On-Line Straight-Phase Liquid Chromatography/Plasmaspray Tandem Mass Spectrometry of Glycerolipids

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Class separation of common glycerolipids on a diol-phase liquid chromatography column with a gradient of the mixed solvents hexane/isopropanol/acetic acid/triethylamine is compatible with on-line plasmaspray ionization mass spectrometry. The positive ion mass spectra exhibit prominent diacylglycerol and monoacylglycerol derived fragments, which can be utilized for quantification. In the selected-ion monitoring mode, the detection limit for phospholipids is in the low nanogram range. The abundance of the diacylglycerol and monoacylglycerol fragments reflects the relative fatty acid composition of the phospholipid classes. Collision-induced decomposition of diacylglycerol fragments with argon as collision gas unambiguously reveals the chain length and degree of unsaturation of the esterified fatty acids in the native lipid. *Lipids 28,* 255–259 (1993).

Modern mass spectrometry (MS) with soft ionization techniques including true thermospray (TSP), "filament on" TSP, fast atom bombardment (FAB) and, most recently, discharge-assisted TSP (plasmaspray, PSP) have opened new possibilities for the characterization of glycerophospholipids with high sensitivity. The ionization techniques now available have permitted detailed investigations which were recently reviewed (1).

Among the soft ionization techniques used for phospholipids, FAB has become especially popular as it can usually provide information on molecular weight and partial fatty acid composition (2–5). Most FAB investigations, however, have been concerned with phospholipid classes isolated by thin-layer chromatography, for example, and not with on-line high-performance liquid chromatography/mass spectrometry (HPLC/MS). Although dynamic FAB is rapidly gaining widespread use and can be utilized in combination with HPLC, the chromatographic procedure is still inconvenient. For example, low solvent flows and ionization matrices, such as glycerol, must be used, calling for splitting of standard HPLC effluents or the use of microbore HPLC columns.

However, by using "filament on" TSP and conventional wide-bore HPLC/MS with ammonium acetate as electrolyte and a high percentage of hydrophobic solvents, Kim *et al.* (6–8) obtained detailed structural information on phospholipids, including the diacylglycerol/monoacylglycerol-derived fragments and acylium ions.

We recently showed (10) that the PSP ionization technique can provide significant advantages over "filament on" TSP as used by Kim and Salem (7) in regard to sensitivity and the range of HPLC mobile phases available. Our approach was to apply an HPLC procedure which would permit a class separation only. We examined two HPLC systems (11,12) of which we chose a cation exchange column and an acetonitrile/methanol/water mixture in an isocratic mode.

Quite recently, Herslöf *et al.* (13) have significantly improved class separations of a variety of glycerolipids, including the common intact glycerophospholipids, on a diol column with a gradient system involving hexane, isopropanol, acetic acid, triethylamine and water as mobile phase. The superiority of this separation procedure over that involving a cation-exchange column (10) led us to investigate its merits in combination with PSP ionization.

As previously pointed out (10), the TSP and PSP ionization methods frequently do not give the fatty acid composition of the intact glycerolipid unambiguously, based on the mass fragments formed. The number of combinations of fatty acids possible for a specific mass number of a diacylglycerol-derived fragment increases substantially with multiple unsaturation. The advantages of using on-line HPLC/MS/MS for the analysis of biological samples are presently being explored in our laboratories. To increase the structural information that can be obtained, we decided to study precursor/product relationships of glycerolipids upon collision of diacylglycerol-derived ions with certain reagent gases. The present paper describes experiments on the use of HPLC/MS/MS for structure determinations of intact glycerolipids.

MATERIALS AND METHODS

Chemicals and solvents. Triolein, 1,2-dilauroyl-3-stearoyl-*sn*-glycerol (12:0/12:0/18:0), 1-*O*-hexadecyl-2-3-dimyristoyl-*sn*-glycerol (16:0Et/14:0/14:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphate [16:0/16:0-phosphatidic acid (PA)], 1,2-distearoyl-*sn*-glycero-3-phosphocholine [18:0/18:0-phosphatidylcholine (PC)], 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine [16:0/18:2-phosphatidylethanolamine (PE)], 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1/18:1-PE) and phosphatidylinositol [soybean phosphatidylinositol (PI)] were used as reference compounds.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphate, 1,2-distearoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine were from Serdary Ltd. (London, Ontario, Canada), triolein and PI were purchased from Larodan (Malmö, Sweden). 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine were purchased from Sigma (St. Louis, MO). 1,2-Dilauroyl-3-stearoyl-*sn*-glycerol and 1-*O*-hexadecyl-2,3-dimyristoyl-*sn*-glycerol were prepared in the laboratory (14). All reference materials were stored under N_2 at −20°C until used.

