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Novel Galactolipids from the Leaves of *Ipomoea batatas* L.: Characterization by Liquid Chromatography Coupled with Electrospray Ionization–Quadrupole Time-of-Flight Tandem Mass Spectrometry

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Sixteen novel and ten known galactolipids have been isolated and characterized from the leaves of *Ipomoea batatas* L. (sweet potato) using an analytical method based on high-performance liquid chromatography coupled with electrospray ionization–quadrupole time-of-flight tandem mass spectrometry. Using this technique, the structures and regiochemistries of the fatty acyl groups and the positions of the double bonds on the acyl chains were determined. Sugar moieties were identified by analysis of one- and two-dimensional nuclear magnetic resonance spectra. The positions of the double bonds of polyunsaturated fatty acids were confirmed, and in some cases their geometries determined, by gas chromatography–mass spectrometry. This is the first report of galactolipids in the leaves of sweet potato.

KEYWORDS: Monogalactosyldiacylglycerol (MGDG); digalactosyldiacylglycerol (DGDG); tandem mass spectrometry; nuclear magnetic resonance spectroscopy; sweet potato; *Ipomoea batatas* L.

INTRODUCTION

Ipomoea batatas (L.) Lam. (Convolvulaceae), commonly known as sweet potato, is a tuberous-rooted herbaceous species cultivated throughout the world for its edible tubers that are either consumed raw or after cooking (*1*). Additionally, the leaves of *I. batatas* are an excellent source of bioactive anthocyanin and polyphenolic constituents (*2*) and may be used as a fresh vegetable or in the form of tea, noodles, bread, or confectionery. A recent study concerning the nutritional composition and physiological functions of the leaves suggested that the sweet potato could represent a beneficial food source by virtue of the presence of polyphenolic compounds (*2*). In this context, 15 anthocyanins and 6 polyphenolics have been identified and quantified in *I. batatas* (*2*), and the leaves are reportedly rich in vitamin B, iron, calcium, zinc, and proteins (*3, 4*).

According to a patent filed by Osamu et al. (*5*), some parts of sweet potato, especially the leaves, runners, stalks, and rhizomes, can be employed as functional foods or drinks presenting carcinostatic activities. The glycolipid fraction of the plant material was shown to suppress proliferation and to promote differentiation of cancer cells and was hence claimed to be responsible for the carcinostatic properties described (*5*).

Glycolipids are the major constituents of plastid membranes and constitute up to 80% of thylakoid membrane glycerolipids, approximately 50% of which are comprised of monogalactosyldiacylglycerols (MGDG) (*6*). Various studies have shown that glycoglycerolipids exhibit specific biological properties including antiviral (*7*), antitumor (*8–13*), and antiinflammatory (*14*) activities. Additionally, they appear to play a role in the inhibition (*15*) and promotion (*16, 17*) of cell growth and in protection against cell death (*8–13, 18*).

The objective of the present study was, therefore, the structural characterization of the galactolipids comprising the nonpolar fraction derived from leaves of *I. batatas*. In the structures of the digalactosyldiacylglycerol (DGDG) and MGDG species present in this fraction, two fatty acyl groups are attached at the *sn*-1 and *sn*-2 positions of the glycerol backbone, and one or two sugar moieties are linked to the *sn*-3 position of the glycerol via acetal linkage. In order to achieve a complete structural characterization of these galactolipids, including the regiochemistries of the fatty acyl groups and the positions of the double bonds on the acyl chains, an analytical method based on high-

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performance liquid chromatography coupled with electrospray ionization–quadrupole time-of-flight tandem mass spectrometry (HPLC-ESI/Q-TOF/MS/MS) was developed. The sugar units and their configurations were assigned using spectroscopic methods including the concerted application of one- and two-dimensional NMR techniques (^{13}C NMR, ^{1}H NMR, selective 1D-TOCSY, HSQC, and HMBC). Additionally, the positions of the double bonds of the polyunsaturated fatty acids attached to the two carbons of the glycerol galactolipid backbones were confirmed, and in some cases their geometries determined, by gas chromatography–mass spectrometric (GC-MS) analysis of the derivatized fatty acids. Using the described strategy, 16 new (**1**, **6**–**14**, **17**, **22**–**26**) and 10 known galactolipids were isolated and characterized. To the best of our knowledge, this is the first report of galactolipids in the leaves of sweet potato.

MATERIALS AND METHODS

Chemicals. HPLC grade methanol was purchased from J. T. Baker (Baker Mallinckrodt, Phillipsburg, NJ), and acetonitrile and formic acid were obtained from Carlo Erba (Milan, Italy). HPLC grade water (18 mΩ) was prepared using a Millipore (Bedford, MA) Milli-Q purification system.

Preparation of Samples. *I. batatas* (L.) Lam. (cultivar Suioh) was cultivated at the National Agricultural Research Center for the Kyusyu Okinawa Region, Japan, and a voucher specimen deposited in the herbarium at the Research Center. Fresh leaves were harvested in July 2004 and were washed, air-dried, powdered, and stored at room temperature until required for analysis.

A sample (3 g) of the powdered leaf material was extracted with 60 mL of methanol for 8 days at room temperature. The extract was filtered and the solvent removed to yield 320 mg of crude extract. In order to separate the nonpolar components from the polyphenols and anthocyanins, a methanolic solution of the extract was passed through a C18 Sep-Pak cartridge (Waters Corp., Milford, MA) that had been preconditioned with methanol, water, and 0.2% formic acid. After being washed with 0.2% formic acid and 0.2% formic acid–methanol (50:50 v/v), the nonpolar components were eluted with methanol, and the methanolic fraction (240 mg) was retained for chromatographic analyses.

HPLC-UV Analyses. The methanolic fraction from the C18 Sep-Pak separation was analyzed by HPLC using a Waters Alliance 2695 liquid chromatograph equipped with a Dual Lambda absorbance detector. Individual galactolipids were eluted isocratically from a Thermo (Bellefonte, PA) Hypersil BDS C18 column (250 × 4.6 mm i.d.; 5 μm) with a mobile phase of methanol–water–acetonitrile (90.5:7:2.5) (elution method A) at a flow rate of 1 mL/min and with detection at 205 nm. In order to improve the separation of the more polar galactolipids, a mobile phase of methanol–water–acetonitrile (82.5:15:2.5) (elution method B) was also employed.

