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Liquid Chromatography/Mass Spectrometry of Phospholipids in Soybean Products Using Particle Beam and Ionspray Interfaces

Maria Careri,* Marco Dieci, Alessandro Mangia and Paola Manini

Dipartimento di Chimica Generale ed Inorganica, Chimica Analitica, Chimica Fisica, Università degli Studi di Parma, Viale delle Scienze, I-43100 Parma, Italy

Andrea Raffaelli

Centro di Studio del CNR per le Macromolecole Stereordinate ed Otticamente Attive, Dipartimento di Chimica e Chimica Industriale, Via Risorgimento 35, I-56126 Pisa, Italy

Particle beam (PB) high-performance liquid chromatography (HPLC)/MS and ionspray (ISP) HPLC/MS were investigated for the separation and mass spectral characterization of the phospholipids phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. Fragmentation patterns obtained in both positive-ion and negative-ion chemical ionization modes using the PB/MS technique were not useful in differentiating phospholipid classes. The HPLC/ISP-MS technique was able to provide direct information about the molecular weights of the individual homologues; more complete characterization of the fatty acid chains and of the polar head group was obtained by using ionspray with tandem mass spectrometry (MS/MS). The HPLC/ISP-MS system was used to characterize the lipid fraction of different soybean products; phosphatidylserine and less polar lipids such as triglycerides were also identified from the on-line mass spectra of eluates. Finally, the potential of the MS/MS selected-reaction monitoring (SRM) technique for the analysis of phospholipids at the nanogram level was explored. The higher selectivity observed with SRM compared with that obtained by using selected-ion-monitoring suggests the potential of the former method for the analysis of these substances in samples with a low content, such as biological and clinical ones.

Phospholipids (PLs) are significant constituents of biological membranes and are found with polar head groups and the ester-linked fatty acids of varying compositions, the nature of the latter two affecting the physical and chemical properties of membranes.[1] PLs invariably occur in all foods of animal and plant origin, and their emulsifying properties can cause serious effects during food processing.

Analysis of these biomolecules has been carried out with chromatographic techniques such as thin layer chromatography (TLC)[2,3] and liquid chromatography (LC).[4–11] High performance (HP)LC methods in normal-phase mode have been proposed for the separation of phospholipid classes,[4,7–11] the selectivity of the retention being based on the adsorption of the polar head to the silanol sites of the stationary phase. Separation of molecular species of phospholipids has also been obtained by reversed-phase (RP)HPLC based on a solvophobic retention mechanism[5–7] and by argentation-HPLC.[12–13] Thus, since not all molecular species are resolved on the RP column, separation based on the degree of unsaturation can be achieved on a silver-coated silica gel HPLC column. Detection of these substances has usually been performed by UV, but serious restrictions are imposed on mobile phase selection, since underivatized PLs absorb near 200 nm with a low extinction coefficient. Alternatively, PLs can be analyzed by HPLC with an evaporative light-scattering detector, which is compatible with gradient elution and permits quantification of amounts of phospholipids at microgram levels.[9,11,14–16]

Mass spectrometry has been demonstrated to be useful for structural elucidation of phospholipids. In the past, field desorption,[17] desorption chemical ionization[18] and fast atom bombardment (FAB)[19–24] have been proved to be suitable for PL analysis, by enabling mass spectra of intact molecules to be obtained. In the FAB mass spectra of phospholipids, acquired using positive-ion detection, few but significant fragments are observed; the protonated molecular ion is usually detected, together with an abundant fragment ion characteristic of the polar head group.[21,25] Munster *et al.* recommended negative-ion FAB for structural analysis of phospholipids, since both the identification of the polar head groups and the determination of the fatty acyl residues were feasible from the fragmentation patterns obtained.[26] FABMS/MS has been shown to be useful for the structural analysis of PLs, offering the potential to achieve complete structural characterization of phospholipid mixtures without a preceding separation.[21,24,27]

LC/MS techniques using a moving belt interface have been applied to the analysis of rat brain phospholipids in the positive-ion chemical ionization mode.[28] For complete HPLC/MS analysis, about 5 μg of an individual PL was injected to give specific fragmentation in the low mass range for each compound.

A rapid and relatively sensitive thermospray LC/MS method has been devised by Kim and Salem for the separation and analysis of the phosphatidylcholine and phosphatidylethanolamine molecular species; reliable structural information for each molecular species was achieved with short analysis times as well as for a complex mixture such as a natural phospholipid preparation from egg yolk.[29] The application of this method to the other PL major classes and also to neutral lipids such as triglycerides has been described by the same authors.[30] More recently, liquid chromatography/thermospray tandem mass spectrometry with plasmaspray ionization has been used for normal-phase analysis of glycerolipids;[31] collision-induced dissociation (CID) of diacylglycerol fragments proved useful for obtain-

* Author for correspondence.

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ing information on the chain length and on the degree of unsaturation of the esterified fatty acids in the native lipid.

Recent papers have reported the application of the LC/MS technique using an electrospray interface for the analysis of phospholipid molecular species[32] and the use of electrospray ionization tandem mass spectrometry for the characterization of bacterial phospholipids.[33]

The present study deals with the mass spectral characterization, including fragmentation processes, of the phospholipids reported in Scheme 1. Two different interfacing systems, viz. particle beam (PB) LC/MS and ionspray (ISP) LC/MS, were used with the aim of verifying the capabilities of these combined techniques with respect to a class of substances of particular interest in analytical chemistry. Product ion spectra following CID of quasi-molecular ions in a triple quadrupole instrument were obtained to identify individual molecular species within each phospholipid. Application of these techniques to the structural determination of molecular species of phospholipids in different soybean products is reported.

