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Characterization of Phospholipid Molecular Species by Means of HPLC-Tandem Mass Spectrometry

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1. Introduction

Phospholipids are the main constituents of the permeability barrier of cells and subcellular organelles. The phospholipid bilayer is the environment in which isolated proteins or dynamic nanoassemblies of sterols, sphingolipids, and proteins, called lipid rafts, act their vital functions, such as energy transduction, signal transduction, solute transport, DNA replication, protein targeting and trafficking, cell-cell recognition, secretion and many others (Lingwood et al., 2010). Christie et al. (2011) reported a detailed description of the general structure of phospholipids. According to Sud et al., 2007, phoshopholipids represent the category of lipids with the highest variety of structures (7775), followed in descending order, by polyketides (6713), fatty acyls (3942), sphingolipids (3936), glycerolipids (3044), sterol lipids (2196) and others.

The positive effects of dietary phospholipids (PL) on hepatic lipid metabolism, atherosclerosis, obesity-related disorders and cardiovascular disease is a consistent experimental evidence (Shirouchi et al., 2007). The daily intake of PL can vary from 2 to 8 g per day and represents 1-10% of total daily fat intake (Cohn et al., 2008). The main natural phospholipid is phosphatidylcholine (lecithin) which is completely absorbed in humans. Natural or synthetic PLs are extracted from eggs and soybean and used as drug delivery systems since decades (Papahadjopoulos, 1978).

During brain development, one of the most efficient forms of supplying ω3-fatty acids such as DHA (docosahexaenoic acid) are phospholipids (Bourre & Dumont, 2002). In animal studies, it was reported that krill oil (rich in ω3-containing PLs) had stronger effects with respect to fish oil (rich in ω3-containing triacylglycerols) in increasing the DHA level in rat brain (Di Marzo et al., 2010). Although ω3 are predominantly linked in the position Sn-1,3 of triacylglycerols (TG) in seal blubber oil, they are esterified in the Sn-2 position of the TGs and PLs of eggs obtained after feeding laying hens with enriched diets. Moreover, more ω3 fatty acids are incorporated in phosphatidylethanolamine (PE) and phosphatidylcholine (PC) than in TGs (Pacetti et al., 2005). Apoptotic events and age-related diseases are strictly related to oxidized phospholipids. Much research remains to be done for the PL characterization by Liquid chromatography (LC) – tandem mass spectrometry (MS) aimed to understand their biological and

physiopathological activity (Domingues et al., 2008). In the food industry, phospholipids are considered among the best emulsifying agents. Commercial preparations derived from soybean and corn oils (commercial *lecithins*) are used extensively in manufactured foods such as bakery items, frostings, non-dairy creamers, confectionery products and ice creams. Staling and off-flavors are often related to the deterioration of the functional lipids in foods (Weihrauch et al., 1983). PLs extracts from eggs and/or fish products can play a pivotal role as an innovative food ingredient (Dumay et al., 2009; Commission Decision, 2000). Recently, a lipid extract rich of ω3-containing PLs from krill (*Euphausia superba*) has been authorised by the EC as novel food/food ingredient (Commission Decision, 2009). Species, geographical origin, and production method of fish (i.e. wild/farmed) are the information which must be labelled in fishery and aquaculture products according to EU labelling regulations (Commission Regulation (EC) No 2065/2001). TGs are considered as markers for wild and farmed fish since their profile reflects the diet lipids (Standal et al., 2010), whereas the phospholipid profile is less affected by the diet and can be related to other variables, such as the species and stock (Joensen et al., 2000).

2. Classes of the main phospholipids and their abundance

The typical range of composition of a mammalian nucleated cell was reported by Vance & Steenbergen (2005). According to these authors, phosphatidylcholine and phosphatidylethanolamine, the quantitatively major phospholipids, range 45–55 % and 15–25 % of total lipids, respectively. Other quantitatively minor phospholipids are phosphatidylinositol (10–15%), phosphatidylserine (2–10%), phosphatidic acid (1–2%), sphingomyelin (5–10%), cardiolipin (2–5%) and glycosphingolipids (2–5).

