Electrospray ionization/mass spectrometry compatible reversed-phase separation of phospholipids: piperidine as a post column modifier for negative ion detection

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Abstract

An electrospray ionization (ESI) compatible separation of phospholipids (PL), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylcholine (PC), was performed on a C18 column by reversed phase High Performance Liquid Chromatography (HPLC) with minimal ESI suppression. The mobile phase, used isocratically, consisted of methanol and water. ESI was used to efficiently transfer the ions present in solution to the gas phase for mass spectrometric (MS) detection. Formation of negative ions was reinforced by incorporating piperidine post column. Limits of detection (LOD) and limits of quantitation (LOQ) were experimentally determined to be 20 and 60 fmol/μl, respectively, when acquiring data in the selected ion monitoring (SIM) mode monitoring three ions with a single quadrupole MS. When acquiring data from m/z 110–900 in the scanning mode, the LOD and LOQ were experimentally determined to be 1 pmol/μl and 3 pmol/μl. When acquiring product ion spectra for m/z 747, the LOD and LOQ were experimentally determined to be 446 attomol/μl and 1.3 fmol/μl, respectively.   © 2000 Elsevier Science B.V. All rights reserved.

1. Introduction

Phospholipids make up a major part of cell membranes and their metabolic lability to endogenous and exogenous phospholipase make them excellent indicators for viability in the determination of biomass (Ringelberg et al., 1997), active community composition (White et al., 1996), nutritional/physiological status (White, 1995), and in defining end points for bioremediation (White et al., 1998). PL contain acyl groups at the *sn*-1 and *sn*-2 position and a polar head group containing a phosphate ester at the *sn*-3 position. The two acyl groups range from saturated, cyclic, hydroxy, to unsaturated fatty acids. The nature of these acyl groups from the total phospholipid fraction is generally obtained without separation by polar head group and has been utilized

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in defining the microbial community composition (White et al., 1996).

Numerous methods for separating PL have been described. Polarity differences have been exploited in normal phase (NP) chromatography to separate PL into classes (Christie, 1985; Christie et al., 1987; Becart et al., 1990; Caboni et al., 1994). More recently, reversed phase (RP) chromatography on non-traditional stationary phases has been employed for the separation of PL. An octadecanoyl poly (vinyl alcohol) phase (Abidi and Mounts, 1997) has been used for the separation of nitrogenous phospholipids, and a perfluorinated stationary phase has been used for the separation of PC, cholesterol, and their degradation products (Miguel et al., 1999).

Phospholipids do not contain strongly adsorbing structural components for easy spectrometric detection. Whereas frequent methods to separate PL have been published, detection has been mainly by light scattering (Christie, 1985; Christie et al., 1987; Becart et al., 1990; Caboni et al., 1994; Abidi and Mounts, 1997). Detection of phospholipids by MS has only been developing recently. Fast atom bombardment (FAB) was one of the first MS techniques used to ionize PL for MS analysis (Heller et al., 1988; Cole and Enke, 1991). Matrix-assisted laser desorption ionization (MALDI) or Fourier transform ion cyclotron resonance mass spectrometry (FTMS) have also been used for structural analysis of PL (Marto et al., 1995). More recently PL have been analyzed by ESI/MS (Smith et al., 1995; Black et al., 1997; Fang and Barcelona, 1998). For ESI to be effective, the mobile phase composition must be compatible with the ESI process. Buffers must be highly volatile and salt content must be kept low to not suppress ESI.

The most sensitive method for detecting PL utilizes the negative ion mode. To do this, an alkaline modifier must be added that is compatible both with the column support and does not suppress ESI. This paper will demonstrate the effectiveness of piperidine as a post column modifier for the analysis of PL in the negative ion mode, after a rapid separation of PL into three distinct classes on a reverse phase C18 column. Detection of PL was performed with both a single and triple quadrupole instrument to compare sensitivity differences.