EXPERIMENTAL

Chemicals

PL standards were obtained from Fluka (Buchs, Switzerland) and were used without further purification: L-α-phosphatidylcholine (PC) from soybean (>99% purity, 100 mg/mL chloroform), L-α-phosphatidylethanolamine (PE) from soybean (>97% purity, 10 mg/mL chloroform) and L-α-phosphatidylinositol (PI) from soybean (>50% purity). Stock standards (1 mg/mL) and dilute standards (200 μg/mL) were prepared monthly; the more dilute working standard (2 μg/mL) was prepared weekly. All other chemicals (acetic acid, ammonium acetate) were of analytical reagent grade and were supplied by Carlo Erba (Milan, Italy). All the solvents were HPLC grade purchased from Lab-Scan (Dublin, Ireland). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). The soybean samples were purchased from a local market.

Liquid chromatography

The liquid chromatograph consisted of a Hewlett-Packard Model 1050 solvent delivery system (Palo Alto, CA, USA), and a Rheodyne 7125 injector fitted with a 20 μL sample loop (Rheodyne, Cotati, CA, USA). For UV detection the Hewlett-Packard HP 1050 variable-wavelength detector was used at 206 nm; the detector signals were monitored using the Maxima Data Acquisition software (Waters, Millipore, Milford, MA, USA).

Isocratic elution was performed using a 200×4 mm Nucleosil 50–5 column (Macherey-Nagel, Düren, Germany) and a mobile phase consisting of *n*-hexane+isopropanol+ammonium acetate buffer 0.2 M, pH 4.2 (47:47:6) at a flow-rate of 1.2 mL/min. The acetate buffer was prepared by mixing 0.2 M acetic acid aqueous solution with 0.2 M ammonium acetate solution up to pH 4.2.

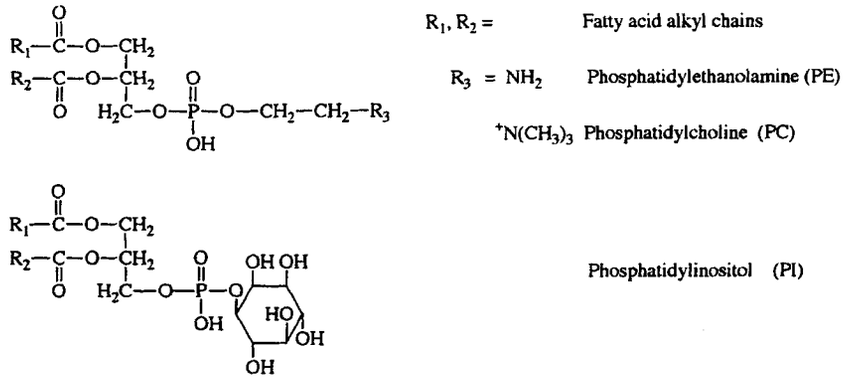
Liquid chromatography/mass spectrometry with a particle beam interface

LC/MS experiments with a Hewlett-Packard particle beam interface (HP 59980A) were carried out with a quadrupole mass spectrometer (HP 5989A) equipped with a dual EI/CI source and 1000 mass units range. The interface was operated with high-purity helium as nebulizing gas (inlet pressure 0.276 MPa), a desolvation chamber temperature of 50 °C and the capillary drawn out about 1 mm from the flush position of the nebulizer. Ionization was normally carried out in the positive-ion chemical ionization (PCI) mode, unless otherwise specified; the source temperature was 250 °C and the source pressure for methane CI was 159.9 Pa. The quadrupole temperature was 100 °C; it was operated with a collision energy of 230 eV. Full-scan conditions were used in all the experiments, with scanning from *m/z* 130 to 900. The HP MS 59940A ChemStation (HP-UX series) was used as an analytical workstation.

The LC system consisted of an HP 1090 chromatograph equipped with an HP 1050 autosampler. Chromatographic separations were carried out under the same conditions as for LC-UV, except for the flow rate which was kept at 0.8 mL/min.

Liquid chromatography/mass spectrometry with an ionspray interface

All ionspray LC/MS experiments were carried out on a PE-Sciex API III+triple quadrupole mass spectrometer (Sciex, Thornhill, Canada), equipped with an atmospheric pressure ionization (API) source and an ionspray interface. The spectra were acquired in positive-ion mode under the following conditions: nitrogen curtain gas flow, 0.8 L/min; nebulizer gas pressure (air), 0.276 MPa; ionspray voltage, 5 kV; orifice voltage, 60 V; scan range, *m/z* 700–950; scan rate, 21 ms/mass unit; no interscan delay; resolution >1 mass unit. Negative ions were also studied in the case of phosphatidylinositol by using the following conditions: ionspray voltage, 4 kV; orifice voltage, −60 V. For LC/MS experiments the column effluent was connected to the fused-silica transfer line (100 μm i.d.×1 m length) of the



Scheme 1. Structures of the phospholipids investigated.

ISP interface via a Rheodyne 7125 injection valve fitted with a 20 μL sample loop. The mobile phase used for all flow-injection experiments was methanol+water (50:50) at a flow rate of 50 μL/min.

Analyses were performed both by selected-ion monitoring, with *m/z* corresponding to quasi-molecular ions $[M+H]^+$ of phosphatidylcholine and phosphatidylethanolamine or $[M+NH_4]^+$ of phosphatidylinositol, and by selected-reaction monitoring, following the reactions *m/z* 758.4→184.0, 716.4→575.2 and 852.2→575.4, characteristic of PC, PE and PI respectively.The collision energy in this case was 25 eV. For selected-ion/monitoring LC/MS experiments a dwell time of 200 ms/mass unit was used. MS/MS measurements were based on collision-induced dissociation within the RF quadrupole at a collision energy of 30 eV. Product-ion LC/MS/MS scans were performed; argon was used as the target gas at an indicated thickness of 230×10^{13} molecules/cm^2.

The HPLC system consisted of a Perkin Elmer series 200 dual solvent delivery system using the column and mobile phase described above and a flow rate of 1 mL/min. The LC effluent was split after the column by a T-piece splitter, leaving a flow-rate of 25 μL/min to be directed to the mass spectrometer. A Macintosh Quadra 950 computer was used for instrument control, data acquisition and data processing using the Sciex API software 2.5 (Tune 2.4, MacSpec 3.22).

Sample preparation

Different soybean products were used as samples: lecithin from soybean, soybean seeds, soybean flour, soybean 'steak' and soybean chunks.

