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Normal-phase Liquid Chromatography Class Separation and Species Determination of Phospholipids Utilizing Electrospray Mass Spectrometry/Tandem Mass Spectrometry

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Membrane phospholipids have been separated into classes by normal-phase chromatography using electrospray (ES) mass spectrometry or with tandem mass spectrometry (MS/MS) for species determination. Biological samples, taken from patients seeking a remedy for gastric catarrh or gastric ulcer were examined. It was found, by altering the cone voltage and changing between positive- and negative-ion modes in ES, that in most cases it was possible to obtain structural information for the molecular species and fatty acids present. MS/MS was employed to achieve accurate species determination.

Phospholipids are major constituents of membranes in plants, animals and microorganisms. In addition to their structural role, some phospholipids also participate in biological processes in various ways. Other phospholipids, such as polyphosphoinositides, are important in cellular signalling systems.[1,2] Phospholipids also serve as a source of arachidonic acid and other polyunsaturated fatty acids that can be metabolized to biologically active eicosanoids such as prostaglandins, thromboxanes, leukotrienes and lipoxins.[3,4] Phospholipids have been given increased attention in many fields, for example as biomarkers in chemotaxonomical studies and in the making of liposomes for drug delivery or cosmetics/detergents. The commercial use of phospholipids is increasing in fields such as biomembranes, skin-care formulations and drug delivery.[5] Accordingly, methods for accurate identification of phospholipid classes and phospholipid molecular species are important in the understanding of the biochemical mechanisms described above.

The possibility of coupling high-performance liquid chromatography (HPLC) on-line with a mass spectrometer, opened new possibilities in the separation and structural analysis of intact phospholipids. Several ionization techniques have been used, such as thermospray (TS) and discharge assisted thermospray (plasmaspray).[6–10] Ma and Kim have described on-line reversed phase HPLC/TS-MS for the separation and detection of glycerophospholipid molecular species in rat brain.[11] However, the HPLC method used gave chromatographic overlapping of different glycerophospholipid molecular species, which may make species identification difficult. Papers describing the analysis of phospholipids with ES-MS and ES-MS/MS using loop injections[12–15] have also been reported. However, since mass spectra of different phospholipids often overlap, and various sphingolipids and neutral lipids may be present in a biological sample the use of loop injections may be unsuitable. Thus, in the analysis of samples taken from a complex biological matrix, there is a need for class separation by LC followed by species identification by mass spectrometry. Soft atmospheric pressure ionization techniques such as atmospheric pressure chemical ionization (APCI) and modern ES offer the possibility of accommodating chromatographic flow rates up to 1000 μL/min.

Modern ES makes the use of post-column splitting unnecessary, in that both narrow bore and standard columns can be used. In addition, APCI and ES combined with MS/MS give structural information for the intact molecule as well as the possibility of obtaining molecule-related fragment ions by in-source fragmentation or by collision-induced dissociation (CID).

The present work describes the determination of all major phospholipid classes with normal-phase HPLC/ES-MS. The method developed has been applied to gastric juice samples taken from patients seeking a remedy for gastric catarrh or gastric ulcer.

EXPERIMENTAL

Standards

The following reference phospholipids were purchased from Sigma (St. Louis, MO, USA): 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid (PA), 1,2-stearoyl-*sn*-glycero-3-phosphatidyl glycerol (PG), 1,2-dipalmitoyl-*sn*-glycero-phosphatidyl ethanolamine (PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl chloline (PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl serine (PS) and phosphatidyl inositol (PI) from soybean.

Biological samples

Gastric juice samples were taken by gastroscopy from patients seeking a remedy for gastric catarrh or gastric ulcer. Typically, gastric juice samples of 2 mL were taken from each patient. Tris buffer was then added to 1 mL of sample to pH 7. After evaporation and freeze-drying of the samples, extraction of total lipid was performed according to the method of Bligh & Dyer.[16] The total lipids were then fractionated on a silica column with solvent elution.[17] The