2. Materials and methods

2.1. Samples

Brain and egg yolk derived PL standards, PG (P0514), PE (P9137), PC (P6638), synthetic PG *sn*-1/*sn*-2 16:0/18:1 (P6956), PG *sn*-1/*sn*-2 16:0/18:2 purified from (P0514) were purchased from Sigma (St. Louis, MO). Standards were prepared at 10 ppm in methanol and diluted accordingly. All solvents were HPLC grade. Water was obtained from an in-house source of Millipore water (Millipore, Bedford, MA). Aqueous NH_4OH (30%) was purchased from Fisher Scientific (Pittsburgh, PA). The soil samples were extracted by a modified Bligh and Dyer extraction and fractionated into general lipid classes (neutral lipid, glycolipid, and polar lipid) by silicic acid column chromatography as previously described (White and Ringleberg, 1998).

2.2. HPLC

Separation of the PL was carried out on an HP 1100 HPLC (Agilent Technologies, Sunnyvale, CA). The mobile phase consisted of 95/5 (methanol containing 0.002% piperidine/water v/v). A HAISIL HL (Higgins Analytical, Mountain View, CA) column, 30 mm×1 mm×3 μ was used for the separation. The column flow rate was 50 μl/min, while 0.02% piperidine in methanol was added post column through a zero dead volume tee at a flow rate of 10 μl/min by Harvard ''33'' Dual Syringe Pump (Harvard Apparatus, Holliston, MA).

2.3. Mass spectrometry

Electrospray mass spectra were obtained using both a VG Platform II single quadrupole MS and a VG Quattro II triple quadrupole MS. Instrument tuning was performed utilizing a synthetic PG standard. Calibration of the Platform II was performed in the negative ion mode with a solution of sodium iodide/cesium iodide. Samples were introduced into the Platform II ESI source at a total flow rate of 60 μl/min. The electrospray capillary was operated at −2.78 kV. The counter electrode was operated at 0.41 kV. The cone was set to −80 V, while the

skimmer lens offset was set to 5 V. The source was operated at 100°C. The calibration of the Quattro II was performed using a solution of horse heart myoglobin in the positive ion mode. Samples were infused into the Quattro II at a flow rate of 20 μl/min. Capillary voltage was optimized at 2.5 kV, counter electrode at 0.50 kV, cone at −80 V and skimmer lens offset to 5 V. The collision energy was optimized at 45 V and the collision cell gas pressure was set to 3.7×10^{-3} mbar. The collision gas was argon.

3. Results and discussion

Valuable information is gained when separating PL into classes (Fang and Barcelona, 1998). The three PL classes utilized for this assay include PG (found in both Gram-negative and Gram-positive bacteria), PE (found largely in Gram-negative bacteria), and PC (found in eukaryotes, i.e. skin, fungi, protozoa and in rare bacteria) (Ratledge and Wilkinson, 1988). Many of the classical methods that have been utilized for the separation of PL have had admitted problems (Becart et al., 1990; Caboni et al., 1994). Additions of NH_4OH as a modifier directly to the solvent reservoir induced backpressure from stationary phase degradation, and retention times decreased. This is due to the addition of NH_4OH to the mobile phase to increase pH. Increased pH dissolves the silica in the analytical column. In the work reported by Becart et al. (1990) a silica saturator column was used to protect the analytical column from the high pH resulting from use of NH_4OH. This extended the lifetime of the column, however, due to the silanol activity, the symmetry factor for PE was not acceptable. When the NH_4OH was added post column to avoid the backpressure increases, the tailing was even more pronounced. An amine-containing compound (triethylamine) was added to ''compete'' for active sites, but was found to contaminate the ESI cone. Since buffers with a high salt content are not compatible with ESI, other solutions were tested. Piperidine (hexahydro-pyridine), $C_5H_{11}N$ is a strong base (pK_b at 25°C = 11.2), which would deprotonate PL seemed a logical candidate. Because it is an amine, it also competes

for active sites thus making the tailing of the PE's less noticeable. Utilization of column packing with little or no silanol activity was also desirable.

With a flow rate of 50 μl/min, the separation of the mixtures of brain and egg yolk derived authentic PL required less than 10 min (Fig. 1A). The system is operated isocratically, thereby avoiding re-equilibration time. Typically, 100% organic solvent is the preferred vehicle for ESI, but the similar retention characteristics of PE and PC required a small amount of water to differentially retain them. Both PG begins to separate into molecular species, PE separates into three clearly separated bands, whereas PC molecular species eluted as a class. Baseline resolution between PE and PC was not required as the MS readily distinguishes the PL classes. Two detection channels were recorded in one analysis, one positive (best for PC) and one in the negative ion mode (for PG and PE). These two ion currents were then overlaid for confirmation.