For the extraction of phospholipids from these products a rapid procedure was applied, as reported in the standardized IUPAC method set up for the analysis of lecithins:[10] 50–250 mg of sample were dissolved in about 3 mL HPLC mobile phase, transferred quantitatively to a 10 mL flask and then made up to volume with the same solvent. After filtration on 0.45 μm membranes (Lida, Kenosha, WI), the extracts were directly injected into the HPLC systems (20 μL) without any other treatment except for appropriate dilutions.

RESULTS AND DISCUSSION

Separation of the individual phospholipids was achieved, using aqueous normal-phase chromatography, by following a standardized IUPAC method set up for the determination of the phospholipid profile of lecithins by HPLC;[10] the separation was preliminarily checked using UV detection and the chromatographic conditions are reported in the Experimental section. Figure 1 shows the PB and the ISP total-ion currents (TIC) obtained from 20 μL injections of a standard mixture (200 μg/mL of each phospholipid). As expected, under ionspray conditions in positive-ion mode, phosphatidylcholine, which has a formal positive charge on the quaternary nitrogen, exhibits the greatest response, followed by phosphatidylethanolamine, whereas the non-ionic phosphatidylinositol gave a weak response.

Positive-ion chemical ionization PB mass spectra of the phospholipids contain similar fragmentation patterns, the quasi-molecular $[M+H]^+$ ions not being detectable (Fig. 2). The spectra contain diglyceride ions at *m/z* 575 and *m/z* 599 resulting from the loss of the phosphate ester head group. In addition, two fragments at *m/z* 239 and *m/z* 263 were detected in the spectra of all phospholipids examined; these

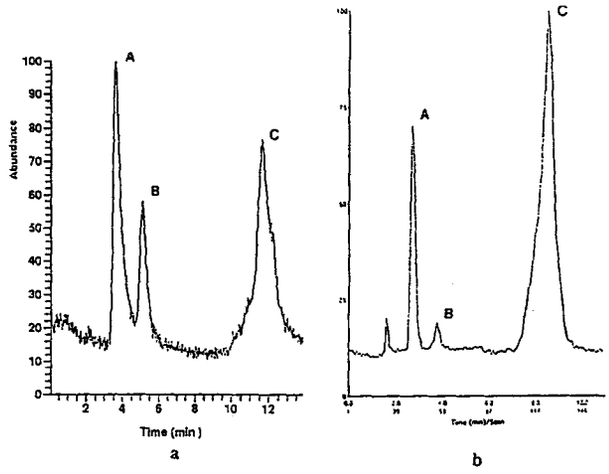


Figure 1. Separation and detection of phosphatidylethanolamine (A), phosphatidylinositol (B) and phosphatidylcholine (C) (4 μg each) using normal-phase chromatography and (a) PB-MS, PCI mode, (b) ISP-MS, positive-ion mode.

ions arise following a simple homolytic cleavage of the ester bond to yield commonly observed acylium ion $[R-C{\equiv}O]^+$ (e.g. *m/z* 239 corresponding to $[R_1-C{\equiv}O]^+$, R_1=C16:0 and *m/z* 263 corresponding to $[R_2-C{\equiv}O]^+$, R_2=C18:2). Abundant signals attributable to protonated fatty acids, C16:0 $[R_1C(OH)OH]^+$ at *m/z* 257 and C18:2 $[R_2C(OH)OH]^+$ at *m/z* 281, which are presumably formed by hydrolysis of the C(O)—O bond, appeared in all instances. These fragments have also been reported in positive-ion thermospray spectra of phosphatidylcholine and phosphatidylethanolamine.[29] Using methane negative-ion CI, intense carboxylate anions at *m/z* 255 ($[R_1-COO]^-$, R_1=C16:0) and *m/z* 279 ($[R_2-COO]^-$, R_2=C18:2) were observed, regardless of the polar head group.

The positive-ion ISP mass spectra of phospholipids are

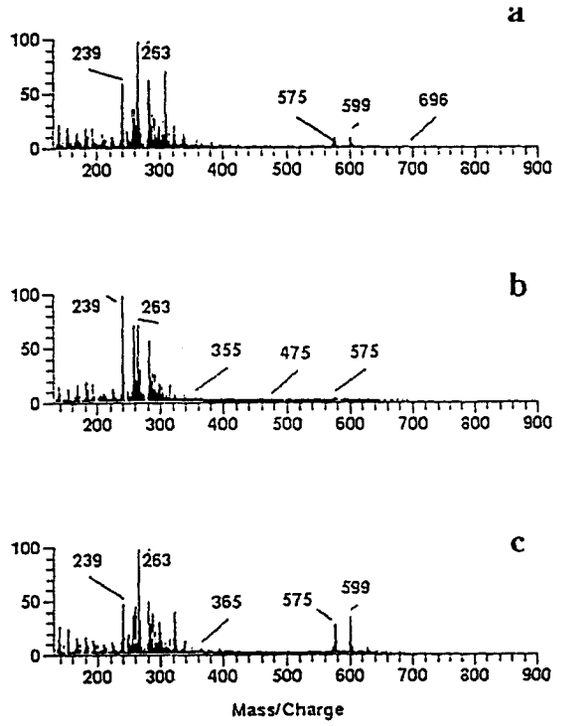


Figure 2. PB PCI full-scan mass spectra of (a) phosphatidylethanolamine, (b) phosphatidylinositol and (c) phosphatidylcholine. Scan range *m/z* 130–900.

quite simple, with few fragment ions observed, as illustrated in Fig. 3. In the spectra of the neutral phospholipids, PC and PE, protonated molecules with two different acyl groups were detected as the major peaks. Considering the plant source of these phospholipids, the materials generating the ions at *m/z* 716 in the spectrum of PE (Fig. 3a) and *m/z* 758 in the spectrum of PC (Fig. 3c) contain 16:0 and 18:2 fatty acids, with the unsaturated moiety most likely esterified at carbon-2 of glycerol. Similarly a PE and a PC with 18:2 and 18:2 acyl groups could produce the *m/z* 740 and *m/z* 782 peaks respectively. Sodium adducts of the 16:0, 18:2 species and of the 18:2, 18:2 species were also detected in the mass spectra of PE (*m/z* 738, *m/z* 762) and PC (*m/z* 780, *m/z* 804).