HPLC-ESI/MS and HPLC-ESI/MS/MS Analyses. The methanolic fraction from the C18 Sep-Pak separation was analyzed by HPLC-ESI/MS "on-line" using a Waters Micro-Hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer coupled with a Waters Alliance 2695 HPLC through a Waters orthogonal Z-spray source interface. Individual galactolipids were separated using the chromatographic conditions described above. The eluate was directly injected into the electrospray ion source, and the MS^1 and MS/MS spectra were acquired in the positive ion mode and interpreted using the Mass Lynx 4.0 software provided by the manufacturer. The desolvation temperature was 180 °C, the source temperature was 100 °C, the capillary voltage was 3400 V, the sample cone voltage was 40 V, the extraction voltage was 1.5 V, and the collision cell voltage was 7 V. MS/MS spectra were obtained in the survey scan mode, using a range of collision energies between 30 and 45 V, and recorded in the *m*/*z* 50–1500 region. Galactolipids were quantified by HPLC-ESI/MS using an external standard calibration method. Samples were analyzed in triplicate and the mean and standard deviation values reported.

NMR Analyses. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker (Bruker BioSpin AG, Fällanden, Switzerland) model DRX-600 MHz spectrometer at 300 K with samples dissolved in CD_3OD (99.96% D; Sigma Aldrich, Milan, Italy) and calibrated using the solvent signal as internal standard (^{1}H, δ = 3.34 ppm; ^{13}C, δ = 49.0 ppm).

Preparation of Fatty Acid Methyl Esters. Galactolipids, previously purified by HPLC, were subjected to direct transesterification by heating at 80 °C for 4 h in glass tubes together with 2.5 mL of 2% sulfuric acid in methanol. After reaction, the tubes were allowed to cool, 2.0 mL of water and 1.0 mL of *n*-hexane were added, and the tubes were sealed and vortex-mixed for 10 min. The tubes were centrifuged for 1 min at 4000 rpm in order to accelerate phase separation, and the *n*-hexane phase was transferred to GC vials. A further volume (1.0 mL) of *n*-hexane was added to the aqueous phase, and a further extraction was carried out as described above: this step was repeated twice.

GC-MS Analyses. Fatty acid methyl esters were analyzed using a Waters GCT orthogonal acceleration time-of-flight mass spectrometer coupled with an Agilent Technologies (Palo Alto, CA) GC6890 series GC system fitted with a DB-1 capillary column (30 m × 0.25 mm i.d; 0.25 μm film thickness; J&W Scientific, Folsom, CA). The carrier gas was helium at a flow rate of 1 mL/min, sample injection was performed in the splitless mode with an injector temperature of 280 °C, the ion source temperature was 200 °C, and the GC-MS interface temperature was 300 °C. The oven temperature was initially held at 40 °C for 5 min, then increased to 60 °C at 4 °C/min, to 150 °C at 15 °C/min, to 280 °C at 3 °C/min, and finally to 300 °C at 20 °C/min; the total run time was 60 min. Mass spectra were recorded at 70 eV ionization energy in the 50–500 mass range with a scan time of 0.5 s.

RESULTS

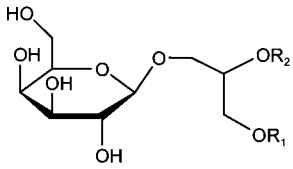
HPLC-UV Analyses. In order to optimize chromatographic conditions, and to isolate pure compounds for NMR and GC-MS analyses, a preliminary HPLC-UV separation was carried out on the nonpolar fraction of the leaf extract of *I. batatas* eluted from the C18 Sep-Pak cartridge. While HPLC with elution method A (as described in Materials and Methods) gave a good separation of the more nonpolar compounds, it was not appropriate for separating the less nonpolar components. For this reason, elution method B, employing a different ratio of organic solvent to water in the mobile phase, was developed. Employing these elution methods, a total of 26 compounds were separated and collected, 16 being obtained with elution method A and 10 with elution method B (see **Tables 1** and **2**).

NMR Analyses. The occurrence of glycerolipid structures was established from the ^{1}H NMR spectra of the purified compounds in which the proton signals associated with H-2 of the D-glycerol units exhibited a characteristic resonance at δ 5.29 indicating a C-2 linkage to an *O*-acyl group (*19*). Further analyses of the sugar regions of the spectra indicated that the compounds were galactolipids, and two groups of molecules (i.e., MGDG and DGDG) could be identified differing only in the number of galactopyranose units O-linked to the di-*O*-acylglycerol backbone.

With respect to the first group of molecules, analysis of the ^{1}H NMR and selective 1D-TOCSY spectra revealed the presence of one β-D-galactopyranosyl unit. β-Glycosylation of the glycerol moiety was confirmed by the low- and high-field chemical shifts exhibited by the anomeric carbon C1′ (δ 105.0) and proton H1′ (δ 4.26), respectively, as well as by the large $^{3}J_{H1'-H2'}$ coupling constant (J = 7 Hz). Moreover, the small coupling constant of the proton H4′ (δ 3.86, J = 2.5 and 4.0 Hz) in the sugar residue, together with the large value of the $^{3}J_{H2'-H3'}$ coupling constant (J = 9 Hz), verified that the sugar unit was galactose.