Many ESI spectra are devoid of fragment ions. When obtaining ESI spectra of PL using a single quadrupole instrument, in-source collision-induced dissociation (CID) is used to fragment the molecule. Fragmentation is induced in the higher-pressure regions between the source and the quadrupole. These ions are accelerated between the counter electrode and the sample cone, resulting in collisions of ions with gas. Fragments produced in the CID spectra are dependent upon the sampling cone voltage. When a high cone voltage is applied, a weak, singly charged $[M-H]^-$ ion is observed whereas ions diagnostic of the two acyl substituents and the polar head group intensify. When the cone voltage is decreased, unfragmented parent ions are collected as ions diagnostic of the two acyl substituents and polar head group are absent. PC is best detected in the positive ion mode due to the positively charged quaternary nitrogen in the head group. Fig. 2 shows the ion abundance of two distinct positive ions of PC, 760 m/z $[M+H]^+$ and 184 m/z corresponding to the polar head group $[H_2PO_4CH_2CH_2N(CH_3)_3]^+$ as the cone voltage is increased. At low cone voltages the higher mass ion is most abundant, whereas at low cone voltages, the ion at m/z 184 is most prominent.

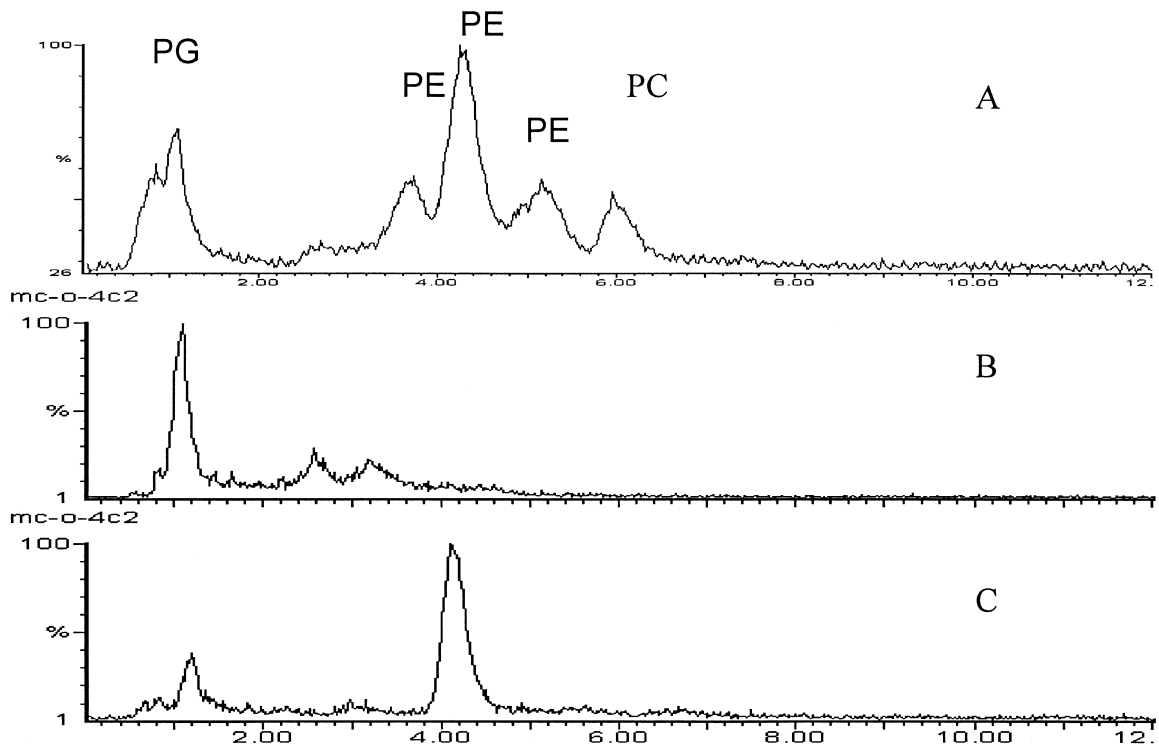Because the polar head groups of different PL are