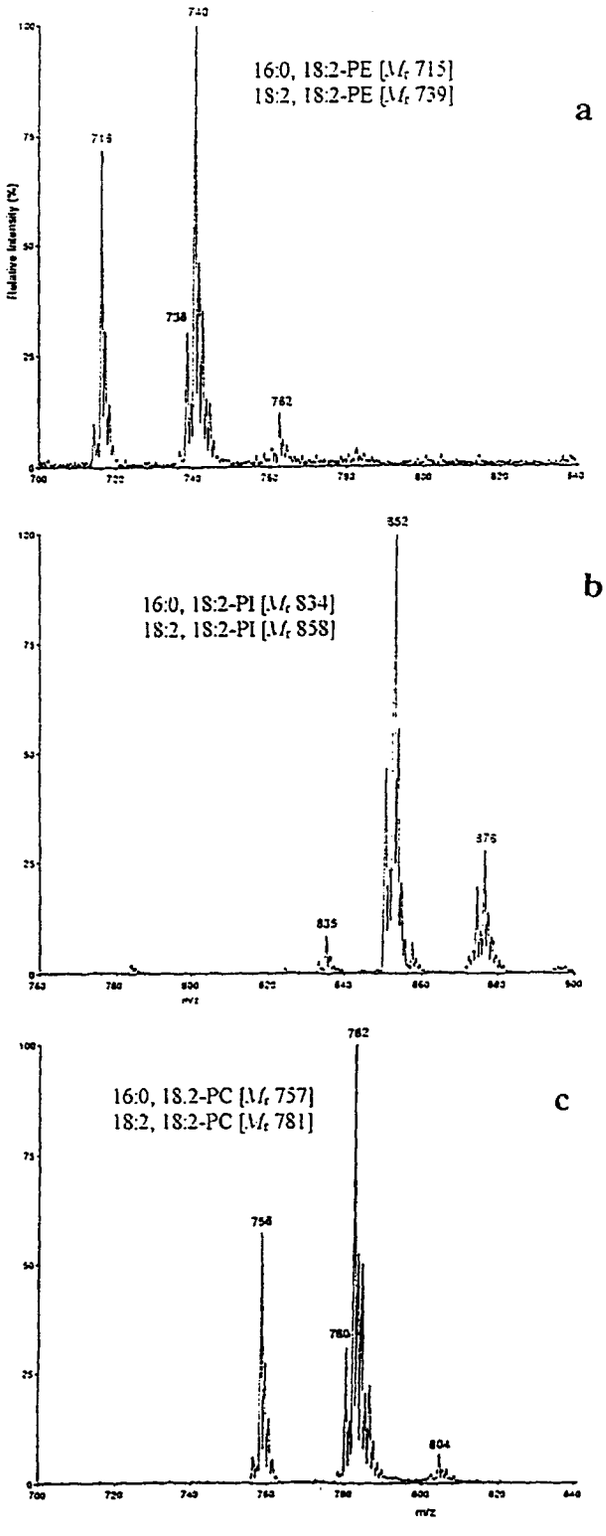


Figure 3. Positive-ion ionspray full-scan mass spectra of (a) phosphatidylethanolamine, (b) phosphatidylinositol and (c) phosphatidylcholine. Scan range *m/z* 700–900.

For acidic compounds such as phosphatidylinositol, which does not have positively charged or amine-containing polar head groups, detection of the negative ions produced by ISP was more successful (Fig. 4). These results are in agreement with those obtained in FAB experiments by other Authors.[34] The negative-ion ionspray spectrum of this compound contains a prominent $[M-H]^-$ at *m/z* 833 for 16:0, 18:2 – PI, whereas the $[M-H]^-$ ion for 18:2, 18:2 – PI gives a much lower intensity peak at *m/z* 857. For both species, fragments arising from the loss of inositol $[M-162]^-$ at *m/z* 672 and *m/z* 696 are observed. The loss of the ketenes (RC=C=O) corresponding to palmitic acid and linoleic acid gives the peaks at *m/z* 571 and *m/z* 595 respectively. In the positive-ion mode, however, ammonium adduct ions at *m/z* 852 and *m/z* 876, corresponding to the acyl substituents 16:0, 18:2 and 18:2, 18:2, are most prominent (Fig. 3b); the low abundance ion at *m/z* 835, which corresponds to the protonated molecular ion of 16:0, 18:2 – PI, can also be seen in the spectrum.

Analysis of product ions produced by collision-induced dissociation (CID) of molecular ion precursors, formed by ionspray, has proved advantageous for phospholipid structure analysis. Product ions obtained following CID of the $[M+H]^+$ ions from 16:0, 18:2 – PC and 16:0, 18:2 – PE and of the $[M+NH_4]^+$ from 16:0, 18:2 – PI are shown in Table 1. CID of the $[M+H]^+$ ions derived both from 16:0,18:2 – PC and from 18:2, 18:2 – PC, results preferentially in the formation of an abundant decomposition product at *m/z* 184, corresponding to the phosphocholine fragment $[(HO)_2P(O)-OCH_2CH_2N(CH_3)_3]^+$ (Table 1). Since this ion unequivocally characterizes this polar head group, it can be a diagnostic ion for phosphatidylcholines. On the other hand, there are additional ions of high mass but of very low intensity, which indicate the presence of diglyceride ions at *m/z* 575 for 16:0, 18:2 – PC (Table 1) and at *m/z* 599 for di-18:2 – PC; these ions reflect release of the polar head group as a neutral species, while the rest of the molecule retains the charge. Low-energy CID fragmentation of PC by FABMS/MS[22] and by IESP/MS/MS[33] usually leads to a formation of a single decomposition product at *m/z* 184. Another specific ion product of PC at *m/z* 86 can be attributed to the fragment $[CH_2=CHN(CH_3)_3]^+$.