In the case of the second group of molecules, examination of the anomeric region of the HSQC spectrum showed that the saccharide portion consisted of two sugars. The selective 1D-

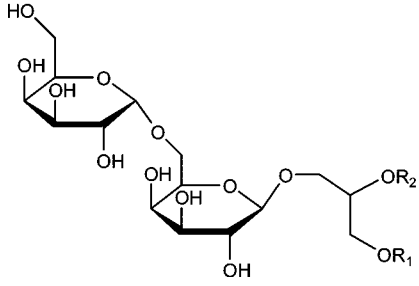
Table 1. Fatty Acid Composition and Quantitative Amounts of MGDG Compounds Isolated from *I. batatas* Leaves



	t_R	M	M+Na$^+$	R_1	R_2	mg g^{-1} plant*
1	69.18[a]	752.54[b]	775.53	16:0	18:3 (*cis* n-3)	3.01± 0.31
2	64.35[a]	778.56	801.55	18:2 (*cis* n-6)	18:2 (*cis* n-6)	2.19± 0.24
3	47.53[a]	776.54	799.53	18:2 (*cis* n-6)	18:3 (*cis* n-3)	5.99±0.57
4	55.77[a]	780.57	803.56	18:3 (*cis* n-3)	18:0	5.08±0.49
5	36.05[a]	774.53	797.52	18:3 (*cis* n-3)	18:3 (*cis* n-3)	11.88±0.67
6	73.35[c]	790.56[b]	813.55	18:3 (*cis* n-3)	19:2	6.89±0.57
7	67.47[c]	790.56[b]	813.55	19:2	18:3 (*cis* n-3)	0.71±0.06
8	17.29[a]	812.64[b]	835.63	20:0	18:1 (*cis* 9)	0.80±0.07
9	16.63[a]	810.62[b]	833.61	20:0	18:2 (*cis* n-6)	0.65±0.05
10	14.26[a]	808.61[b]	831.59	20:1(*trans* n-9)	18:2 (*cis* n-6)	1.85±0.16
11	71.59[c]	806.59[b]	829.58	20:1(*trans* n-9)	18:3 (*cis* n-3)	11.92±0.69
12	15.45[c]	822.62[b]	845.61	20:1(*trans* n-9)	19:2	0.63±0.05
13	15.97[c]	838.65[b]	861.64	20:1(*trans* n-9)	20:1(*trans* n-9)	2.17±0.23
14	15.32[c]	822.62[b]	845.61	21:0	18:3 (*cis* n-3)	0.17±0.01

[a] According to elution method A. [b] Novel galactolipid. [c] According to elution method B. * The results are reported as mean ± standard deviation.

Table 2. Fatty Acid Composition and Quantitative Amounts of DGDG Compounds Isolated from *I. batatas* Leaves



	t_R	M	M+Na$^+$	R_1	R_2	mg g^{-1} plant*
15	71.87[a]	916.61	939.60	16.0	18:2 (*cis* n-6)	1.89±0.17
16	53.90[a]	914.59	937.58	16:0	18:3 (*cis* n-3)	4.66±0.45
17	16.33[a]	930.63[b]	953.62	16:0	19:2	0.43±0.03
18	97.78[a]	942.63	965.62	18:0	18:3 (*cis* n-3)	0.56±0.04
19	52.64[a]	940.61	963.60	18:2 (*cis* n-6)	18:2 (*cis* n-6)	0.72±0.06
20	37.16[a]	938.59	961.58	18:2 (*cis* n-6)	18:3 (*cis* n-3)	1.20±0.13
21	27.53[a]	936.58	959.57	18:3 (*cis* n-3)	18:3 (*cis* n-3)	6.26±0.53
22	53.73[c]	952.61[b]	975.60	19:2	18:3 (*cis* n-3)	2.21±0.21
23	15.19[a]	946.66[b]	969.65	20:1(*trans* n-9)	16:0	1.04±0.11
24	53.08[c]	968.64[b]	991.63	20:1(*trans* n-9)	18:3 (*cis* n-3)	3.25±0.33
25	13.10[c]	984.67[b]	1007.66	20:1(*trans* n-9)	19:2	0.22±0.01
26	12.62[c]	1000.70[b]	1023.69	20:1(*trans* n-9)	20:1(*trans* n-9)	0.20±0.01

[a] According to elution method A. [b] Novel galactolipid. [c] According to elution method B. * The results are reported as mean ± standard deviation.

TOCSY spectra indicated the presence of one α-D-galactopyranosyl unit and one β-D-galactopyranosyl unit. The anomeric signal at δ 4.28 (J = 7.3 Hz), together with the proton signals of H4′ at δ 3.91 (J = 2.5 and 4.0 Hz) and of H3′ at δ 3.53 (J = 9 Hz), established that the β-galactopyranose sugar was linked to the D-glycerol moiety. The anomeric signal at δ 4.90 (J = 3.6 Hz), together with the proton signals of H2″ at δ 3.82 ($^3J_{H1''-H2''}$ = 3.6 Hz, $^3J_{H2''-H3''}$ = 9.5 Hz) and of H4″ at δ 3.94 (J = 2.5 and 4.0 Hz), implied the presence of an α-galactopyranose sugar. Finally, the HMBC spectrum revealed the crucial correlation between C1″ of the external α-galactose and the geminal H-6′ protons of β-galactopyranose, thus establishing a (1″→6′)-O-glycosidic linkage.

Within each group of galactolipids, the individual components differed only with respect to the length of the acyl chains of the fatty acids, their degree of unsaturation, and the positions of the double bonds. These structural features were not immediately definable through examination of the NMR data, and hence more detailed analyses were performed in order unambiguously to identify each galactolipid and to determine the regiochemical distribution of the acyl chains.

GC-MS Analyses of Fatty Acids. With the aim of identifying the fatty acids linked to the glycerol backbone, the pure galactolipids previously isolated by HPLC were subjected to direct transesterification, and the fatty acid methyl esters were analyzed by GC-MS. The results indicated the presence of palmitic (C16:0), stearic (C18:0), oleic (C18:1 n-9 *cis*), linoleic (C18:2 n-6, 9 *cis*), α-linolenic (C18:3 n-3, 6, 9 *cis*), nonadecadienoic (C19:2), arachidic (C20:0), 11-eicosenoic (C20:1 n-9 *trans*), and heneicosanoic (C21:0) acids, and the distribution of these acids in each HPLC fraction is reported in **Tables 1** and **2**. The identities of all fatty acids (except for C19:2) were confirmed by comparison of their retention times with those of corresponding standards, and in some cases it was possible to define the geometries of the double bonds in the polyunsaturated fatty acids. The presence of nonadecadienoic acid (C19:2) was confirmed on the basis of molecular weight and the MS fragmentation pattern (**Figure 1**), which indicated the presence of double bonds at C-10 and C-13 of the acyl chain.