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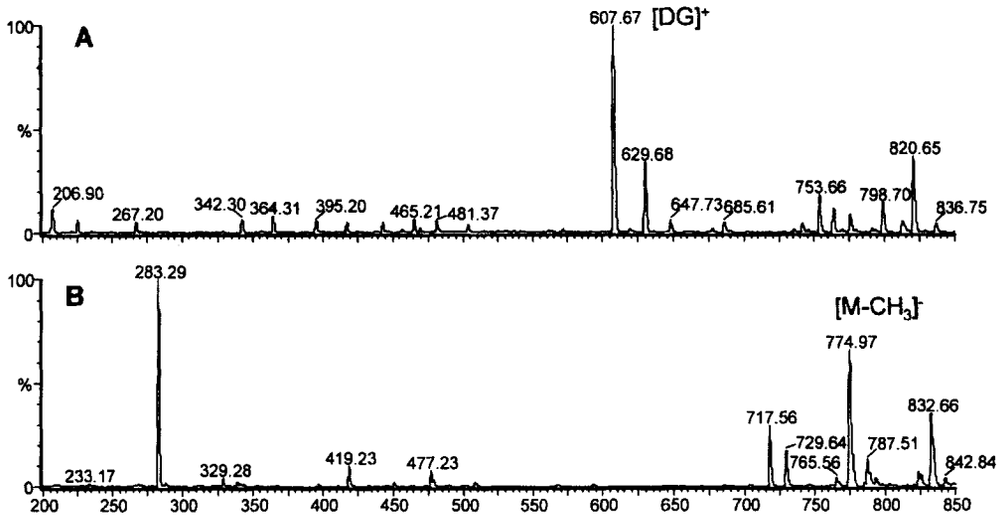


Figure 1. APCI mass spectra of PC-di18:0 (a) in positive-ion mode and (b) in negative-ion mode.

Table 1. Linear gradient composition

Time (minutes)	%A	%B
0	100	0
25	20	80
30	20	80
40	100	0
65	100	0

polar lipid fraction was evaporated, freeze-dried and dissolved in hexane + 1-propanol + water: 40/54/5 (v/v/v). The final concentration was abut 200 ng/μL.

Liquid chromatography

A Varian model 9012 gradient pump (Varian Chromatography Systems, Walnut Creek, CA, USA) using a 10 μL loop was used for sample introduction. The LC separations were performed on a diol column, 250 × 3.0 mm i.d., packed in our laboratory with diol modified silica (5 μm, Lichrospher 100 DIOL, Merck, Germany). The flow rate was 0.4 mL/min and the column temperature 55 °C. Prior to use of the column a blank gradient was run. The linear solvent gradient is shown in Table 1. Solvent mixture A: hexane + 1-propanol + formic acid + triethylamine; 79/20/0.6/0.08 (v/v), solvent mixture B: 1-propanol + water + formic acid + triethylamine; 88/10/0.6/0.08 (v/v).

Mass spectrometry

Mass spectrometry was carried out on a VG QUATTRO II mass spectrometer (Fisons Instruments, VG Organic, Altrincham, UK) equipped with pneumatically-assisted electrospray and atmospheric pressure chemical ionization sources. Data handling was performed with a VG Masslynx NT32 data handling system. In all modes, full scan spectra, between *m/z* 200 and 900, were obtained at a scan speed of 250 mass units/s with a mass resolution of 1 u at half peak height. In the ES experiments the LC effluent entered the mass spectrometer through an electrospray capillary set at