CID of the $[M+H]^+$ ion from two molecular species of phosphatidylethanolamine, viz. 16:0, 18:2 – PE and 18:2, 18:2 – PE, is dominated by cleavage of the phosphate–glycerol bond, with charge retention on the lipid portion of the molecule. Hence, in the product ion mass spectra of these molecular species, prominent peaks at *m/z* 575 (parent ion is *m/z* 716) (Table 1) and at *m/z* 599 (parent ion is *m/z* 740) corresponding to diacyldiglyceride ions 16:0, 18:2 and di-18:2 are observed. In addition, monoglyceride ions containing 16:0 and 18:2 fatty acids appear at *m/z* 313 and *m/z* 337 respectively; monoglyceride ions serve to identify the fatty acyl groups esterified at the *sn*-1 and *sn*-2 positions of the phospholipids. Product ions from the *m/z* 716 ion of 16:0, 18:2 – PE also include both the acylium ions, $[R_1-C\equiv O]^+$ at *m/z* 239 and $[R_2-C\equiv O]^+$ at *m/z* 263, whereas among the product ions generated from CID of the ion at *m/z* 740 ($[M+H]^+$ of 18:2, 18:2 – PE) only the $[R_2-C\equiv O]^+$ ion appears at *m/z* 263, as expected. The ions

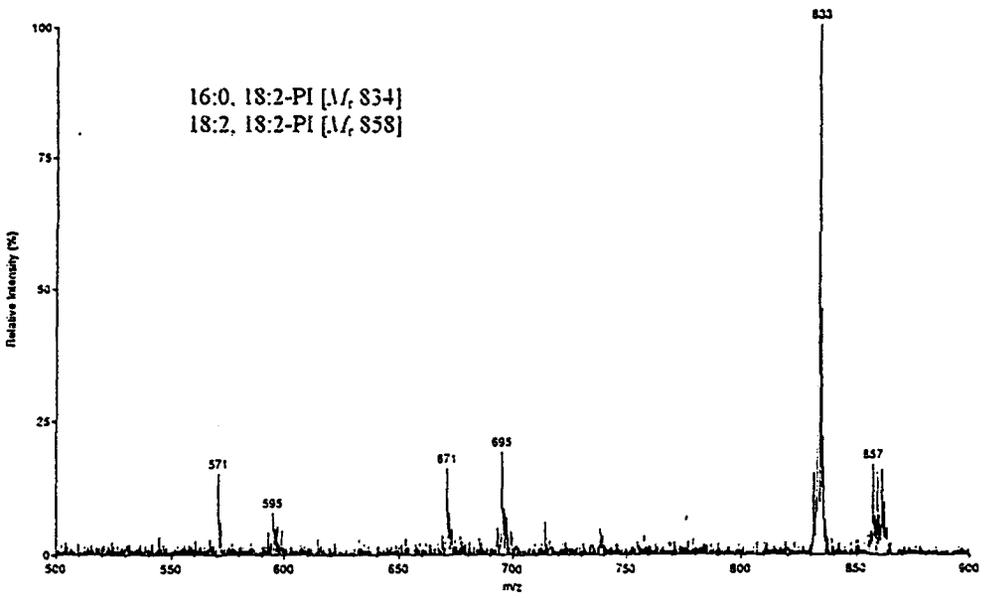


Figure 4. Negative-ion ionspray full-scan mass spectrum of phosphatidylinositol. Scan range *m/z* 500–900.

at low mass are dominated by a characteristic series of homologous peaks, of odd mass numbers down the entire length of the unsaturated 18:2 fatty acid chain which is present in the acyl substituents at *sn*-2. These ions at *m/z* 67, 81, 95, 109, 123, 137,..., have the structure $[(CH_2)_nCH{=}CH{-}CH_2{-}CH]^+$. It is noteworthy that, so far, the CID of protonated molecular species of phospholipids have been reported only using FAB ionization.[21,25]

Phosphatidylinositol behaves in a similar fashion. In fact, CID of the positive ammonium adduct ion $[M+NH_4]^+$ (*m/z* 852), derived from 16:0, 18:2 – PI, results in an intense ion corresponding to the diglyceride ion at *m/z* 575, whereas the presence of the less abundant fragment ions at *m/z* 239 and 263, attributable to the acylium ions $[R-C{\equiv}O]^+$, confirms the fatty acid assignment of a 16:0 and of an 18:2 species (Table 1). A fragment appearing at *m/z* 754 accounts for the loss of the (–O–P(O)–OH) moiety from the intact molecule.

Finally, a comparison between analyses of phospholipid standards carried out both by selected ion monitoring (SIM), using an *m/z* value corresponding to a protonated or an ammoniated molecular ion, and by selected-reaction monitoring (SRM) following the reactions described in the Experimental section, was performed. In the chromatographic trace obtained using SIM for 40 ng of PLs, phosphatidylinositol was not detectable (Fig. 5a). With phospholipids injected at the same level, the use of the SRM technique (Fig. 5b) provided better results in terms of selectivity for all the compounds analyzed, owing to the higher signal-to-noise ratio. The selectivity observed with SRM on phospholipids suggests a possible application of this technique to the analysis of low-level samples, such as those of biological and clinical origin. In fact, from the signal-to-noise ratio shown in Fig. 5b, it is evident that even lower amounts could be easily detected by HPLC/ISP/MS/MS operating in SRM mode.