Cardiolipin (CL) can be found predominantly in the mitochondrial inner membrane (Xiao et al., 2011). CL shows different physiological roles, including the maintainance of the structure of membrane-embedded proteins and proton trapping during oxidative phosphorylation (Houtkooper & Vaz, 2008). Abnormal cardiolipin metabolism was related to a life-threatening inherited disease, the Barth syndrome, as well as other important diseases, such as ischemia/reperfusion injury, heart failure, neurodegeneration, and diabetes (Chicco & Sparagna, 2007). Anticardiolipin (aCL) antibody of IgG and/or IgM isotype in serum or plasma, present in medium or high titer, is one of the classification criteria for the antiphospholipid syndrome (APS). APS is associated with thrombosis, heart valve lesions, renal small-artery vasculopathy, chronic renal ischemia and thrombocytopenia (Miyakis et al., 2006).

Phosphatidic acid (PA) has recently been reported as a lipid messenger involved in various cellular functions in plants, animals, and microorganisms. PA is linked to different regulatory processes, such as signaling pathways in cell growth, proliferation and reproduction, as well as in responses to hormones and biotic and abiotic stresses (Wang et al., 2006).

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant phospholipids in eukaryotic cells and thus have major roles in the formation and maintenance of vesicular membranes (Henneberry et al., 2001). In mammalian cell membranes, PC constitutes 40–50% of total phospholipids and can be synthesized by either of two pathways, the methylation pathway or the CDP-choline pathway. PE is the second most abundant mammalian membrane phospholipid and constitutes about 45% of total phospholipids in the brain, but only about 20%of total phospholipids in the liver (Morita et

al., 2010). Both PC and PE are considered a source of arachidonic acid (together with phosphatidylinositol), which is liberated by phospholipase A2. Arachidonic acid is converted via the cyclo-oxygenase and lipoxygenase pathways to eicosanoids, including prostaglandins, thromboxanes, prostacyclins, leukotrienes and lipoxins (Billah & Anthes, 1990). Although many prokaryotes lack it, PC can be found in significant amounts in membranes of rather diverse bacteria. It is assumed that more than 10% of all bacteria possess PC (Sohlenkamp et al., 2003). In prokaryotic cells, in which PE is the most abundant membrane phospholipid, all of the PE is derived from PS decarboxylation (Vance & Steenbergen, 2005).

Lysophospholipids such as **Lysophosphatidylcholine (LPC)** are released by phospholipase A2 together with the fatty acid. According to Qiao et al., 2006, LPC is proinflammatory and atherogenic. In the vascular endothelium, LPC increased permeability and expression of proinflammatory mediators such as adhesion molecules and cytokines. However, saturated LysoPC in high concentrations reduced melanoma cell adhesion *in vitro* (Jantscheff et al., 2011). Certain lysophospholipids such as ether-linked lysophosphatidylcholine are acetylated by a specific acetyltransferase to form platelet activating factors (Hanahan, 1986).

Ether-linked PE includes plasmanylethanolamine and plasmenylethanolamine which are characterized by an ether bond (not an ester) at the *sn*-1 position of the glycerol moiety. Plasmenylethanolamine is also called **phosphatidylethanolamine plasmalogen (pPE)** and displays a *cis* double bond on the alkyl chain, adjacent to the ether bond, forming a vinyl-ether linkage. Plasmalogens of PE and PC range approx. 18% of phospholipids in humans, but the plasmalogen content of individual tissues or cell types varies widely. It was demonstrated that liver cells export plasmenylethanolamine with lipoproteins. In erythrocytes, kidney, lung, testes and skeletal muscle, the plasmenyl forms account for 20-40% of the total amount of ethanolamine phosholipids. These values were found to be higher in brain, heart, lymphocytes, spleen, macrophages and polymorphonuclear leukocytes.

Phosphatidylinositol (PI) is the precursor of phosphatidylinositol phosphates upon the activation of PI-kinases, which are implicated in thrombin-mediated signaling processes in platelets (Holinstat et al., 2009). Phosphatidylinositol transfer proteins (PITPs) bind and facilitate the transport of phosphatidylinositol (PI) and phosphatidylcholine between membrane compartments; they are able to sequester PI in their hydrophobic pocket. Their knockdown affects signal transduction, membrane trafficking, stem cell viability, neurite outgrowth and cytokinesis (Cockroft et al., 2011).