The solvents used were from FSA Lab Supplier (Loughborough, England), or from May and Baker Ltd. (Dagenham, England). They were of HPLC grade and were used without further purification.

Bacterial samples. Pseudomonas fluorescens cells were grown, harvested and extracted for total lipids by Bligh

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Abbreviations: CID, collision induced decomposition; FAB, fast atom bombardment; HPLC/MS, high-performance liquid chromatography/mass spectrometry; MS, mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PSP, plasmaspray; TIC, total ion current; TSP, thermospray.

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and Dyer (9) extraction as described previously (10). Freeze-dried cells (600 mg) yielded phospholipids which were dissolved in 1 mL of mobile phase (A) (see below).

Liquid chromatography. Class separations were performed by using a column packed with diol-modified silica (E. Merck, Darmstadt, Germany; Li-Chrosphere 100 Diol, 5 μm, 25 cm × 4 mm i.d.). The mobile phase was a modification of the method developed by Herslöf *et al.* (13) and consisted of (A) hexane/isopropanol/acetic acid/triethylamine (75:25:7.5:0.05, by vol) and (B) hexane/isopropanol/acetic acid/triethylamine (21:78:1.5:0.05, by vol) in a linear gradient from 100% of (A) to 100% of (B) during 25 min at a flow rate of 0.8 mL/min (13).

A Waters (Milford, MA) model 600-MS HPLC pump equipped with a variable-volume Waters model U6K injector was used. Samples injected were typically 10–20 μL from solutions containing 1 μg/μL of phospholipids.

MS. The effluent from the column was introduced into a Trio 3 (VG Masslab, Altrincham, United Kingdom) tandem quadrupole mass spectrometer through the standard liquid chromatography/MS thermospray/plasmaspray probe. The vaporizer capillary was at a temperature of 240–260°C, the ion source was at 280°C, the discharge current 500–700 μA and the repeller voltage typically between 95–110 V depending on source conditions. Collision-induced decomposition (CID) was achieved by using helium, argon or xenon at pressures between 1×10^{-4} to 5×10^{-2} mbar (measured in the collision cell) and at energies between 0-20 eV. A standard VG 11/253 data system configuration was employed for mass spectral data acquisition.

A cold trap (liquid nitrogen) was installed between the source diffusion pump and the backing rotary pump to prevent contamination of the pump oil by the hydrophobic HPLC solvents used.

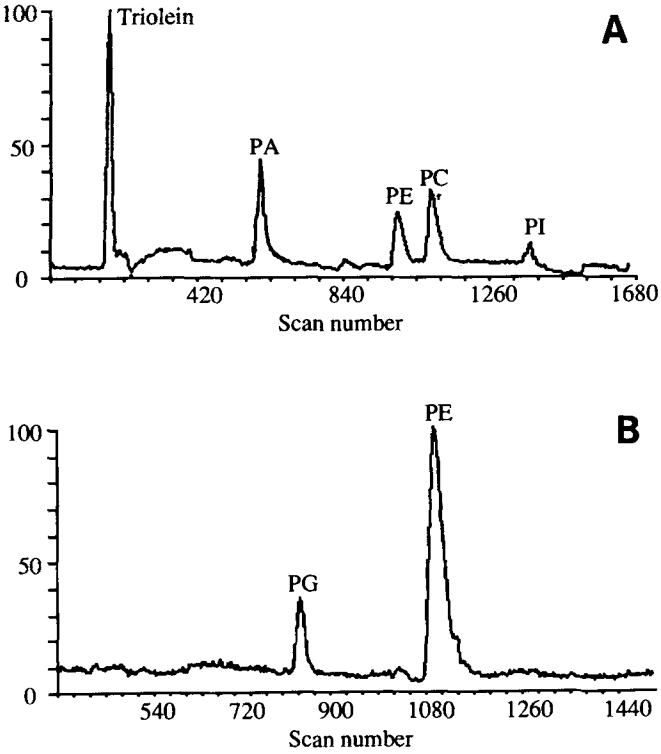

FIG. 1. A. Positive ion plasmaspray mass chromatogram of a mixture of glycerolipids consisting of: triolein, 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (PA), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (PC) and phosphatidylinositol (PI from soybean). B. Positive ion plasmaspray mass chromatogram of a total Bligh and Dyer (9) lipid extract from *Pseudomonas fluorescens* cells showing two peaks containing phosphatidylglycerol (PG) and PE species.