Table 1. Intensity of product ion abundances from CID of the ISP-generated $[M+H]^+$ ions derived from 16, 0: 18:2 – PC and 16,0:18:2 – PE and of $[M+NH_4]^+$ ions derived from 16,0:18:2 – PI

Molecular species 16:0, 18:2 – PC		Molecular species 16:0, 18:2 – PE		Molecular species 16:0, 18:2 – PI	
Product ion (*m/z*)	Intensity	Product ion (*m/z*)	Intensity	Product ion (*m/z*)	Intensity
60	667	57	1200	239	600
86	9667	67	1000	263	500
104	1556	81	2300	575	30 700
125	5778	95	3400	754	400
184	228 589	109	1967	852	200
316	867	123	1300		
478	1222	137	1300		
496	1778	149	900		
575	444	161	1000		
758	5689	175	600		
		239	2200		
		263	2300		
		306	1900		
		313	1700		
		337	1200		
		575	41 800		
		601	200		

Identification of phospholipids in soybean products

Fig. 6 shows the total ion current LC/PB-MS (PCI mode) and the LC/ISP-MS (positive-ion mode) chromatograms of a crude soybean lecithin sample. From the TIC profile obtained using PB-MS (Fig. 6a), five peaks were detected, three of which eluted at the retention times of the phospholipids under investigation; however, these substances could not be differentiated on the basis of the PCI mass spectra, and thus retention times were confirmed by injection of pure materials. Additional components eluted at 2.05 and 2.55 min but did not give clearly interpretable PCI spectra; they contained fragment peaks at *m/z* 239, 257 and 263, which are also present in the spectra of the phospholipids discussed above, in the latter case being attributable to ions from the esterified fatty acid groups at the *sn*-1 and *sn*-2 positions of the phospholipids. Because of the presence of a signal at *m/z* 313, corresponding to the monoglyceride ion of C16:0 fatty acid, the structure of an acylglycerol was hypothesized, but, as fragment ions in the high-mass region were lacking, identification was difficult. Another problem arising in the experiments with the PB interface was the

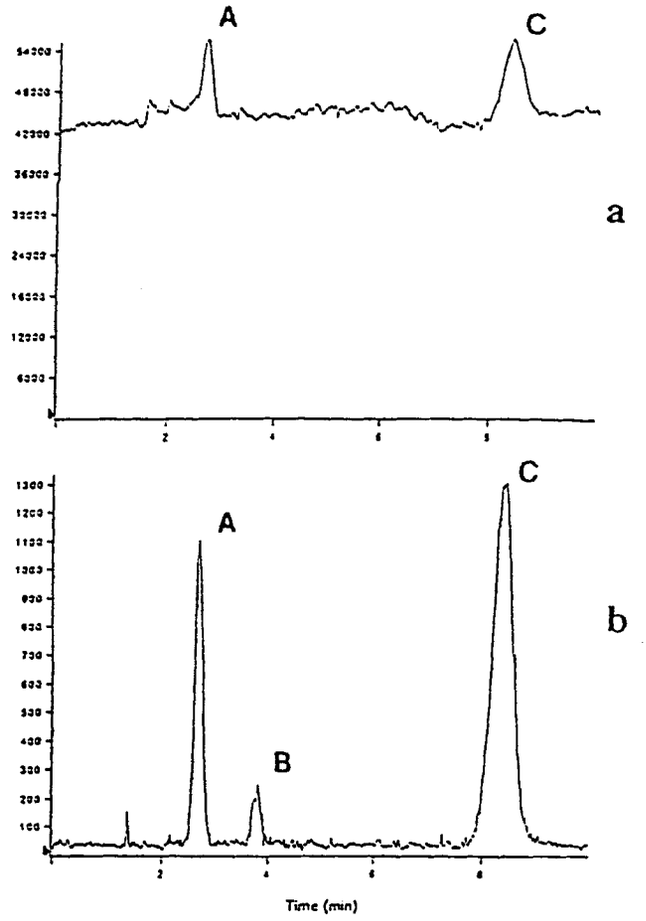


Figure 5. LC/ISP-MS analysis of a mixture of phospholipid standard (40 ng each): (a) selected-ion monitoring chromatogram (positive-ion mode) at *m/z* 716 for PE (A), *m/z* 852 for PI (B) and *m/z* 758 for PC (C); (b) LC/MS/MS selected-reaction monitoring chromatogram; conditions as in Experimental.

observation of considerable instrument contamination, with an accompanying decrease in the overall performance, after injection of crude soybean extracts into the LC/PB-MS system. In particular, clogging of the skimmers in the interface and contamination of the EI/CI repeller in the MS source were observed, thus indicating that an LC/MS system with a PB interface is not adequate for the analysis of this class of substances in real samples. As a consequence of all these observations, only ISP mass spectra in the samples investigated will be discussed from now on.

The ISP total-ion current trace (Fig. 6b, *m/z* 700–950) shows the presence of the phospholipids under investigation, whose spectra display strong quasi-molecular ions, as mentioned above. In addition, two prominent peaks at retention times 1.58 and 8.43 min are visible. The peak which eluted at 1.58 min gave the ISP mass spectrum reproduced in Fig. 7a, which shows a series of abundant high-mass ions representing quasi-molecular ions from a mixture of triglycerides. The signals at *m/z* 849, *m/z* 873 and *m/z* 897 were attributed to ammonium adducts $[M+NH_4]^+$ corresponding to the triacylglycerols 16:0 18:2 16:0, 16:0 18:2 18:2, and 18:2 18:2 18:2 respectively, based on the composition of triglycerides from soybean oil.[35] Ionspray ionization of triglycerides in the presence of an ammonium salt in the LC mobile phase provides the same results that are obtained by performing desorption chemical ionization analysis of soybean triglycerides with ammonia as a reagent gas.[35] Also Murphy[25] reported