Phosphatidylserine (PS) is a quantitatively minor membrane phospholipid. It is produced both by prokaryotic and eukaryotic cells. PS is not simmetrically exposed across the cell membrane. It is generally exposed on the outside surface of cell membranes and is widely believed to play a key role in the removal of apoptotic cells and in initiation of the blood clotting cascade. Upon decarboxylation, PS is also converted into phosphatidylethanolamine in bacteria, yeast and mammalian cells. PS is present in brain in higher amounts with respect to other mammalian tissues such as the liver, particularly in molecular species containing docosahexaenoic acid, which accounts for 30-40% of the total fatty acid content of the human grey matter. Moreover, PS is a cofactor of important enzymes, such as some protein kinases (Vance et al., 2005).

Sphingomyelin (Sph) is one of the main phospholipid component of the lipid rafts (Simons et al., 1997) which affect the membrane fluidity and membrane protein trafficking. Sphingolipid-cholesterol nanoassemblies are essential to the activity of some membrane

proteins because they regulate the subcompartmentalization propensity of the phospholipid bilayer at little energetic cost (Lingwood and Simons, 2010).

3. Methods of analysis of phospholipids

The characterization of the phospholipid profile is a strategic tool in order to evaluate the nutritional properties of several food products, food ingredients, nutraceuticals, functional foods, cosmetics and pharmaceutical products containing polar lipids (Cohn et al., 2008) or liposomes (Henna Lu et al., 2010). Moreover, the increased commercial use of phospholipids as ingredients for functional food, baby-food and pet food, has lead to the development of specific analytical methods in order to separate and identify phospholipids (Le Grandois et al., 2009; Standal et al., 2010; Winther et al., 2010).

For these reasons, the terms 'phospholipidomics' and 'phospholipidomic analysis' have been recently introduced to define the study of the phospholipid classes in their biological and natural environment in order to detect characteristic fingerprints of metabolic processes (Viola et al., 2007). FTIR was used for the determination of the total content of phospholipids in *biceps femoris* of pigs (Villé et al., 1995). However, the fingerprint of the phospholipid classes and possibly of the molecular species of PL is often the necessary premise for biological, medical and food research studies.

3.1 Extraction

The isolation of phospholipids at an analytical scale is usually accomplished upon solvent/solvent extraction of the total lipids of a sample and further purification of the PL fraction. In food products, the main fraction of total lipids is usually formed by neutral lipids, i.e., triacylglycerols. Phospholipids are the minority of the total lipids, ranging from less than 0.1% in some plant oils, to about 5-25% in some dairy products, 20-30% of the total lipids in egg derived products and more in lean meats. In soy, lecithin is bound to proteins. A detailed survey of the phospholipid content of several kinds of food was reviewed by Weihrauch & Son (1983). **Table 1** reports the total phospholipid content of egg, dairy and meat products as reported by more recent literature.

For analytical purposes, the extraction of the total lipid fraction according to the procedures firstly described by Folch et al. (1957) and Bligh & Dyer (1959) and their successive modifications more than 50 years ago are still extensively used, because the extraction yield is practically quantitative. Thus, these methods are preferred to the extraction with petroleum ether or Soxhlet extraction. Novel methods of extraction of total lipids are based on pressurized liquid extraction (PLE) (Boselli et al., 2001) and even neat supercritical CO_2 (Boselli et al., 2000).

3.2 Isolation and purification

The clean-up of the PL fraction is needed prior to HPLC analysis in order to increase the sensitivity of the determination when the PL content is relatively low compared to the neutral lipids. Usually, membrane lipids (lean meat or fish or egg products and cell membranes) are supposed to contain a PL content (Table 1) which is suitable for direct HPLC analysis. This is also the case of commercial lecithins (AOCS, 2007; ISO, 2009). However, if a better sensitivity is needed, the main ancillary techniques used for PL cleanup prior to HPLC analysis are column chromatography, solid phase extraction (SPE) and thin layer chromatography (TLC) with a normal phase approach.