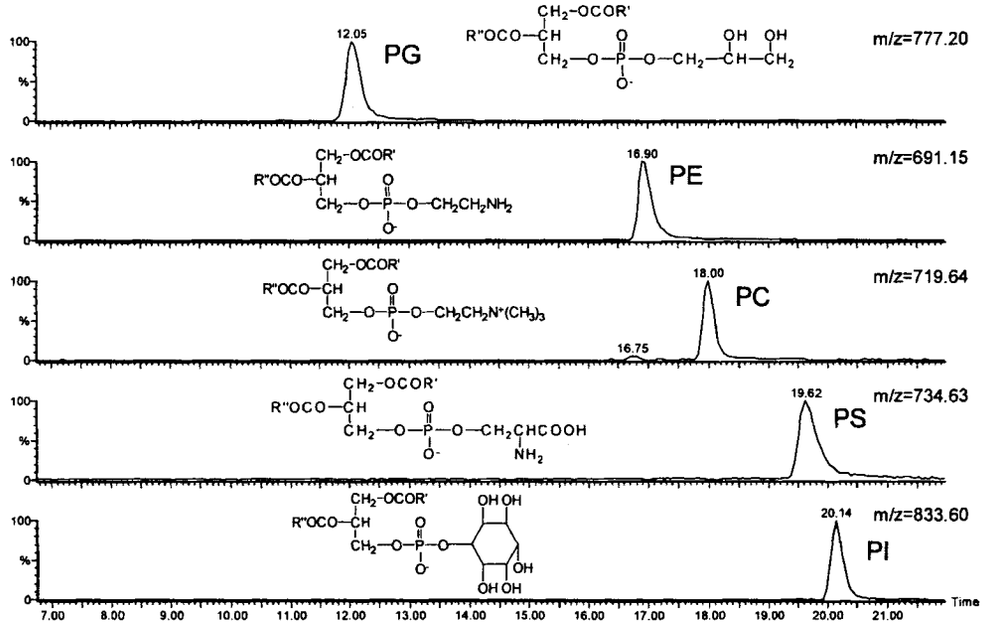


Figure 2. Normal-phase HPLC/MS separation of a standard mixture of phospholipids, represented as the mass chromatogram of each phospholipid class in negative-ion ES. Conditions given in the Experimental section.

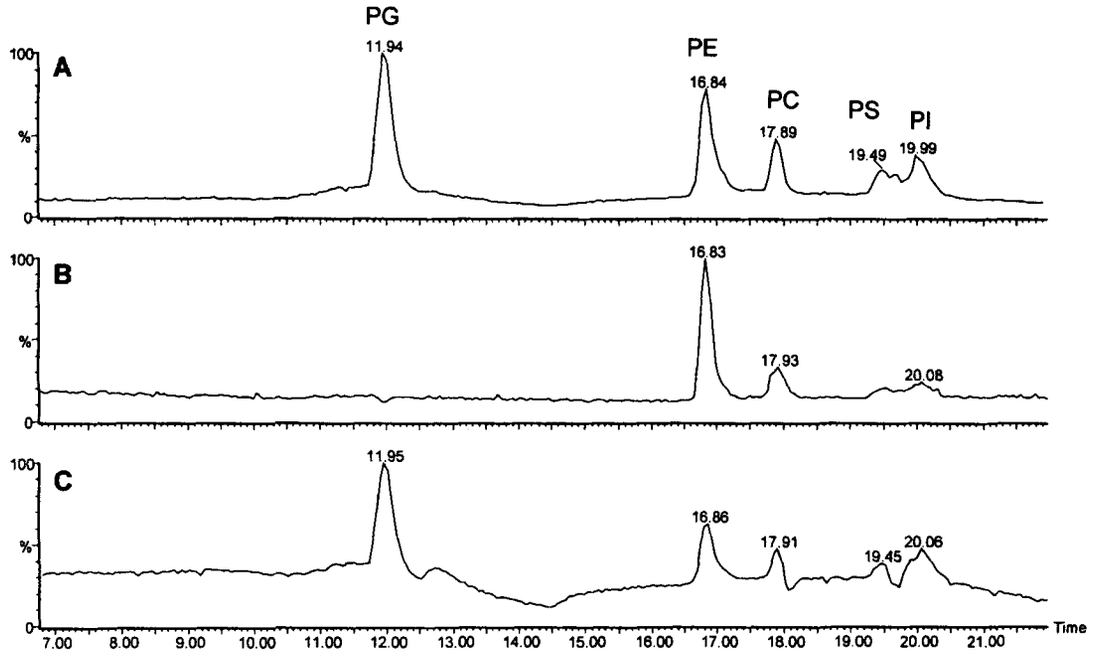


Figure 3. Total-ion current from: (a) molecular ion region, ES^-, CV=60 V, (b) diacylglyceride related fragment ion region, ES^+, CV=110 V, and (c) fatty acid fragment ion region, ES^-, CV=110 V.

−2.4 kV (ES^- and +2.9 kV (ES^+), in both cases at a source temperature of 160 °C. Nitrogen was used both as drying gas and nebulizing gas at flow rates of 250–300 L/hr and 20 L/hr respectively.