RESULTS AND DISCUSSION

Separation of glycerolipid classes. The positive ion mass chromatogram in PSP [total ion current (TIC) scanning over the range of *m/z* = 120–600] using the diol column and gradient mobile phase system as described above is shown in Figure 1A. The chromatographic peaks represent 76, 24, 25, 25 and 10 μg for triolein, PA, PE, PC and PI, respectively. Good chromatographic peak shape is obtained for all glycerolipid classes studied, including PI which is a natural complex species mixture (see below) isolated from soybeans. Moreover, the gradual change of solvent composition during the chromatographic run does not require modification of the ion source PSP parameters.

A phospholipid extract from bacterial cells *(P. fluorescens)* was also studied by PSP under the same chromatographic conditions (Fig. 1B). Only two glycerophospholipid classes are distinguishable, phosphatidylglycerol (PG) and PE, in agreement with the previously-reported phospholipid composition (15). As both mass chromatograms indicated excellent class separation characteristics with negligible or no apparent molecular species separation (that is PI in Fig. 1A, PG and PE in Fig. 1B), the ions forming the PE peak (Fig. 1B) from *P. fluorescens* (scan numbers 1100–1150) were studied in some detail. The abundances of the diacylglycerol-derived fragment cluster (*m/z* = 540–610) in all spectra within the PE peak were plotted three-dimensionally as a function of the scan numbers (Fig. 2). The diacylglycerol-related portion of the PSP mass spectrum averaged over the entire peak is shown on top. The results indicate a slightly shorter retention time of phospholipids with longer fatty acid chains (*i.e.*, *m/z* = 604 corresponding to 36 fatty acid carbon atoms) compared to phospholipids with shorter fatty acids (*m/z* = 550 corresponding to 32 fatty acid carbon atoms).

Relative fatty acid composition. The fatty acid composition of a PI from soybean was calculated by measuring the mean ion intensities of the monoacylglycerol and diacylglycerol related fragments from a series of full-scan spectra of variable amounts (5, 12.5, 25 and 32 μg) of phospholipid. In Figure 3, the amount of fatty acids present represented as wt% of the total fatty acids, has been reproduced as calculated either from the monoacylglycerol related ions or from the diacylglycerol related ions. For comparison, results from conventional gas chromatographic fatty acid analysis after transesterification (Larodan, Malmö, Sweden) have been incorporated in the figure. Apart from minor components (*i.e.*, 18:3) which fall below the detection limit in full-scan, there is a good agreement between MS measurements on intact phospholipids and gas chromatography measurements of total fatty acid methyl esters.

PSP spectra of triacylglycerol and 1-O-alkyl-2,3-diacyl-sn-glycerols. We have also studied the MS fragmentation of some nonphosphatidic glycerolipids using PSP

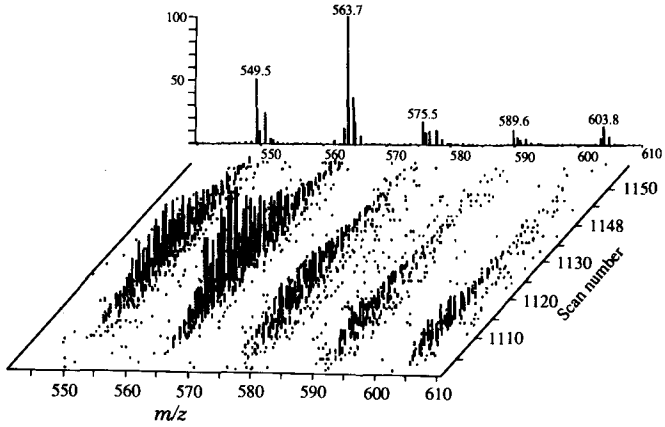

FIG. 2. Three-dimensional presentation of the diacylglycerol-derived fragments (m/z = 540–610) extracted from the phosphatidylethanolamine (PE) peak in Figure 1B. The upper spectrum shows the average intensities of the peak.