	Food product	mg PLs/100 mg total fat	mg PLs/1000 mg food product	Reference
Egg products	Egg yolk powder	31		Aro et al., 2009
Dairy products	Milk (in vat)	0.4	0.09	Avalli et al., 2005
	Raw milk	0.71	0.29	Rombaut et al., 2005
	Butter	0.2	1.6	Avalli et al., 2005
	Butter	0.22	1.81	Rombaut et al., 2005
	Cream (natural)	0.9	1.98	Avalli et al., 2005
	Cream	0.35	1.39	Rombaut et al., 2005
	Cream (centrifuged)	0.5	0.9	Avalli et al., 2005
	Buttermilk	4.5	0.9	Avalli et al., 2005
	Buttermilk	21.8	0.91	Rombaut et al., 2005
	Whey (Cheddar)	5.3	0.18	Rombaut et al., 2005
	Quarg	24.7	0.32	Rombaut et al., 2005
	Cheese (Cheddar)	0.47	1.5	Rombaut et al., 2005
	Cheese (Grana Padano)	0.16-0.59	n.r.	Caboni et al., 2000
Meat	Goat meat	n.r.	0.062-0.082	Almeida et al., 1997
	Rabbit meat	9-19	n.r.	Cambero et al., 1991
	Pork muscle (glycolitic)	n.r.	4.8	Leseigneur-Meynier et al., 1991
	Pork muscle (oxidative)	n.r.	8.6	Leseigneur-Meynier et al., 1991
	Pig (commercial meat)	56.1	8.8	Villé et al., 1995
	Pig (biceps femoris)	74.6	10.5	Villé et al., 1995
	Beef (steer)	4.4-22.2	4.6-5.6	Duckett et al., 1993
	Lambs (*longissimus toracis*)	24.7-41.8	5.8-6.6	Aurousseau et al., 2004

Table 1. Phospholipid content of some food products of animal origin (n.r., not reported).

Hoischen et al. (1997) described a procedure aimed to separate the main lipid classes by using column chromatography. Total lipids dissolved in chloroform were fractionated on a silica gel stationary phase into neutral lipids (eluted with chloroform), glycolipids (acetone-chloroform, 95:5 by vol.), and phospholipids (methanol- chloroform 90:10 by vol.).

An example of a SPE clean-up procedure used for both the neutral lipids and polar lipids of vegetable origin is reported by Pacetti et al. (2007). The Bligh& Dyer lipid extract (10 mg) was dissolved in 200 μl of chloroform/methanol (2:1, v/v) and subjected to SPE by using a silica gel stationary phase packed in a cartridge of 6-ml volume (1 g of adsorbent). The purification was performed with sequential elution of 5ml hexane/diethyl-ether (4:1, v/v), 5ml hexane/diethyl ether (1:1, v/v), 5ml of methanol and 5ml of chloroform/ methanol/water (3:5:2, v/v/v). The fractions of hexane and diethyl ether, containing neutral lipids (NL), were combined, dried and used for fatty acid (FAME) analysis after transmethylation. The fractions of methanol and chloroform/methanol/water, containing polar lipids (PoL), such as glycolipids and phospholipids, were combined, dried and used for HPLC/MS and FAME analysis of the polar fraction. Caboni et al., 1996 applied SPE columns packed with several different stationary phases (silica, aminopropyl, C18 and C8) to the extraction of PL from fatty foods.

A simple TLC procedure described by Ferioli & Caboni (2010) for the purification of PL prior to GC analysis of FAME can be potentially applied to purify the PL fraction prior to HPLC injection. These authors dried 20 mg of lipids under nitrogen. Successively, the lipids were dissolved in 100–150 μL of chloroform and were loaded on a Silica gel TLC plate. The eluent was a mixture of n-hexane/diethyl ether 3/2 (v/v). The total PL band was located at the origin and was visualised under UV light (254 nm) by spraying the TLC plate with a 0.02% (w/v)

ethanolic solution of 2,7-dichlorofluorescein (sodium salt) and then was scraped off and collected. Successively, PL were extracted three times with chloroform (1 mL each).

4. HPLC Separation, detection and quantitation

4.1 HPLC separation

The separation of PL into different classes can be accomplished either with normal phase (NP) or reversed phase (RP) high performance liquid chromatography.