For the APCI experiments the probe temperature was held at 500 °C and source temperature at 120 °C. Collision-induced decomposition (CID) spectra were recorded at a collision energy of 25 eV with a mixture of xenon+argon (25:75 v/v) as collision gas at a pressure of 1.5×10^{-3} mBar. Dose response curves were constructed in single-ion monitoring (SIM) and multiple-reaction monitoring (MRM) modes, with a mass resolution of 1 u at half peak height, a dwell time of 0.08 s and an interchannel delay of 0.02 s.

Nomenclature and abbreviations

Fatty acids are referred to by the number of carbons together with the number of double bonds in the aliphatic chain.

RESULTS AND DISCUSSION

Standard samples

Initially, it was of interest to compare APCI with discharge assisted thermospray (plasmaspray (PSP)), due to the similarity of the ionization process using these techniques. In the positive-ion mode, APCI produced molecular-related ions and diacylglyceride-related fragment ions (Fig. 1(a)) whereas PSP only produced the latter. In the APCI negative-

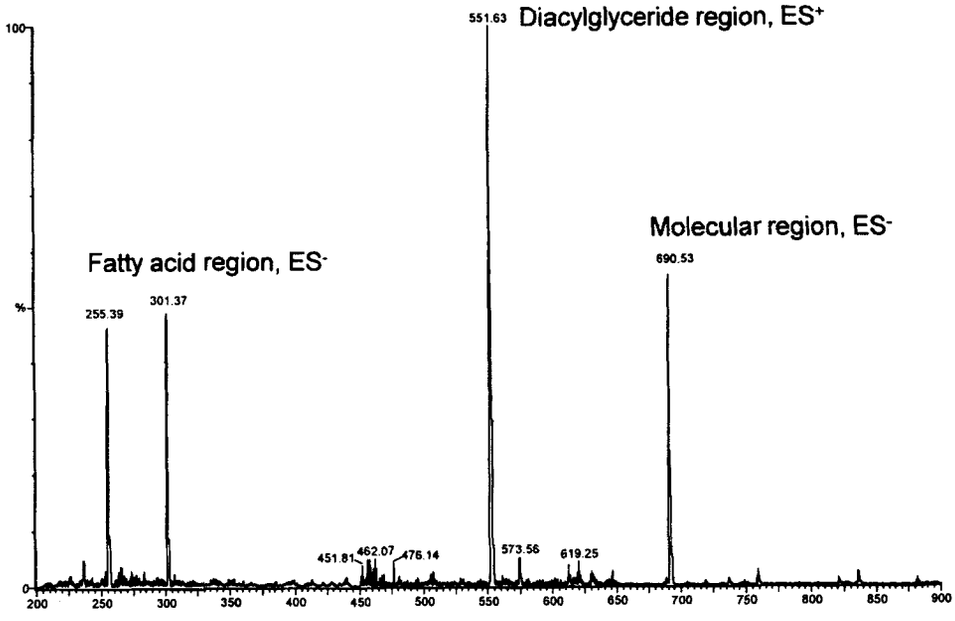


Figure 4. Combined ES^- and ES^+ mass spectra of PE-di16:0, obtained from Fig. 3. m/z=301.37 represents a fatty acid adduct ion.