HPLC-ESI/MS and HPLC-ESI/MS/MS Analyses. The methanolic fraction from the C18 Sep-Pak cartridge was subjected to HPLC-ESI/MS analysis in order to determine the definitive structure of each galactolipid. Elution systems with different percentages of organic phase (methods A and B as described in Materials and Methods) were used to separate galactolipids with diverse polarities. **Figure 2** presents the HPLC-ESI/MS profile of the nonpolar fraction from leaves of *I. batatas* obtained using elution method A.

Analyses were carried out in the presence or absence of 0.2% formic acid in the mobile phases in order to investigate the effect of acidic conditions on the separation of the galactolipids and on their behavior in the electrospray source. The positive ion HPLC-ESI/MS of the galactolipid-enriched methanolic fraction showed different MS profiles according to the acidic strength

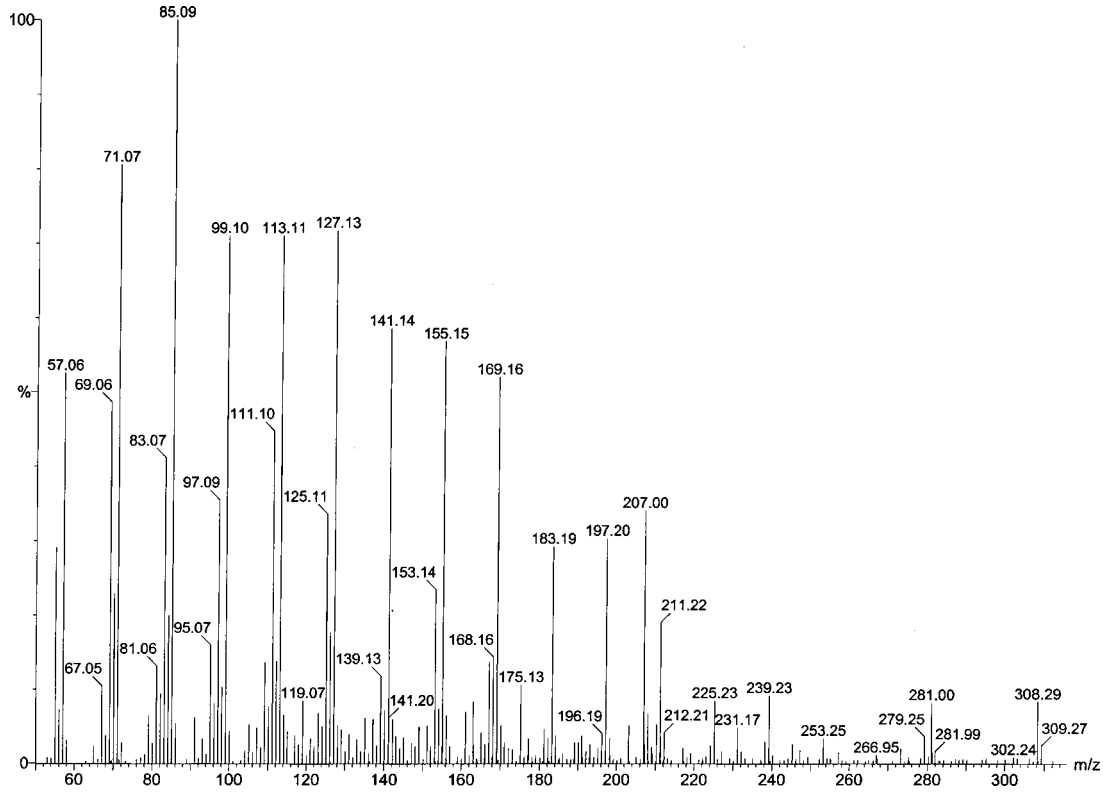


Figure 1. Electron impact mass spectrum of methyl nonadecadienoate (*m*/*z* 308) derived from compound **22** by transesterification.

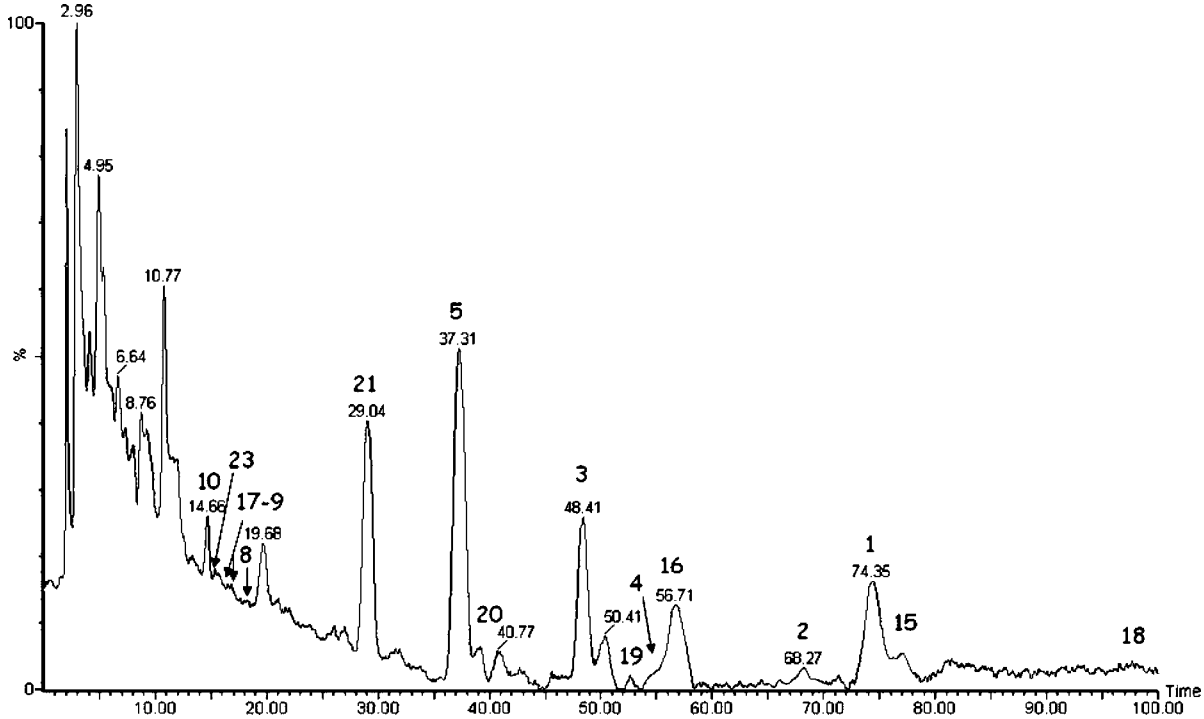


Figure 2. HPLC-ESI/MS analysis (using elution method A) of the methanolic extract of leaves of *I. batatas*. The more polar galactolipids (compounds **6**, **7**, **11**–**14**, **22**, **24**–**26**) were eluted in the first 13 min.