NP-HPLC is more extensively applied than RP-HPLC to characterize PL from biological tissue and food products. With NP-HPLC, phospholipids are separated essentially on the basis of the polarity of the head group. They elute by order of increasing polarity: phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (Sph). The lyso forms elute after their corresponding parent phospholipid. Almost all the published methods use a porous silica stationary phase, usually 100 or 250 mm in length, with an internal diameter ranging from 2.0 to 4.6 mm. Recently, monolithic silica gel columns have been used increasingly more often for the separation of lipids, and several applications have been published (Jakab & Forgács, 2002; Merlin et al, 2006). Graeve and Janseen (2009) improved the HPLC–evaporative light scattering (ELSD) method, which permits the separation and quantification of lipid classes ranging from squalene (with a very low polarity) to highly polar lysophosphatidylcholine, using a monolithic silica stationary phase (100mm×4.6mm I.D. column). Other methods use silica gels that are chemically modified with diol (Silversand & Haux, 1997; Sas et al, 1999), cyanopropyl or amminopropyl moieties to alter the polarity. However, the acidic phospholipids will not be recovered using an amminopropyl column with the same efficiency as can be achieved by using an unmodified silica column. Yoon and Kim (2002) investigated the HPLC separation of phosphatidylcholine originated from egg yolk. Column temperature, mobile phase composition (isopropanol/hexane/methanol; methanol/water; methanol) and kinds of stationary phase (silica, bonded phases like C1, NH_2, CN and diol) were varied to understand the effectiveness of PC separation. Pure methanol as mobile phase, silica as a stationary phase and temperature of 45°C represented the best HPLC operating condition. When the NH_2-bonded and diol column are used, the peaks of PC and Sph overlapped. Anyway, Neron at al. (2004) successfully applied the diol column in order to separate and quantitate the wheat flour phospholipids during dough mixing in the presence of phospholipase. They showed that the diol column allowed an efficient separation in less than 16 min of all PL classes of wheat flour (N-acyllysophosphatidylethanolamine, N-acylphosphatidylethanolamine, PE, PC, PG, Lyso-PC and PI). Pang et al (2009) achieved the optimal separation and quantification of seven major phospholipid classes in human blood (PS, PE, PG, PI, PC, Lyso-PC and Sph) by using elution with mobile phase hexane (A) and 2-propanol with water, formic acid and ammonia as modifiers (B) and using a HPLC diol column (250mm×3.0mm, i.d., 5.0 µm, particle size). An isocratic elution method (A, 30%; B, 70%) was used for better repeatability and no balance time.

The choice of mobile phase is strictly dependent on the method of detection. The mobile phases containing phosphate or sulfate buffer salts are incompatible with electrospray ionization. The selectivity of the solvents used in the mobile phase can exert a marked effect on the separation of individual phospholipids, and in particular it can change the order of elution of specific components. Specific examples of NP-HPLC separation of PL in biological