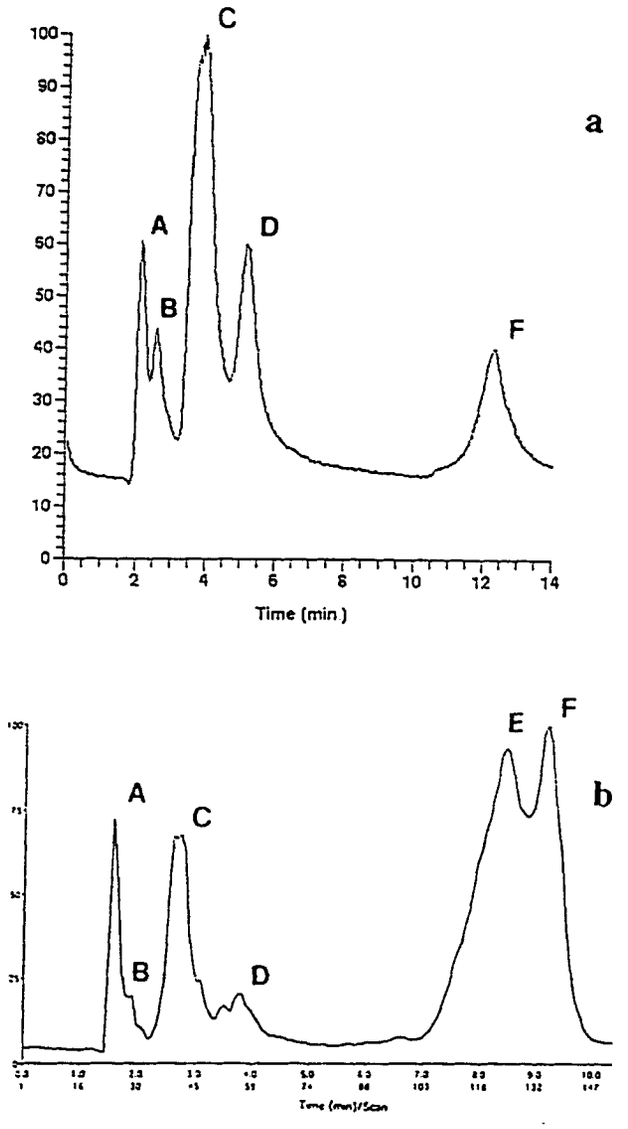


Figure 6. Total-ion current LC/PB-MS (a) and LC/ISP-MS (b) chromatograms of an extract from soybean lecithin.

abundant $[M+NH_4]^+$ ions for ammonia chemical ionization of these lipids, as expected on the basis of the proton affinity of esters relative to that of ammonia. Electrospray (ESP) ionization of triglycerides has previously been reported by Duffin *et al.*,[36] who highlighted the potential of ESP-MS analysis in discriminating these compounds from fatty acids and mono- and diglycerides because of the absence of fragmentation of triacylglycerols. Conversely, other techniques such as FABMS or plasma desorption are known to produce fragmentation, making discrimination impossible. As for the signal at 8.43 min (Fig. 6b), although this peak partially coeluted with that of phosphatidylcholine, the structural information provided on-line by the LC/ISP-MS technique allowed the identification of this substance as phosphatidylserine (PS). Protonated molecules at *m/z* 760 and *m/z* 784 were detected as the major peaks for both the molecular species 16:0, 18:2 – PS and 18:2, 18:2 – PS respectively; correspondingly, formation of sodium adducts produces peaks at *m/z* 782 and *m/z* 806 (Fig. 7b).

In the case of the crude extract of a variety of soybean seeds, a full-scan positive-ion ISP-MS chromatogram shows a series of different peaks, some of which were identified from the on-line mass spectra (Fig. 8). In this case, the most intense signal was that of the compound at

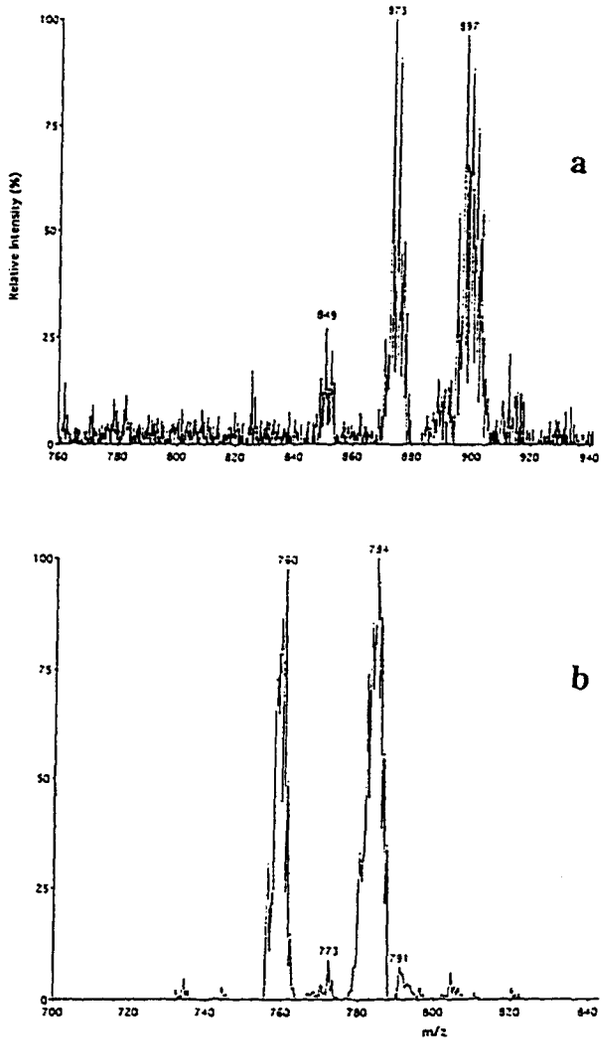


Figure 7. Positive-ion ISP mass spectra for (a) triglycerides and (b) phosphatidylserine detected in lecithin from a soybean sample.

retention time 1.57 min, whose ISP spectrum contains ions at *m/z* 849, *m/z* 873 and *m/z* 897 which are characteristic of a mixture of soybean triglycerides. In the ISP spectra of the compounds eluting at 2.65 min, 4.60 min and 9.39 min, the molecular masses confirm the presence of the expected compounds, PE, PI and PC respectively. Other minor peaks are apparent in the chromatogram at retention times 5.51 min and 6.78 min; two specific ions at *m/z* 884 ($[M]^+$) and at *m/z* 902 ($[M+NH_4]^+$) clearly identify the former peak as the triacylglycerol 18:1 18:1 18:1. The spectrum acquired during the elution of the peak at 6.78 min is characterized by two ions, including the molecular ion, by a mass difference of 18; the base peak at *m/z* 927 presumably corresponds to the $[M+NH_4]^+$ ion from the triacylglycerol 18:1 18:1 20:4. This chromatographic peak was detected only in this particular variety of soybean seed extract and in the extract of soybean flour. The ISP mass spectrum acquired on-line for the peak at 8.27 min in Fig. 8 displays protonated molecular ions at *m/z* 760 and *m/z* 784, indicating the additional presence of phosphatidylserine in this sample.