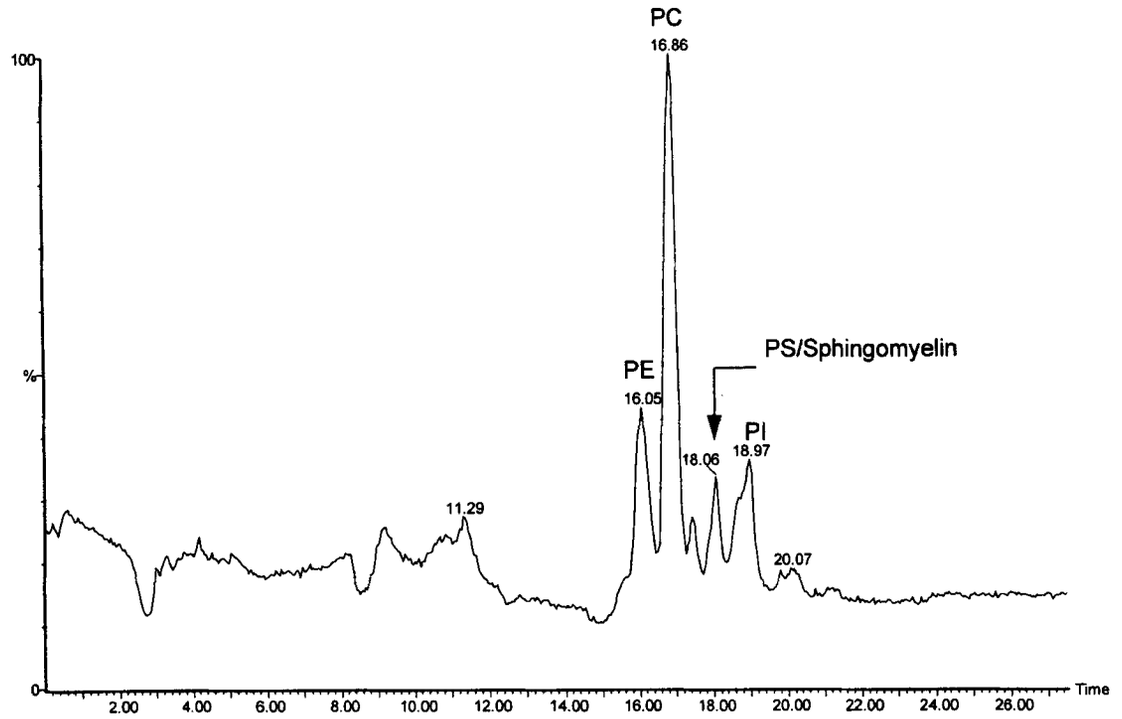


Figure 5. Mass chromatogram, total-ion current, of gastric juice sample, 20 ng/uL obtained by HPLC/MS.

ion mode both molecular ions and fatty acid fragment ions are produced (Fig. 1(b)). All phospholipid classes, with the exception of PC, gave prominent $[M-H]^-$ ions. For PC, a molecular-related $[M-CH_3]^-$ ion was produced, due to the thermal fission of a methyl group from the choline moiety. However, with APCI the response of both PS and PI are lower compared with the other glycerophospholipid classes. Full scan sensitivity in APCI was found to be the same as with PSP but was about two orders of magnitude less than with ES. Thus, our further studies were performed with ES.

The normal-phase LC separation system used in our laboratory for many years, has been optimized to separate all major phospholipid classes (including sphingomyelins) from neutral lipids, Figure 2 shows a typical separation of all the major glycerophospholipid classes of a standard mixture. The mass spectrometry software offers the possibility of altering the cone voltage and changing between positive- and negative-ion mode respectively in the same run. Thus, the cone voltage was optimized to give negative molecular related ions for all phospholipids. In ES^-, for example, at a cone voltage of 60 V, molecular ions $[M-H]^-$ were produced for all classes except PC, from which molecular related ions $[M-15]^-$ were produced.[18] At a high cone voltage, 110 V, in-source fragmentation produced diacylglycerol related fragments in ES^+ which

