoyl-3-octadecadienoyl-*sn*-glycerol and 1-*O*-octadecyl-2-octadecadienoyl-3-hexadecanoyl-*sn*-glycerol are shown in Figs 4 and 5 respectively. Of the five ions representing the DAGE species, the best diagnostic ions were the $[M - alkyl]^+$, $[M - sn\text{-}2]^+$ and $[M - sn\text{-}3]^+$ ions, since these relatively abundant ions characterize the alkyl and the two acyl groups, and thereby establish the molecular weight and structure of the DAGE. Combining the three diagnostic ions with one of the molecular adduct ions, i.e. $[M + NH_4]^+$ and $[M + Na]^+$, will of course be important when characterizing DAGEs in complex mixtures. Increasing the CapEx changed the intensities of the fragment ions. At CapEx +210 V, all three diagnostic ions of DAGEs have relatively high abundances, and thus this CapEx is optimal for regiospecific analysis of reference compounds, as well as of natural mixtures of DAGEs. However, for quantification purposes, fragmentation can be completely avoided by analyzing the ether lipids at CapEx +120 V.

The positional location of the two fatty acids in DAGE did not influence the chromatographic resolution (Figs 4A and 5A). However, the resolution was affected, as expected, by the species of the two fatty acids in DAGEs, i.e. the total number of carbon atoms and double bonds. The mass spectra in Figs 4C and 5B (CapEx +210 V) revealed a positional effect, as the loss of *sn*-2 fatty acids was favored over the loss of *sn*-3 fatty acids (and also over the loss of the alkyl group). The $[M - sn\text{-}3]^+/[M - sn\text{-}2]^+$ ratios (Table 2) reveal that the loss of *sn*-2 fatty acids is, in general, favored over the loss of *sn*-3 fatty acids in the DAGE species, at least in the CapEx voltage range of +170 to +250 V.

Table 2 also reveals the effect of the fatty acid species on the fragmentation pattern, *viz*. that the loss of fatty acid is promoted with the degree of unsaturation. For instance, at CapEx +170 V, the 18:2*n*-6/16:0, 18:1*n*-9/16:0, and 16:0/18:2*n*-6 DAGE species show an increasing tendency to fragment the *sn*-3 fatty acid, relative to loss of the *sn*-2 fatty acid, indicating that the fatty acids are fragmented from the DAGE molecule in the following order: 18:2*n*-6, 18:1*n*-9, and 16:0.

Hence, the method presented may perform regiospecific characterization of DAGE species mainly by the mass spectroscopic analysis described, but also by the chromatographic retention times obtained.

Verification of structure of synthetic DAGE

The pancreatic lipase hydrolysis of the synthetic DAGE reference compounds revealed a very high regiospecificity of the synthetic DAGEs and a low degree of isomerization of 2-MAGEs into the corresponding 3-MAGEs. The fatty acid in 2-MAGE was, therefore, much more stable than in corresponding 1,2-diacyl-*sn*-glycerols, which readily isomerize to the 1,3-diacyl-*sn*-glycerols.[28] An explanation for this observation may be found in the relative stabilization of the *sn*-2 acyl group by the *sn*-1 alkyl function, which is unable to shift position.

Table 2. Calculated ratios of loss of *sn*-2 and *sn*-3 fatty acids in selected synthetic DAGE species

Fatty acid & position		CapEx(V)	$[M - sn\text{-}3]^+ / [M - sn\text{-}2]^+$ ion ratio[a]
sn-2	*sn*-3		
18:2*n*-6	16:0	+170	0.15
		+210	0.30
18:1*n*-6	16:0	+170	0.40
16:0	18:2*n*-6	+170	0.70
		+210	0.65
		+250	0.25

[a] $[M - sn\text{-}2]^+$, loss of *sn*-2-fatty acid; $[M - sn\text{-}3]^+$, loss of *sn*-3-fatty acid.

Comparison of analyses of the synthetic neutral ether lipids

For all the compounds analyzed, the intensities of the two molecular adduct ions moved in the opposite direction when the CapEx was altered. The $[M + NH_4]^+$ ion had the highest relative abundance at low CapEx, whereas the $[M + Na]^+$ ion had the highest relative abundance at high CapEx. The cationic sodium must originate from impurities in the 30% ammonium hydroxide aqueous solution, which was used as a mobile phase additive and ionizing agent. It was generally more difficult to obtain reproducible ion intensities for the ammonia molecular adduct ion than for the other characteristic ions of the ether lipid classes studied, which was probably due to loss of ammonia from the HPLC solvent reservoirs. Therefore, the variation in the relative ion intensities of the ammonia molecular adduct ions made this ion the least reliable.

The fragment ions produced from the GE, MAGE, and DAGE compounds analyzed showed some similarities, e.g. the loss of the alkyl group and the low relative abundance of this ion at low CapEx (Figs 1–5). However, DAGE was extremely difficult to ionize, in contrast to GE and MAGE.

Interestingly, loss of a water molecule or the alkyl group in MAGE was preferred for loss of the fatty acid (Figs 2 and 3). Actually, loss of fatty acids could not be verified, although the formation of acylium ions was observed in all spectra obtained at high CapEx voltages. The high polarity of the hydroxy group, compared with the long-chain fatty acid in MAGE, probably rendered it much more ionizable and thus favored loss of water. However, this does not explain why the loss of the alkyl group was also preferred for the long-chain acyl esters.

CONCLUSION

The LC/ESI/CID/MS method presented in this paper was excellent for the regiospecific analysis of synthetic neutral ether lipid reference compounds. Characteristic fragmentation patterns, full mass spectra, and diagnostic ions combined with chromatographic RTs were obtained for each of the synthetic reference compounds: GEs, 2-MAGEs, 3-MAGEs, and DAGEs. Thus, the method may prove a valuable tool in analysis of neutral ether lipids from various natural sources.

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