of the eluent employed. Mass spectra acquired following acidic elution displayed, in addition to $[M + Na]^+$ ions, fragment ions at $[M + H - 162]^+$ and $[M + H - 180]^+$ associated with the preferred loss of a galactosyl moiety. Additionally, in-source collision activation of these species produced further fragmentation arising from the neutral loss of a fatty acyl group as the free fatty acid from the diacylglycerol structure, formally represented as $[R_xCO + 74]^+$ ions. Under the experimental conditions employed, simple $[R_xCO]^+$ ions were also present in which R_1 and R_2 indicate the fatty acyl groups linked at the *sn*-1 and *sn*-2 positions of the glycerol backbone, respectively. In contrast, spontaneous in-source fragmentation was not preferred under nonacidic conditions, and the mass spectra displayed only $[M + Na]^+$ ions with minor signals originating from $[M + H - 162]^+$ and $[M + H - 180]^+$ fragments.

These results confirmed the fatty acid composition of each galactolipid, as already established by GC-MS analyses, and indicated variously the presence of C16:0 (palmitic or hexadecanoic acid), C18:0 (stearic or octadecanoic acid), C18:1 (octadecenoic acid), C18:2 (octadecadienoic acid), C18:3 (octadecatrienoic acid), C19:2 (nonadecadienoic acid), C20:0 (arachidic or eicosanoic acid), C20:1 (eicosenoic acid), and C21:0 (heneicosanoic acid). Unfortunately, these analyses did not allow the regiochemical distribution of each fatty acid to be established.

The positional distribution of the acyl chains in the galactolipids could be determined from the intensities of the peaks produced by the loss of carboxylic acids linked to the glycerol backbone, in agreement with previous literature. Thus, Guella et al. (*20*) reported that the loss of the carboxylic acid linked to the *sn*-1 glycerol position always produced a more intense peak than that derived from the loss of the *sn*-2-linked acyl chain. In the present case, consideration of the relative intensities of the $[M + Na - R_1CO_2H]^+$ and the $[M + Na - R_2CO_2H]^+$ fragments derived from the $[M + Na]^+$ precursor ion in the HPLC/ESI/MS/MS experiments enabled the regiochemical distribution of the acyl chains to be determined and the galactolipids occurring in the mixture to be identified (**Tables 1** and **2**).

The HPLC-ESI/MS/MS data recorded for these compounds under the analytical conditions described in Materials and Methods showed a preferred pathway of fragmentation leading to the formation of $[M + Na - 162]^+$ ions together with product ions originating from the fragmentation of the galactose moiety. Moreover, since DGDG compounds contained two galactosyl units, product ions associated with the loss of the second sugar from $[M + Na - 162]^+$ ions were also detectable in the MS/MS spectra. Additionally, spectra obtained under neutral conditions from both groups of galactolipid displayed primarily the loss of the neutral fatty acid giving rise to $[M + Na - R_1CO_2H]^+$ and $[M + Na - R_2CO_2H]^+$ fragments.

Determination of the degree of unsaturation and of the position of the double bonds in the fatty acids on the galactolipid acyl chains was achieved by analysis of the MS/MS spectra in the high-mass regions. In general, for the higher series of MGDG and DGDG compounds, i.e., those with at least one C20:0, C20:1, or C21:0 fatty acid linked to carbons of the glycerol backbone, more complex MS/MS spectra were obtained.

In the case of compound **11,** which eluted (method B) at a retention time of 71.59 min, the HPLC-ESI/MS exhibited a peak at *m/z* 829.9 corresponding to the sodium adduct of an MGDG containing eicosenoic (C20:1) and linolenic (C18:3) acids linked to the glycerol backbone. In the corresponding MS/MS spectrum (**Figure 3**), two abundant product ions were observed at *m/z* 519.6 and 551.5, referred to as G_1 and G_2, respectively, according to the nomenclature used by Kim et al. (*21*). These ions resulted from the neutral loss of the fatty acids, C20:1 and C18:3, respectively (**Figure 3**), thus allowing the fatty acid composition to be defined. The regiochemistry of the MGDG molecule could also be established from the relative intensities of the $[M + Na - R_xCO_2H]^+$ ions, on the basis of which the *sn*-1 and *sn*-2 positions were ascribed to the eicosenoic (C20:1) and linolenic (C18:3) fatty acyl chains, respectively.

In agreement with a previous report (*21*), fragment ions originating from the sugar moiety were detected in the low-mass region. Thus, cleavage of the glycosidic bond generated the sodium adduct ions at *m/z* 185.1 (B) and 203.1 (C), in which the sugar ring lost or retained the hydroxyl group, respectively (**Figure 3**). The fragment ion at *m/z* 243.2 (E) was correlated with the concomitant cleavage of the two neutral fatty acids from the galactosylglycerol backbone. In addition, fragment ions at *m/z* 261.3, 293.2, and 335.4, previously indicated as $[R_2CO]^+$, $[R_1CO]^+$, and $[R_2CO + 74]^+$ product ions, were also present. The sodium adduct ions at *m/z* 301.3 ($^2D_{1,2}$) and 315.3 ($^3D_{1,2}$) were formed by the simultaneous neutral losses of one fatty acid and a part of the other fatty acid, respectively, according to the fragmentation pathways reported in **Figure 3**.