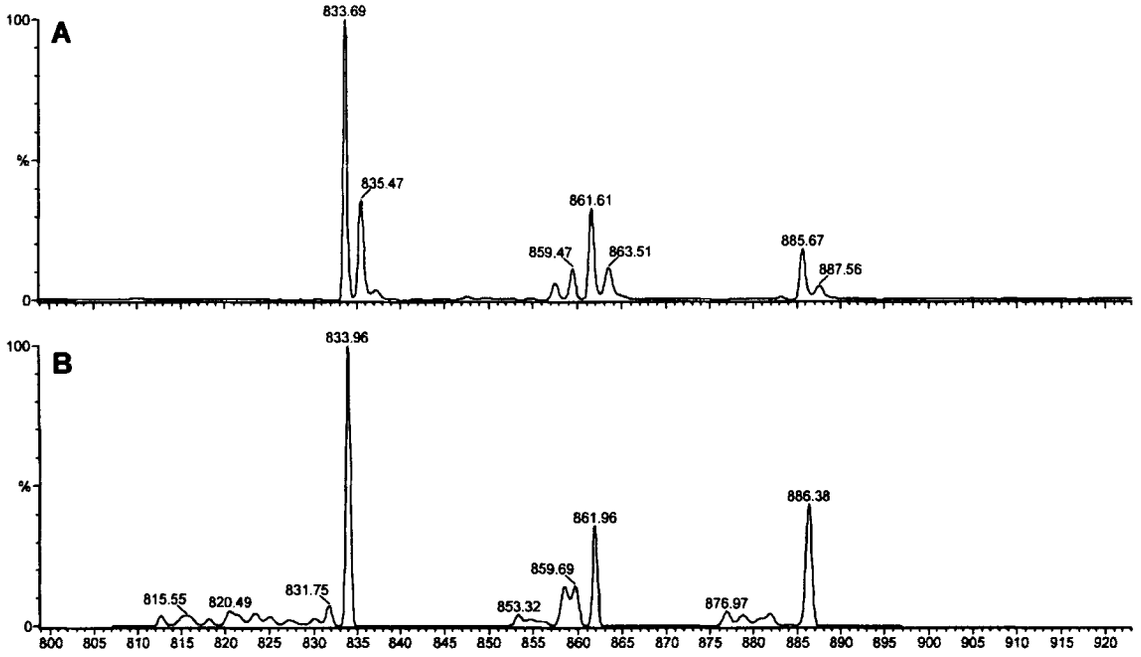


Figure 6. (a) Mass spectrum of glycerophospholipid species of PI and (b) parent ion MS/MS spectrum of $m/z=241$. The spectra were obtained in single runs.

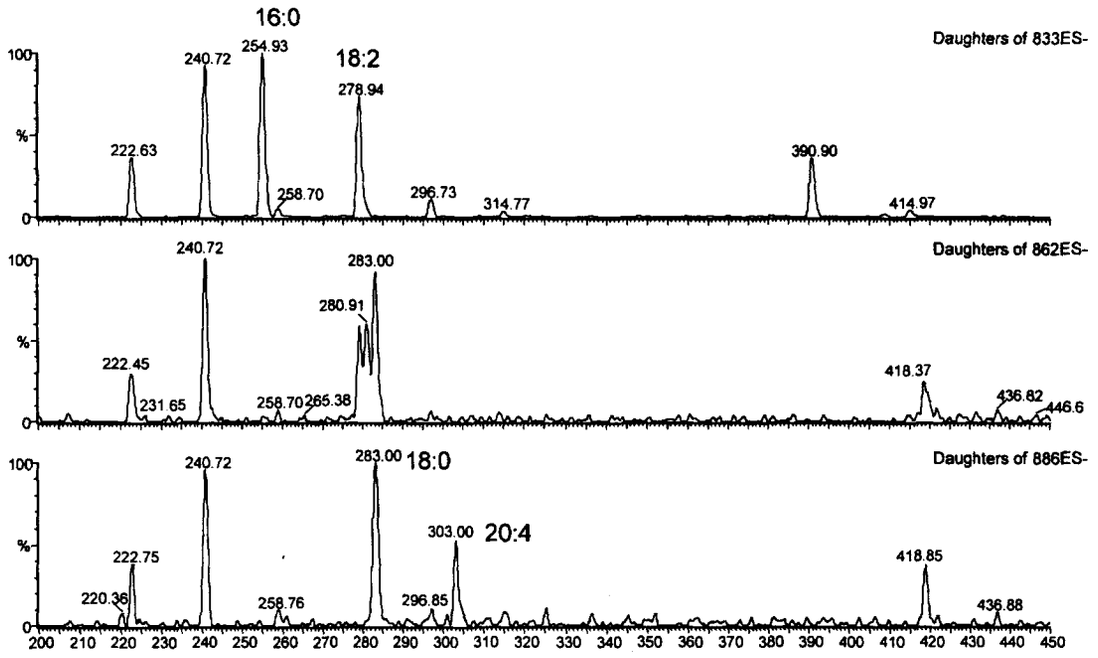


Figure 7. MS/MS daughter-ion spectra of major PI species in a gastric juice sample. Major fatty acids are indicated.

correspond to the molecular species composition whereas ES^- produced fatty acid fragment ions, cf. Fig. 3. By combination of the mass spectral data obtained from Fig. 3, a 'combined' mass spectrum was created (Fig. 4). By altering the cone voltage, in combination with switching from positive- to negative-ion mode and class separation by HPLC, it was possible to obtain detailed information of an unknown sample regarding class, species and fatty acid composition in a single run.