samples were provided by Hayakawa et al (2004) using acetonitrile/methanol/1M ammonium formiate. Kakela et al. (2003) used hexane/isopropanol/formic acid/triethylamine. Malavolta et al. (2004) used chloroform/methanol/water/ammmonium hydroxide. Commonly, NP HPLC requires an eluent gradient or one or more changes of the eluent mixture (Becart et al 1990; Fagan & Wijesundera, 2004; Hemström & Irgum, 2006; Bang et al, 2007). Good separation of all PL classes is possible by using gradient elution starting with a solvent of low polarity and ending with a solvent mixture of higher polarity. Anyway, the isocratic condition is often used in order to reduce time-consuming analysis and to avoid column reconditioning between subsequent runs. Kivini et al (2004) studied qualitative and quantitative HPLC chromatographic methods to determine the effects of feed supplements on hen egg yolk phospholipids. They used silica as stationary phase and they tested the isocratic elution using methanol/acetonitrile/concentrated sulphuric acid (30:100:0.05, v/v) and the ternary gradient elution using n-hexane, isopropanol:chloroform (4:1) and isopropanol:water (1:1). The isocratic condition allowed for a faster elution of Sph, PE and PC classes with respect to gradient elution (6 min vs 26 min, respectively) but the peaks obtained were too wide for reliable area calculation. So, the isocratic method was suitable for qualitative separation of PL while the gradient elution was found to be useful in the quantitative determination. Pacetti et al. (2005) employed NP-HPLC coupled with a tandem mass spectrometric detector in order to characterize the molecular species of PL of egg from hens fed diets enriched in seal blubber oil. A silica column was used as stationary phase. The mobile phase was a gradient of solvent A (CHCl$_3$/MeOH/NH$_3$ 80:19.5:0.5, v/v) and solvent B (CHCl$_3$/MeOH/H$_2$O/NH$_3$, 60:34:5.5:0.5, v/v). The total lipid fraction was injected without prior clean-up. This procedure reduced analysis time, manipulations, solvent amounts and consumable parts (e.g., TLC plates and derivatization reagents) to a minimum. The separation of five main classes from the egg lipid extracts was obtained as shown in **Fig. 1**. The retention time for the different classes increased in the order PE, PI, PC, Sph and Lyso-PC. All classes eluted within 18 min. The chromatographic system mainly separated the different phospholipid classes and also allowed some minor separation among different species within a given class. Thus, it is possible to highlight that a binary eluent system consisting of chloroform, methanol and water allowed a separation of five PL classes whereas the ternary eluent system (Kivini et al, 2004) allowed the separation of three PL classes, although the separation was faster.

Some care must be exercised in the use of normal phase HPLC because the presence of water in the solvent system can alter the affinity of PL for silica in replicated runs. Although the order of elution typically remains the same, the exact retention time can be difficult to reproduce (Pulfer & Murphy, 2003). In addition, in NP separations often rather extreme pH conditions (basic or acidic) are required to obtain baseline separation whereby potential hydrolysis can occur during the separation process. In order to avoid this disadvantageous condition, recent studies have developed a method for separation of PL classes based on a silica hydrophilic interaction liquid chromatography (HILIC). This LC technique is typically employed for polar compounds. It uses a polar stationary phase that can retain polar compounds on the column without the disadvantages of using solvents that are immiscible with water, as used in traditional normal phase HPLC. The highly polar organic mobile phase (often acetonitrile) used in HILIC is especially compatible with ESI-MS, resulting in high sensitivity. The HILIC separation is based on hydrophilicity of compounds: amphiphilic molecules like phospholipids elute in order of increasing hydrophylicity. Schwalbe-Herrmann et al. (2010) developed an isocratic HILIC method to separate the
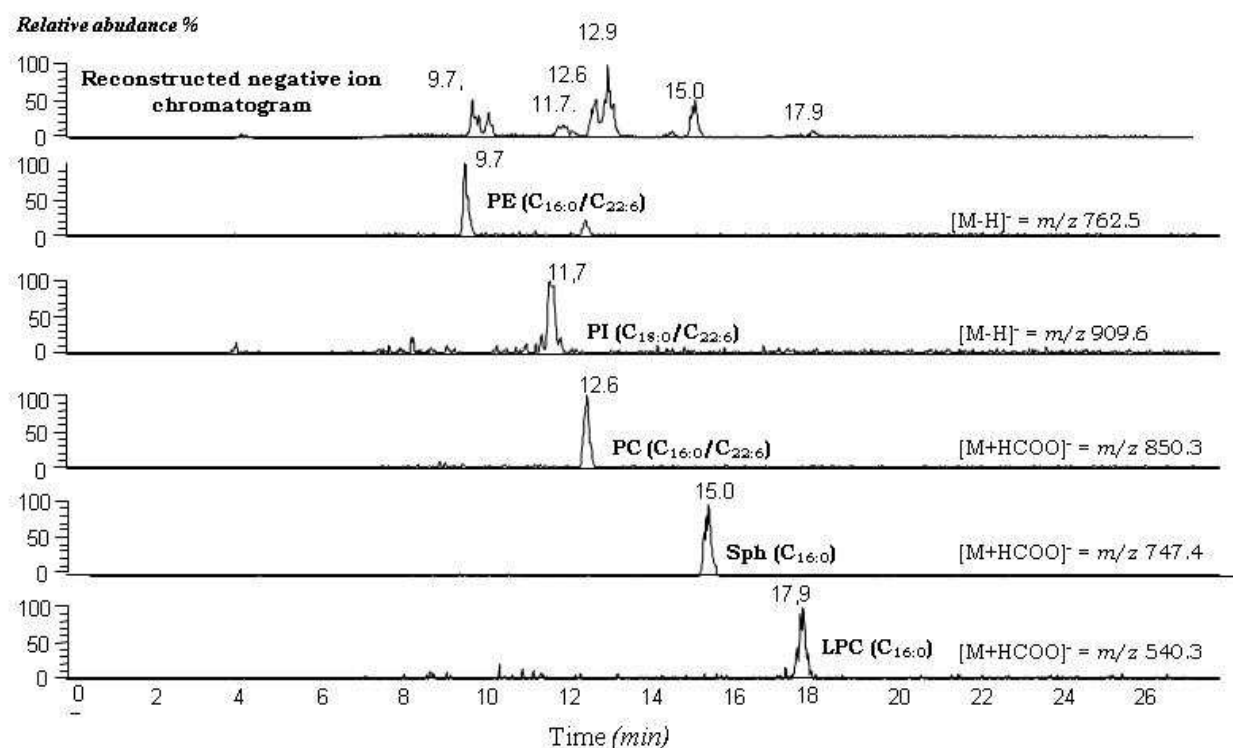
Fig. 1. Negative ion HPLC-ESI-MS analysis of PL of eggs from hens fed diets enriched in seal blubber oil with the MS operating in scan mode.