Information about the position of the double bonds on the fatty acyl chains could be obtained from the analysis of the high-mass region of the MS/MS spectrum. In the typical fragmentation pathway of a saturated fatty acyl chain, only ions arising from the neutral losses of C_nH_{2n} and C_nH_{2n+2} from the parent ion can be detected, with the neighboring peaks in the series being separated by 14 units. However, for unsaturated acyl chains, neutral losses of C_nH_{2n-2} are also reported, while the presence of a double bond in the chain reduces the neighboring peak separation to 12 units (*21*). In the case of compound **11**, detailed analysis of the high-mass region of the MS/MS spectrum of the $[M + Na]^+$ ion at *m/z* 829.9 revealed a peak at *m/z* 811.9 associated with the $[M + Na - 18]^+$ ion and two other peaks at *m/z* 783.8 and 771.8 resulting from the above-mentioned losses. The latter two peaks indicated that the first double bond was located at C-15 of the *sn*-2 fatty acyl chain, in agreement with data obtained from the GC-MS analyses. From a consideration of the separation (i.e., 12 or 14 units) between neighboring peaks in the mass range from *m/z* 771.8 to *m/z* 551.5, it was possible to locate the double bonds on the *sn*-2 acyl group at C-9 and C-12, confirming the identity of 9,12,15-octadecatrienoic acid, and the double bond on the *sn*-1 acyl chain at C-11, confirming the presence of 11-eicosenoic acid, both assignments being in agreement with GC-MS analysis.

For compound **24**, which eluted (method B) at a retention time of 53.08 min, the HPLC-ESI/MS exhibited a peak at *m/z* 991.9 corresponding to the sodium adduct of a DGDG compound. This component presented the same pattern of fatty acids as the MGDG species analyzed above, but with two galactosyl units linked to the *sn*-3 position of the glycerol backbone. Moreover, the MS/MS spectrum of **24** (**Figure 4**) showed a fragmentation pattern that was very similar to that described for the associated MGDG. The relative abundance of the two product ions, $[M + Na - R_1CO_2H]^+$ and $[M + Na - R_2CO_2H]^+$, at *m/z* 681.7 (G_1) and 713.7 (G_2), respectively, was informative about the regiochemical distribution and the composition of the two fatty acids present in the molecule. It was concluded that this DGDG presented a 20:1 acyl chain at the C-1 glycerol position and an 18:3 acyl chain at the C-2 position. A detailed study of the fragmentation pattern in the high-mass region verified the presence of one double bond at C-11 of the *sn*-1 fatty acyl chain, establishing the presence of 11-eicosenoic acid, and of three double bonds at C-9, C-12, and C-15 of the *sn*-2 acyl chain, corresponding to 9,12,15-octadecatrienoic acid, both assignments being in agreement with GC-MS analysis.

The presence in the MS/MS spectrum of **24** of two fragment ions at *m/z* 829.9 and 667.7, originating from two sequential neutral losses of 162 units associated with each galactose moiety, verified that the structure of DGDG was related to that of MGDG **11**. Analysis of the low-mass region of the MS/MS spectrum of **24** provided additional information confirming this result. In particular, cleavage of the internal glycosidic bond produced the sodium adduct of the digalactosyl ion at *m/z* 363.4 ($C_2 - 2H$) and a sodium adduct ion at *m/z* 347.4 (B_2) arising from the loss of the hydroxyl group linked to the 1′ position of the 6-O-substituted galactose (**Figure 4**). Additionally, a sodium