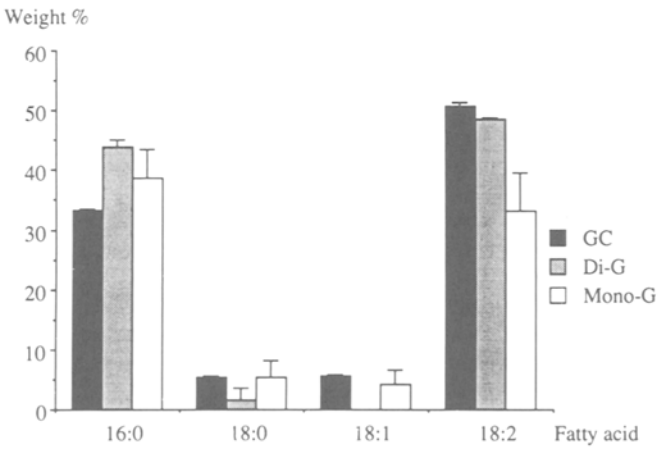

FIG. 3. Fatty acid composition of phosphatidylinositol from soybean determined by gas chromatography (GC) and by plasmaspray mass spectrometry calculated from the total counts of the intensities of the diacylglycerol-derived (Di-G) and the monoacylglycerol-derived (Mono-G) fragments.

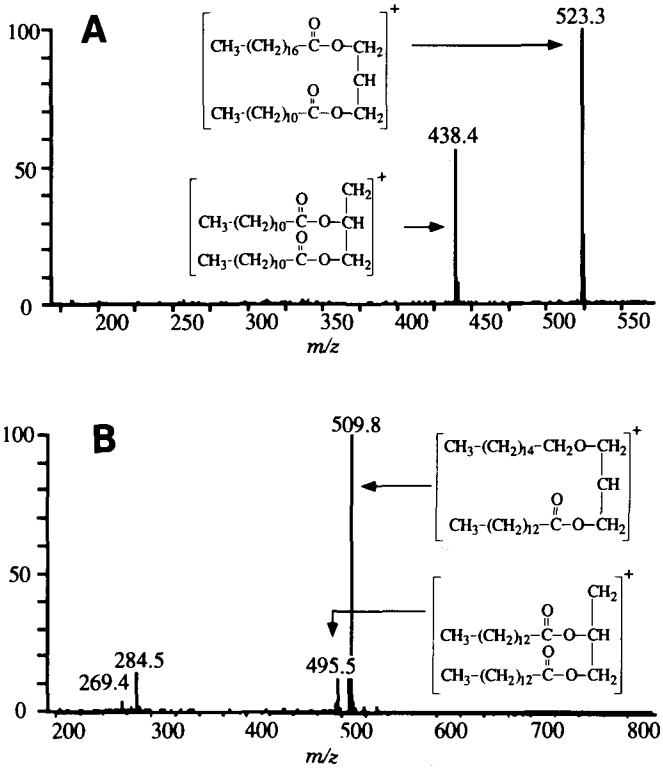

FIG. 4. Positive ion plasmaspray spectrum of A, 1,2-dilauroyl-3-stearoyl-*sn*-glycerol, and B, 1-*O*-hexadecyl-2-3-dimyristoyl-*sn*-glycerol.

ionization. Figure 4A shows the positive ion PSP spectrum of 10 μg of 1,2-dilauroyl-3-stearoyl-*sn*-glycerol. As for the phosphatides, no $(M + H)^{+1}$ ions are seen in PSP. The spectrum shows only the two possible diacylglycerol related fragments, formed by loss of a fatty acid from either position of the protonated molecule.

In Figure 4B, a spectrum of 1-*O*-hexadecyl-2-3-dimyristoyl-*sn*-glycerol has been reproduced. Similar cleavage mechanisms as operate for the triacylglycerol are seen. Notably, cleavage with loss of the carboxylic acid (α- and β-carbon atom) from the protonated molecule (m/z = 509) is a more prominent process than that involving formal loss of the alcohol group (m/z = 495).