Other samples investigated were soybean flour, soybean

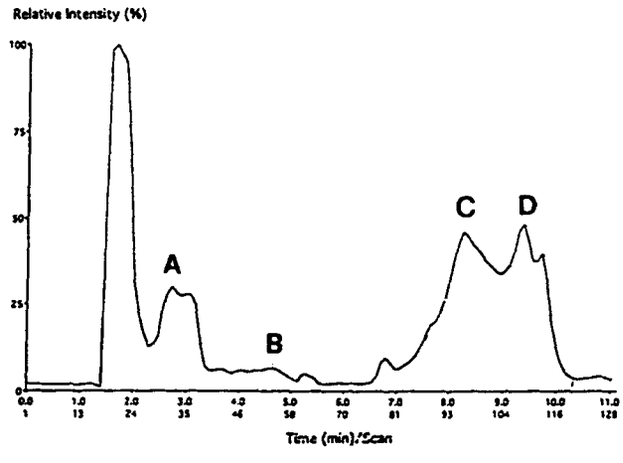


Figure 8. Total-ion current LC/ISP-MS chromatogram of the extract from soybean seeds. Peaks: A, phosphatidylethanolamine; B, phosphatidylinositol; C, phosphatidylserine; D, phosphatidylcholine.

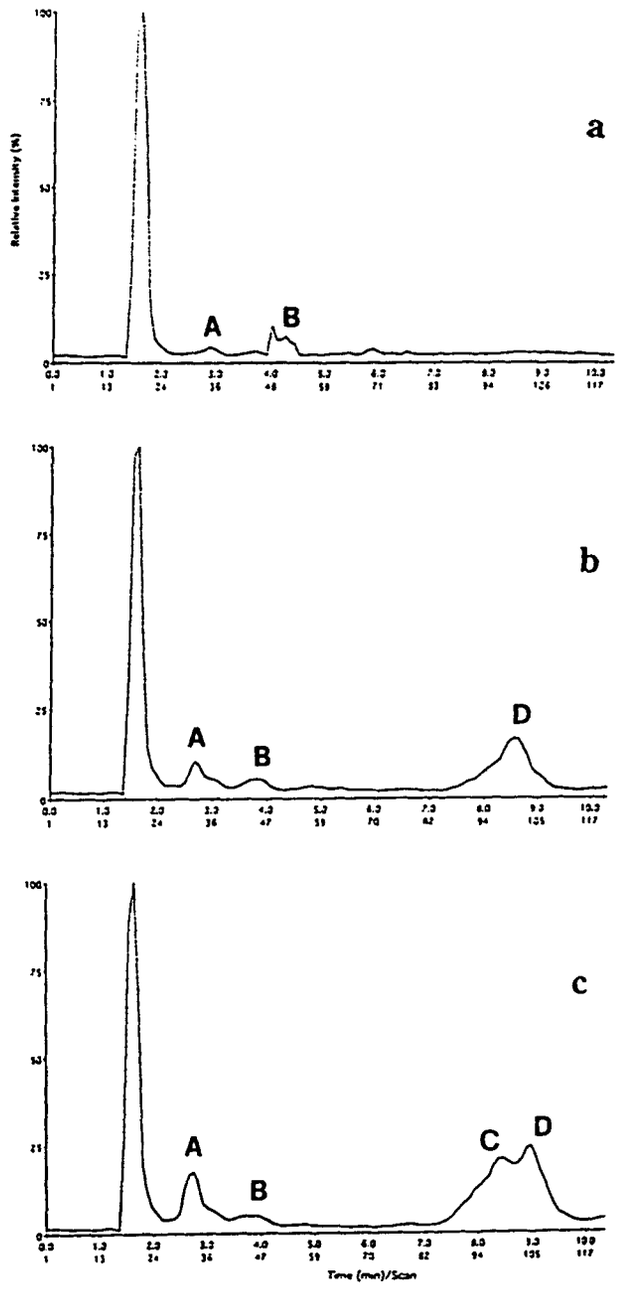


Figure 9. Total-ion current LC/ISP-MS chromatograms of the extracts from (a) soybean flour, (b) soybean 'steak' and (c) soybean chunks. Peaks: A, phosphatidylethanolamine; B, phosphatidylinositol; C, phosphatidylserine; D, phosphatidylcholine.

'steak' and soybean chunks, whose ISP total ion chromatograms are displayed in Fig. 9. In all these samples, the earlier-eluting peak, whose ISP-MS spectrum contained ammonium adduct ions of soybean triacylglycerols, was the most abundant. In the TIC of soybean flour extract (Fig. 9a), the phospholipids identified were phosphatidylethanolamine (retention time 2.91 min) and phosphatidylinositol (retention time 4.02 min). Crude extracts of the other two soybean products show similar TIC profiles; in addition to triglycerides, peaks of other expected compounds are also visible. The additional signal at retention time 8.38 min in Fig. 9c, showing ions at *m/z* 760 and *m/z* 784, is due to phosphatidylserine.

CONCLUSION

Normal-phase HPLC/ISP-MS was proved to be valuable for the analysis of different phospholipid classes also in complex matrices, such as crude extracts of soybean products. Considering the polar head group, the method of choice for the analysis of PE and PC is ionspray ionization in the positive-ion mode, whereas better results were achieved for PI by ionizing in the negative-ion mode. Positive-ion CID mass spectra provided structural information on the nature of the fatty acyl groups esterified at the *sn*-1 and *sn*-2 positions of phospholipids and, in the case of PC, on the nature of the phosphate ester head group. As for the HPLC/PB-MS system, fragmentation patterns of phospholipids obtained under both positive-ion and negative- ion chemical ionization conditions were not helpful in characterizing different classes of these substances. In addition, instrument contamination with a decrease in the overall performance after injection of crude extracts of soybean products into the HPLC/PB-MS system was observed.

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