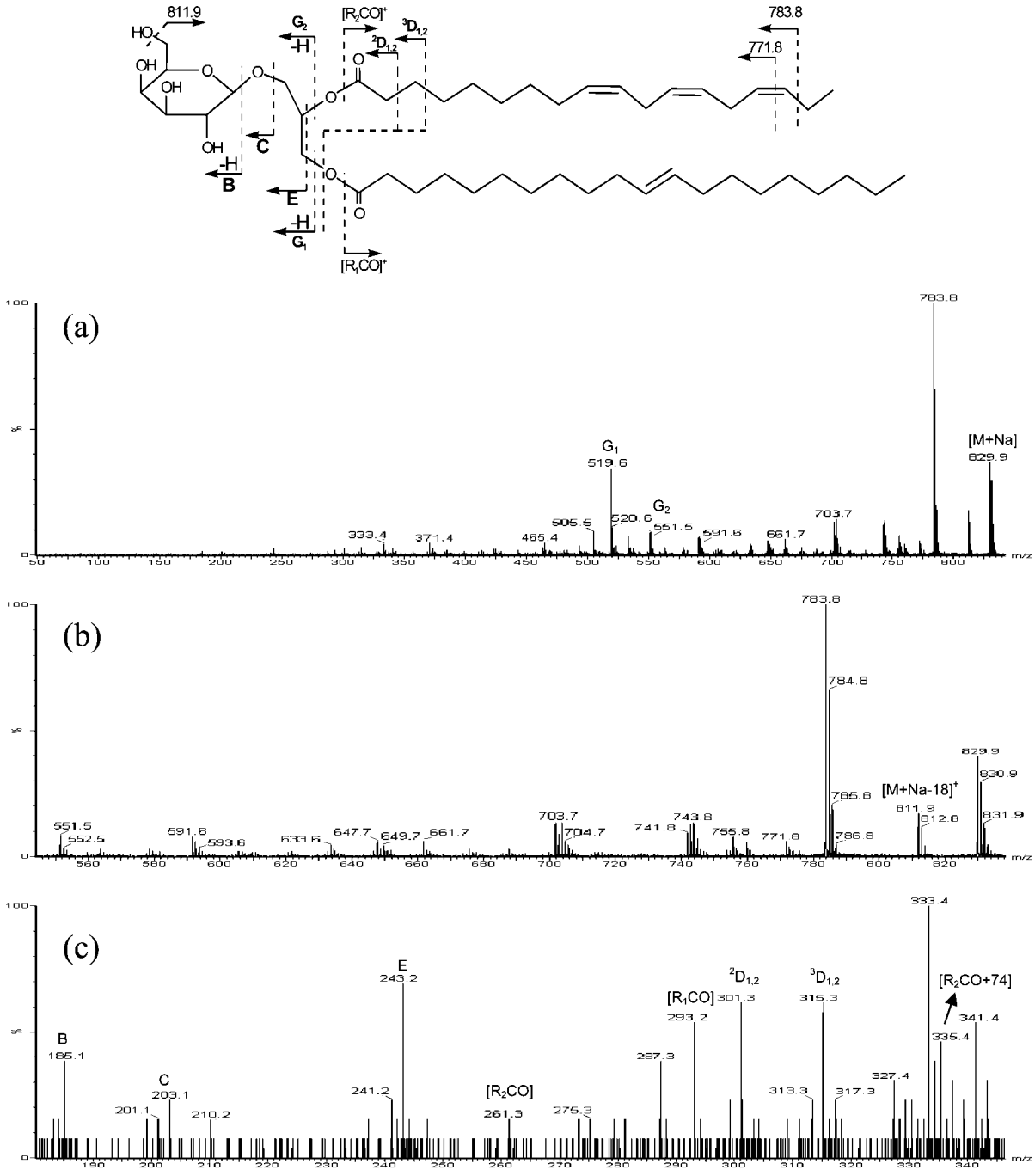


Figure 3. Positive HPLC-ESI/MS/MS spectrum and fragmentation pathway of the $[M + Na]^+$ ion at *m/z* 829.9 derived from MGDG compound **11** showing the total spectrum (a), the high-mass region (b), and the low-mass region (c).

adduct ion at *m/z* 405.5 (E) was generated by the simultaneous cleavage of the two neutral fatty acids, while further sodium adduct ions at *m/z* 463.5 ($^2D_{1,2}$), 477.5 ($^3D_{1,2}$), *m/z* 533.7 ($[M + Na - 162 - R_1CO_2H]^+$) and 551.7 ($[M + Na - 162 - R_2CO_2H]^+$) originated from the fragmentation pathway depicted in **Figure 4**. Finally, the MS/MS spectrum also displayed ions at *m/z* 293.3, 335.5, and 367.1 corresponding respectively to the fragment ions $[R_1CO]^+$, $[R_2CO + 74]^+$, and $[R_1CO + 74]^+$.

The MS/MS spectra obtained for the galactolipids belonging to the lower series of MGDG and DGDG compounds, i.e., those characterized by C16:0, C18:0, C18:1, C18:2, C18:3, and C19:2 fatty acids linked to the glycerol backbone, were less informative than those recorded for the higher series. Nevertheless, comparison of the mass spectra of the higher galactolipids with those belonging to the lower series permitted the structural characterization of the latter. According to this methodology, and applying the two elution methods (A and B) in order to improve the separation of galactolipids with different polarities, it was possible to identify all galactolipids present in the analyte mixture in terms of fatty acid composition, regiochemistry, and position of double bonds on the respective acyl chains (**Tables 1** and **2**). The pure isolated galactolipids were quantified by HPLC-ESI/MS, and the results obtained are reported in **Tables 1** and **2**. From these data it was estimated that MGDG compounds comprised approximately 70% of the total amount of galactolipids present in the nonpolar fraction.

DISCUSSION

The present study has demonstrated that galactolipids are accumulated in the leaves of sweet potato, and 16 novel

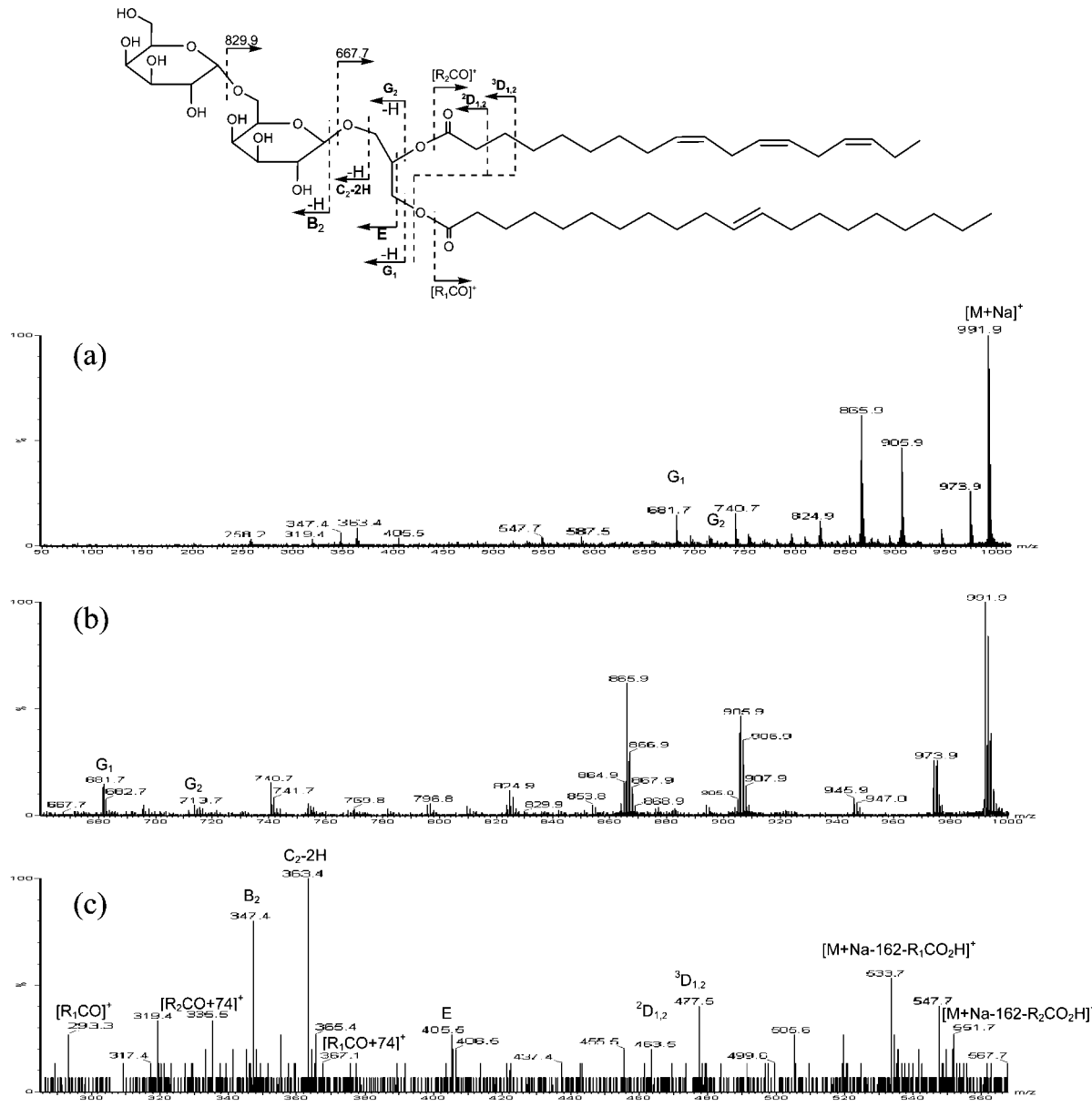


Figure 4. Positive HPLC-ESI/MS/MS spectrum and fragmentation pathway of the $[M + Na]^+$ ion at *m*/*z* 991.9 derived from DGDG compound **24** showing the total spectrum (a), the high-mass region (b), and the low-mass region (c).