Fig. 1. (A) Chromatogram of purified brain and egg yolk derived authentic PG, PE and PC; (B) extracted ion chromatogram (EIC) of PG from soil containing 15:0, 16:0, 16:1, 17;0, 17:1, 18:1, 19:1 (see Fig. 5); (C) EIC for ions diagnostic of PE from the soil used in B.
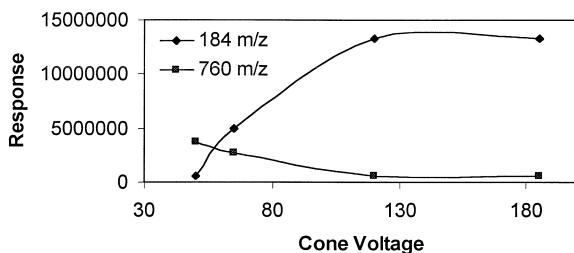

Fig. 2. Ion abundance of m/z 760 and m/z 184 of PC at increasing cone voltages in the ESI.

Table 1
Low mass ions diagnostic polar head groups of PG, PE, and PC

Head group	m/z	Corresponding ion
PG	153	$[CH_2C(OH)CH_2HPO_4]^-$
	171	$[HPO_4CH_2CH(OH)CH_2OH]^-$
	227	$[CH_2C(OH)CH_2PO_4CH_2(OH)CH_2OH]^-$
PE	140	$[HPO_4CH_2CH_2NH_2]^-$
	196	$[CH_2C(OH)CH_2PO_4CH_2CH_2NH_2]^-$
PC	168	$[HPO_4CH_2CH_2N+(CH_3)_2]^-$
	224	$[CH_2C(OH)CH_2PO_4CH_2CH_2N+(CH_3)_2]^-$

different in structure, low mass ions diagnostic of each polar head group can also be identified. Table 1 shows the low mass negative ions diagnostic of PG, PE, and PC. Fig. 3 shows the ESI spectrum for a synthetic, 130 picomoles/μl, PG with 16:0 (palmitic) fatty acid at the sn-1 position and 18:2 (linoleic) fatty acid at the sn-2 position. Note the relative

intensities with the sn-1 PLFA is roughly a third the sn-2. This difference can be used to determine the positional specificity.

Tandem mass spectrometry, MS/MS, provides great advantages in the structural analysis of PL. Product ion spectra and multiple reaction monitoring (MRM) were used to investigate PG containing a 16:0 fatty acid at the sn-1 position and 18:1 fatty
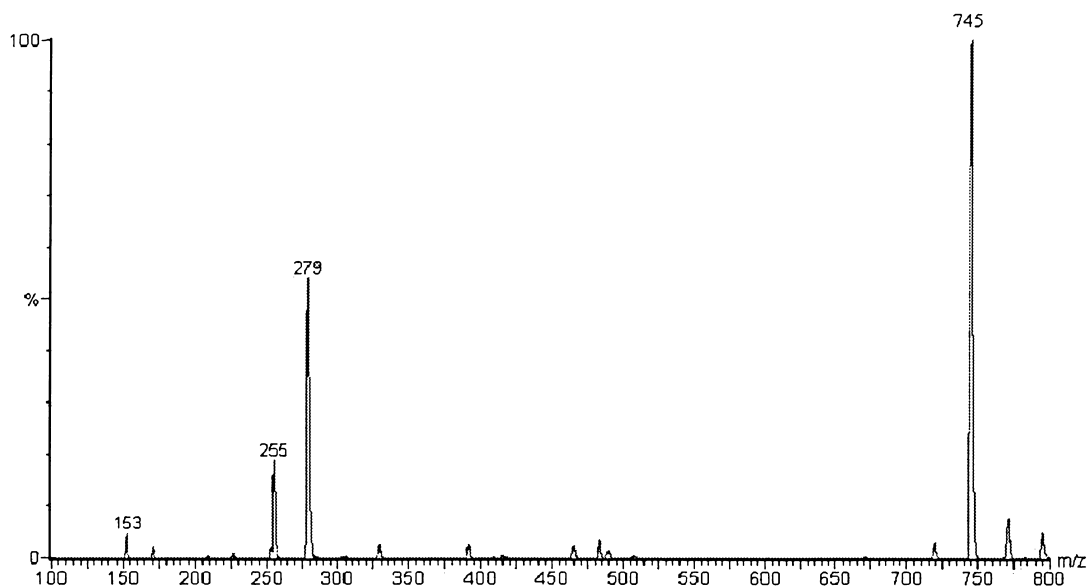
Fig. 3. ESI spectrum for 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phospho-rac-[1-glycerol].