Quantification. Previous preliminary studies on the quantification of phospholipids by using PSP and the cation HPLC column with an acetonitrile/methanol/water mixture indicate that straightforward procedures can be used to give linear dose-response curves. Such studies were repeated in some detail for several phospholipid classes by using the diol column and the hydrophobic solvent mixtures used in this work. Figure 5 shows dose response curves for PA, PC and PE (all dipalmitoyl substituted) measured by single ion monitoring of the m/z = 551 ion (diacylglycerol related fragments). The responses exhibit good linearity for all molecular species ($r^2 > 0.99$), and an intercept at the origin indicates negligible adsorption and/or breakdown in the chromatographic and interface system. At a signal-to-noise ratio of 3:1, the detection limit is approximately 3 ng. The ionization tendencies for the phospholipid classes investigated are in the same order of magnitude. Sensitivity in selected ion monitoring (SIM) is comparable with that obtained in the previously used liquid chromatographic system, providing a detection limit in the low nanogram range (10).

In another experiment, we compared the sensitivity of two PC molcules with different chain-lengths (dipalmitoyl and dioctanoyl PC). We found that the relative sensitivity between the long and short chain lengths is dependent on the experimental conditions, *i.e.*, tuning of the ion source, the cleanness of the source and the capillary vaporizer, but reproducibility under set conditions was found to be satisfactory.

CID measurements. PSP ionization of a phospholipid with a defined fatty acid composition was studied. Figure 6A shows the PSP positive ion mass spectrum of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine. As expected, diacylglycerol related fragments are present at m/z = 575 (18:2 + 16:0). The spectrum further shows two monoacylglycerol related fragments at m/z = 337 (18:2) and m/z = 313 (16:0).

The mass spectrometer was arranged for MS/MS measurements in the precursor/product mode. A CID mass

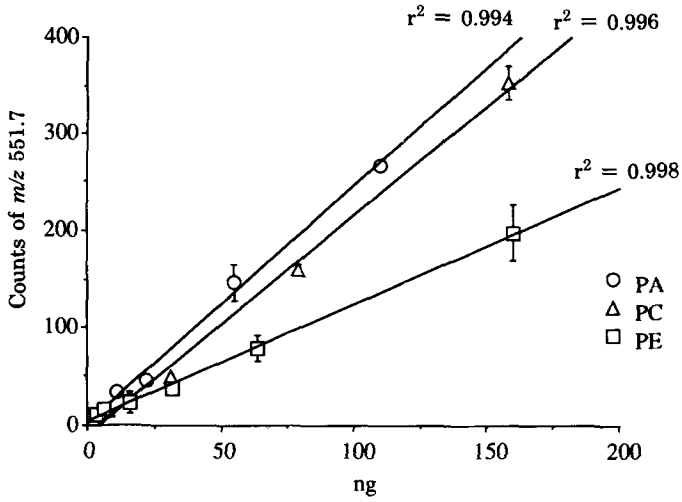

FIG. 5. Dose response curves of the diacylglycerol related fragments (*m/z* = 551) from 1,2-dipalmitoyl-*sn*-glycero-3-phosphate [16:0/16:0-phosphatidic acid (PA)], 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine [16:0/16:0-phosphatidylcholine (PC)], and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine [16:0/16:0-phosphatidylethanolamine (PE)] measured in single ion mode.

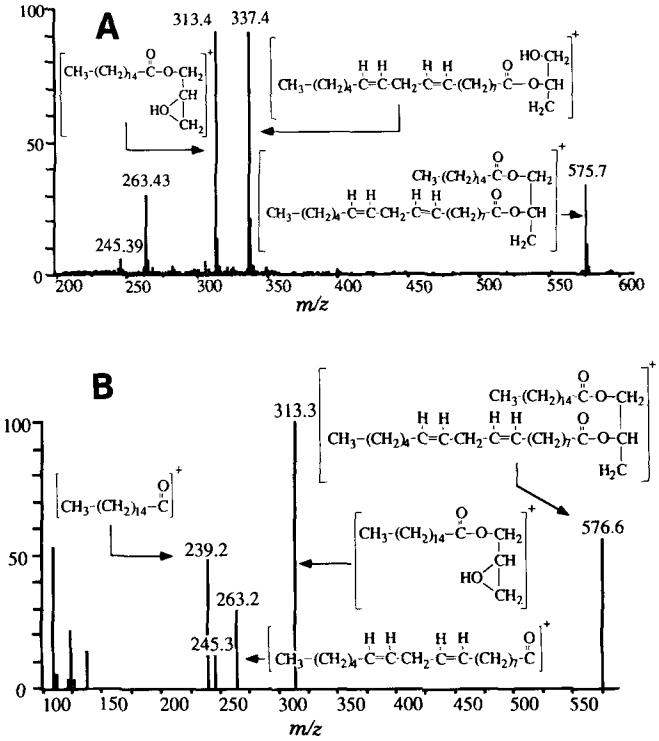

FIG. 6. A. Positive ion plasmaspray of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine. B. Collision-induced decomposition of the diacylglycerol-derived fragment *m/z* = 575.5, showing the most abundant product ion, *m/z* = 313.3