galactolipids (**1**, **6**–**14**, **17**, **22**–**26**) together with 10 known galactolipids were unambiguously identified (**Tables 1** and **2**) by detailed spectrometric analysis.

Two main classes of MGDG compounds are found in higher plants (*22*). In the first (and major) class, C18:3 fatty acids are located at both the *sn*-1 and *sn*-2 positions of the glycerol backbone: such C18:3/C18:3 galactolipids are found in all eukaryotic lipids. In the second class, C18:3 fatty acids are located at the *sn*-1 position, and a C16:3 fatty acid is present exclusively at the *sn*-2 position of the glycerol: these C18:3/C16:3 galactolipids are similar in structure to the glycerolipids of the cyanobacteria and are referred to as prokaryotic type. Almost all plants studied so far contain MGDG compounds with the eukaryotic (C18:3/C18:3) structure, and some plants, such as pea or cucumber, contain only eukaryotic MGDG compounds and are known as "18:3 plants". On the other hand, species of several genera including *Spinacia* and *Arabidopsis* also contain prokaryotic (C18:3/C16:3) MGDG compounds and are known as "16:3 plants" (*22*). Following this classification, *I. batatas* should be defined as an 18:3 plant on the basis of the fatty acid composition of the MGDG and DGDG spoecies present in the leaves.

The abundance of galactolipid compounds characterized by high contents of α-linolenic (C18:3 n-3, 6, 9 *cis*) and linoleic (C18:2 n-6, 9 *cis*) acids explains, to some extent, the potential interest in *I. batatas* as a nutraceutical plant. α-Linolenic and linoleic acids are essential fatty acids that serve as precursors for all n-3 and n-6 fatty acids, respectively. The essential nature of α-linolenic acid has been well demonstrated (*23*, *24*), and it is known that deficiency leads to anomalies in the composition of nerve membranes (*25*, *26*) and in their architecture and function (*27*, *28*). Also noteworthy is the presence (confirmed by GC-MS analysis) in sweet potato of the fatty acids C21:0 and C19:2, since these are rarely reported as constituents of plant lipids.

The successful isolation and characterization of the galactolipid classes in leaves of *I. batatas* was achieved in the present study by developing an adapted extractive method and an

analytical strategy based on mass spectrometry and NMR. Several mass spectrometric approaches have been applied in the determination of the structures of the fatty acyl groups in glycerolipids. Thus, Orgambide et al. (*29*) employed methanolysis of glycerolipid mixtures, followed by GC-MS analysis of the fatty acid methyl ester derivatives, to determine fatty acid composition in *Rhizobium leguminosarum*. An alternative strategy used the collision-induced dissociation (CID) spectral pattern of the carboxylate ion ($RCOO^-$) obtained by negative ion fast atom bombardment mass spectrometry (FAB/MS) of glycerolipids in order to determine the structures of the fatty acids (*30, 31*). However, using these methods it was not possible to assign the detected fatty acid methyl ester derivatives or the free carboxylate ions to the associated galactolipids present in the mixture or to ascertain their positions on the glycerol backbone. In contrast, Kim et al. (*32*) were able to characterize the structures of the MGDG and DGDG species in wheat flour from the positive ion CID spectra of the sodium adduct ions $[M + Na]^+$ of galactolipids desorbed by FAB ionization. Although it was possible to define the positions of the double bonds on the acyl chains using this technique, the experiment was carried out on pure compounds and thus does not represent a reliable method for the analysis of mixtures. Moreover, while electrospray ionization–quadrupole ion trap tandem mass spectrometry coupled with HPLC has been applied to the regiochemical determination of the acyl chains on the glycerol backbone of MGDG and DGDG compounds, this approach is not suitable to clarify the positions of the double bonds on the acyl chains (*20, 33*).

In the present study, a combination of two different mass spectrometric techniques permitted the determination of the fatty acid composition of each galactolipid compound by providing information about acyl chain length, regiochemical distribution, and positions of the double bonds. In this way, all of the structural features of the galactolipids present in the leaves of *I. batatas* were elucidated. The present study describes the first application in this field of ESI/Q-TOF/MS combined with HPLC.

As already discussed, galactolipids differing only in the length of their acyl chains showed two different types of MS/MS fragmentation patterns. In order to explain this unusual behavior, a preferential conformational state for the higher series of galactolipids is postulated, perhaps involving a relaxed form of the acyl chains that renders them more prone to fragmentation than their shorter counterparts. The apparent complexity of the high mass region in the relative MS/MS spectra of these galactolipids can, in fact, be simply resolved, allowing the positions of the double bonds on the acyl chains to be determined. These results, in turn, permitted the full characterization of the lower galactolipid series by comparison of their fragmentation patterns with those obtained for the higher series.

ABBREVIATIONS USED

1-D, one dimensional; CD_3OD, deuterated methanol; DGDG, digalactosyldiacylglycerol; GC-MS, gas chromatography–mass spectrometry; HMBC, heteronuclear multiple bond correlation; HPLC-ESI/Q-TOF/MS/MS, high-performance liquid chromatography coupled with electrospray ionization–quadrupole time-of-flight tandem mass spectrometry; HSQC, heteronuclear single-quantum correlation; MGDG, monogalactosyldiacylglycerol; NMR, nuclear magnetic resonance; TOCSY, total correlated spectroscopy; UV, ultraviolet.

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