acid at the *sn*-2 position. Fig. 4A shows the ESI spectrum for 1 ppm PG when scanning from m/z 110–900; Fig. 4B shows the product ion spectra for m/z 747 as the parent ion selected in the first quadrupole with collisionally assisted dissociation (CAD) in the second quadrupole in the presence of
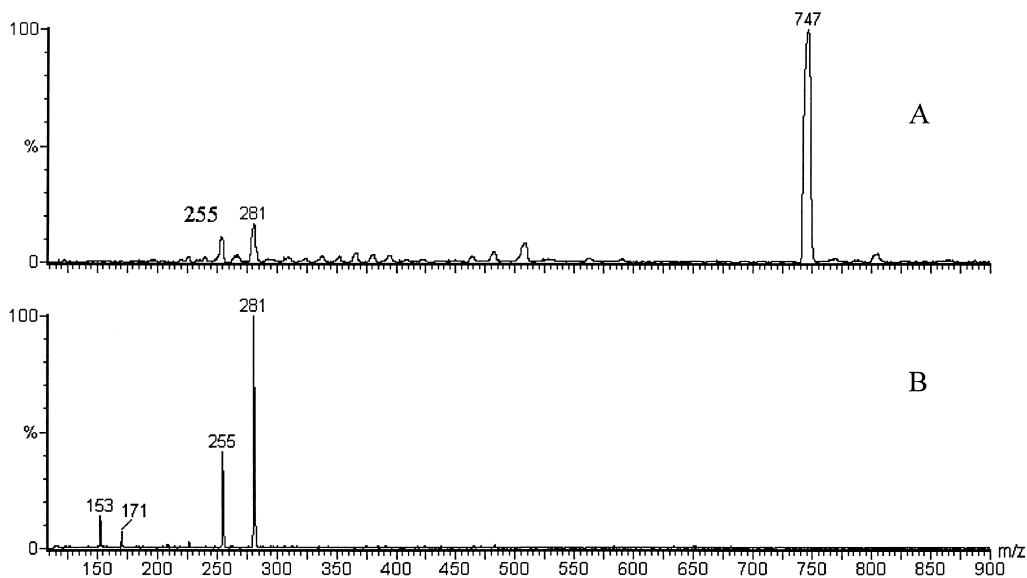

Fig. 4. (A) ESI spectra for 1 ppm PG 16:0/18:1 when scanning from m/z 110–900. (B) ESI product ion spectra for m/z 747 of 1 ppm PG 16:0/18:1 when scanning from m/z 110–900.

3.7×10^{-3} mbar Ar, yielding the product ions that were analyzed by scanning between m/z 110–900 in the third quadrupole. MS/MS decreased chemical noise in the product ion spectra thereby increasing the signal-to-noise (s/n) ratio and the resulting sensitivity. Comparing the s/n ratio of the *sn*-1 and *sn*-2 fatty acids in Fig. 4A to the product ion spectrum from CAD in the second quadrupole, the product ion spectrum for PG is more sensitive by a factor of 50 (Fig. 4B). By scanning the third quadrupole over a narrower range, the sensitivity can be increased. Acquiring product ion spectra by scanning the dissociated fragments of the parent ion, the LOD and LOQ were experimentally determined to be 446 attomol/μl and 1.3 fmol/μl, respectively. When the third quadrupole was scanned at m/z 281, as a product of m/z 747, a roughly 3-fold additional gain in sensitivity was achieved. This multiple reaction monitoring (MRM), m/z 747→m/z 281 represents the most sensitive application of HPLC/ ESI/MS/MS.