spectrum of the diacylglycerol related fragments (*m/z* = 575) using argon as collision gas at a pressure of 10^{-3} torr, and a collision energy of 3 eV is shown in Figure 6B. The spectrum indicates in the high mass range the presence of undecomposed precursor ions at *m/z* = 575 and prominent monoacylglycerol related fragments at *m/z* = 313. Acylium ions at *m/z* = 263 and 239 of both fatty acids are observed in the spectrum corresponding to 18:2 and 16:0 fatty acids, respectively. The spectrum further indicates subsequent fragmentation of the fatty acid residues leading to a series of low mass fragments. The CID experiment thus unequivocally reveals fatty acid composition.

As an application, the fatty acid composition of PI from soybeans was examined. The three most abundant diacylglycerol related fragments as indicated from the full spectrum (Fig. 7) were analyzed by using precursor/product MS/MS as described above.

The three MS/MS product ion spectra of the precursor diacylglycerol related ions as shown in Figure 7 give information on the fatty acid species. The diacylglycerol related ions of *m/z* = 575.6 consist of an 18:2 fatty acid moiety (acylium ions of *m/z* = 263.3) and a 16:0 fatty acid (monoacylglycerol related ion of *m/z* = 313.3). Analogously, the H_2O adduct of the diacylglycerol related ions of *m/z* = 591.7 consists of an 18:3 acylium ion (*m/z* = 261.2) and a 16:0 acid (*m/z* = 313.3). The diacylglycerol-related ions of *m/z* = 603.3 have an 18:0 acid (*m/z* = 341) and a 18:2 acid (*m/z* = 263.3).

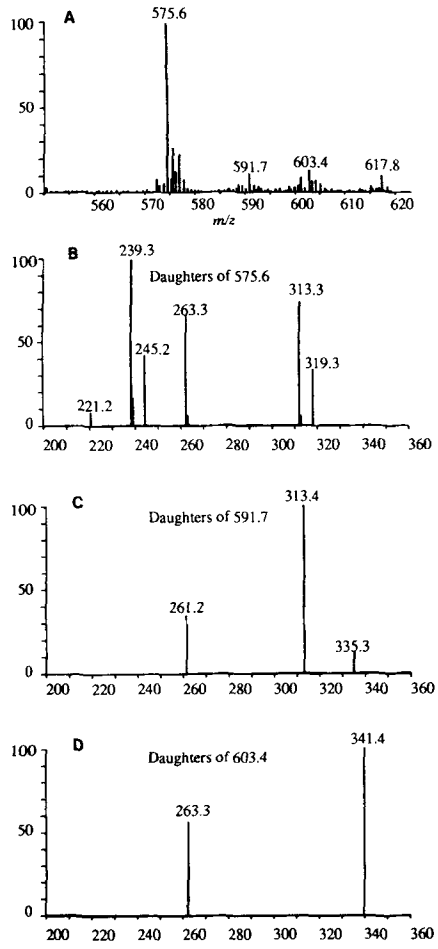

FIG. 7. A. Mass spectrum of phosphatidylinositol from soybean, and B, the collision-induced decomposition product ion spectra of the precursor ions of *m/z* = 575.6, C, of *m/z* = 591.7, and D, of *m/z* 603.4.

Changing the collision energy indicates that useful information was obtained only within a rather narrow ion energy range. For example, at 3 eV there is practically no decomposition of the precursor fragments whereas at 20 eV the CID spectrum only showed unspecific fragments of low mass. There are no major qualitative differences in the spectra when the collision gas Ar was replaced by He or Xe as long as the collision energy and pressure are adjusted accordingly for each. Argon was found to be the collision gas of choice, because adequate fragmentation was obtained at convenient gas pressures and collision energies.

CID after PSP ionization offers excellent possibilities to study glycerophospholipid molecules in detail. It is possible to obtain information on the fatty acid composition of individual species within phospholipid classes.

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