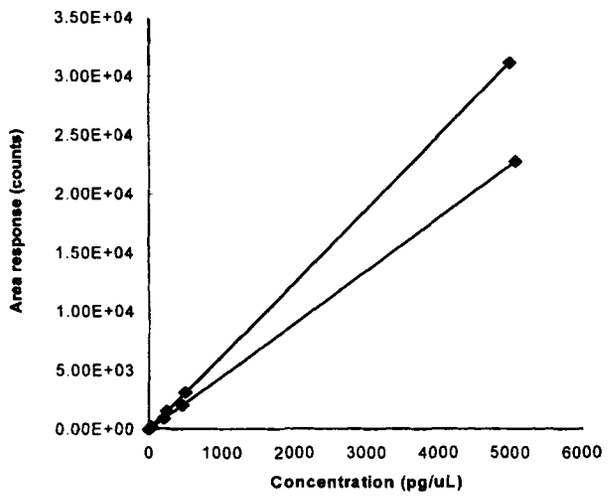


Figure 8. SIM dose response curves of PC (upper) and PI.

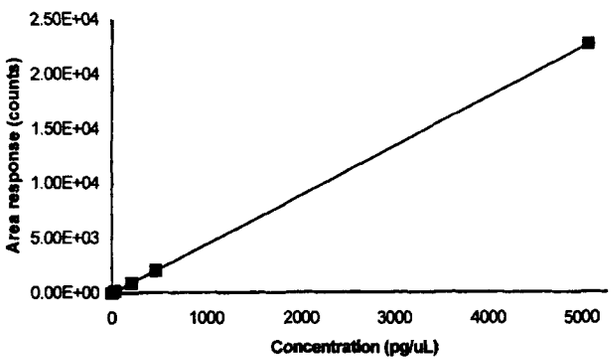
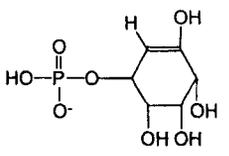


Figure 9. MRM (*m/z* 775→283) dose response curves of PC.

Biological samples

The approach described above was applied to a number of gastric juice samples (Fig. 5). In these samples even-numbered, saturated and unsaturated fatty acids were found. From PI, a prominent ion of $m/z=241$ was observed, which could correspond to the presence of the odd-numbered fatty acid 15:0, which indicates the presence of bacterial phospholipids. This is not surprising, since bacteria are present in the gastric region. However, parent ion MS/MS experiments showed that this fragment was present in all PI species, which suggested that it could be a fragment ion from the inositol head group. The in-source induced *m/z* 241 fragment was further analysed by MS/MS. Ions of $m/z=79$ were thereby observed indicating the loss of a phosphate group from a phosphoinositol fragment. Tentatively, the $m/z=241$ ion may have the following structure:



However, in a sample containing bacteria the presence of a 15:0 fatty acid can not be excluded. The glycerophospholipid species seen in Fig. 6(a) were submitted to MS/MS analysis so that the species fatty acid combination(s) of 16:0, 18:0, 18:1, 18:2 and 20:4 fatty acids could be established (Fig. 7). PC and PE also showed various species combinations of 16:0, 18:0, 18:1, 18:2 and 20:4 acids indicating that these phospholipids originate from a human source.

The medical results from the gastric juice samples will be published elsewhere.

Sensitivity

In the full-scan mode under the conditions described (flow rate 0.4 mL/min and using three different cone voltages), the detection limit was in the range of 150–450 fmol/μL depending on the phospholipid. In the SIM and MRM modes the sensitivity was found to be 7–10 fmol/μL at a signal-to-noise ratio of 3:1. Figures 8 and 9 show good linearity (r^2=0.999985), indicating straightforward potential for quantification in the low fmol range.

CONCLUSIONS

If the unknown sample consists of only a few phospholipid classes, MS/MS could be employed directly without LC separation, bearing in mind that suppresson effects in the ion source can occur and make quantification difficult. Moreover, mass spectra of different phospholipid classes often overlap. With HPLC class separation, the tedious task of correctly identifying a phospholipid class is avoided, making class identification straightforward. Furthermore, the possibility of altering the cone voltage and changing between positive- and negative-ion mode operation in the same run, makes it possible to attain information on molecular weight and fatty acid composition for all glycerophospholipid classes in a single LC/MS analysis. If additional information is desired, for example exact fatty acid combination in the molecular species, MS/MS analysis can be employed.

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