3.1. Analysis of environmental samples

Soil samples were collected, the polar lipids extracted, and analyzed using the single quadrupole instrument. Fig. 1B is an extracted ion chromatogram (EIC) for ions diagnostic of PG from soil microbiota while Fig. 1C is an EIC for ions diagnostic of PE from the soil sample. Fig. 5 shows the ESI mass

spectrum and the associated fatty acids for the PG (Fig. 1B) eluting at 0.95 min. The most abundant fatty acid of PG in the soil sample was 15:0. All of the fatty acid structures detected and labeled in Fig. 5 were confirmed by GC/EI/MS to be 14:0, 15:0, 16:1, 16:0, 17:1, 17:0, 18:1, and 19:1. Data from GC/MS can be used to identify individual fatty acids from the total ester-linked fatty acids of the polar lipids (PLFA), whereas LC/MS data can be used to determine both the nature of the polar head group of the intact phospholipid, as well as the localization and composition of the associated fatty acids. Despite the considerably lower sensitivity of GC/MS analysis (pmoles/μl), the high resolution of capillary gas chromatography to separate methyl esters of the total phospholipids enables the position and chirality of methyl branching, unsaturation, hydroxylation, cyclopropyl ring formation to be determined. These features of the phospholipids give insights into the community composition and physiological status of the microbial communities (White et al., 1996). With tandem mass spectrometry is possible to form the fatty acid negative ions in the instrument at high sensitivity and to determine the position of unsaturation, branching and other structures (Zirrolli and Murphy, 1993). Unfortunately the determination of *trans*/cis conformations of monoenoic PLFA cannot be done by MS/MS. The increases in *trans/cis* ratios is a valuable measure of exposure to toxic conditions (White et al., 1996).
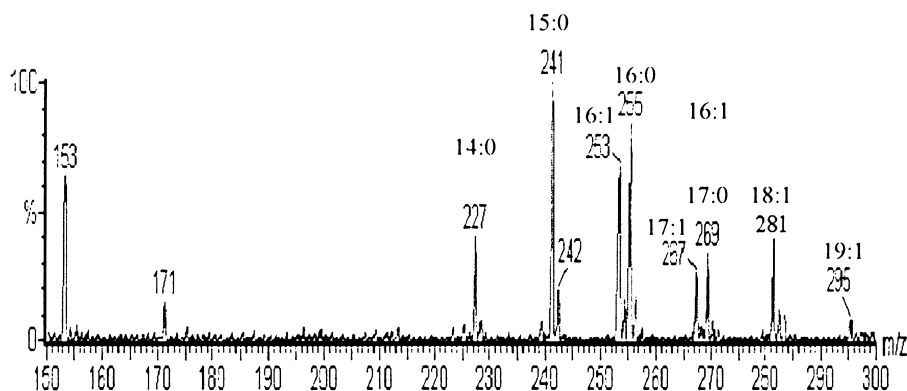

Fig. 5. ESI mass spectrum for peak at 0.95 min PG in Fig. 1B showing fatty acids and polar head groups.

4. Conclusion

The chromatographic system described herein for the rapid separation and identification of phospholipids by HPLC/ESI/MS/MS is a very sensitive and specific analytical system. Many methods are available for separating phospholipids into distinct classes, but very few are compatible with ESI/MS. The few methods that are available are not robust, and tend to degrade the column life. Piperidine has shown to be an excellent post column modifier for the detection of PL in the negative ion mode. It is a strong base, thus aiding in deprotonation of the analytes of interest. Piperidine also helps maintain the chromatographic integrity of amine containing compounds by competing for active sites. Decreasing the tailing of the PE increased the signal-to-noise, resulting in lower limits of detection and quantitation. From the data presented herein, the LOQ of specific fatty acids by HPLC/ESI/MS is at least two orders of magnitude lower than with capillary GC/MS. Analysis of intact PL by HPLC/ESI/MS provides analysis of a much greater mass range, does not require derivatization, and is much more sensitive than the traditional GC/MS analysis of the phospholipid fatty acids from the total polar lipid fraction. GC/MS does however provide conformational analysis of the fatty acids that cannot be done with the HPLC/ESI/MS/MS. Analysis of intact phospholipids by HPLC/ESI/MS provides much greater specificity than GC/MS (Fang and Barcelona, 1998). The utilization of HPLC/ESI/MS/MS with product ion scans offers the greatest sensitivity, in addition true neutral loss or neutral gain scans may promise still greater sensitivity and specificity.

Acknowledgements

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