phospholipid classes in human blood plasma and in tissue extract (swine brain). The detected classes in both cases were PE, PC, Sph and LPC and their chromatographic baseline separation was achieved with a mobile flow of acetonitrile, methanol and ammonium acetate 10mM (55:35:10) at a flow of 0.6 mL/min instead of 1 mL/min which is the typical flow used with silica columns. Zhao et al. (2011) reported a good separation and quantification of 11 lipid classes including all major choline-containing compounds in egg yolk, such as acetylcholine, betaine, choline, glycerophosphocholine, lysoPC, lysoPE, PC, PE, PI, phosphocholine and Sph. They varied the mobile phase using a binary gradient elution with acetonitrile (phase A) and 10 mM ammonium formate in water at pH 3.0 (phase B) as eluents.

RP-HPLC systems are used for PL separation as well (Nakanishi et al. 2010; Ogiso et al. 2008; Lin et al 2004). The separation is based on the lipophilicity of the combined chain length and number of double bonds present in the fatty acid side chains (Peterson et al., 2006). An advantage of RP chromatography is the high resolving power. Two lipid species that only differ in the intramolecular position of a single double bond can be separated (Brouwers et al, 1998). Because of the complexity of closely related structure with ionic charges, mixture of PL molecular species have not been readily resolved by conventional RP with a simple solvent. In the absence of mobile phase electrolyte additives, negatively charged PL, such as fosfatidic acid, elute through an alkylsilyl RP column with little retention (Abidi, 1991), while neutrally charged PL (i.e PC, PE) are partially adsorbed on the alkylsilica phase (e.g. octadecylsilica – ODS) resulting in unusually long retention times and peak broadening. (Abidi, 1992). This problem can be avoided by means of the incorporation of electrolytes into mobile phases, such as quaternary ammonium phosphates or by using reversed phase ion-pair and ion-interaction (Abidi, 1996; Ma et al, 1995; Dodbiba et al, 2011).

However using ESI with ion pair reagents in the mobile phase is not desirable because of their strong suppression effect on the analyte response and possible contamination of the ion source. Recently, Barroso et al. (2005), enhanced the RP-HPLC performance in the separation of PL in human bronchoalveolar lavage fluid. They used a C8 pellicular packing material at elevated temperature with an increasing gradient of acetonitrile containing 0.1% formic acid. A good separation of phospholipids (PC, PG, PI) and their lysophospholipids was achieved without peak tailing. Vernooij et al. (2002) obtained a good chromatographic resolution for egg yolk PC molecular species by means of RP-HPLC system consisting of a Licrospher end-capped RP-18 and acetonitrile/methanol/triethylamine (550/1000/25) as mobile phase. The end-capping of the stationary phase in combination with the use of triethylamine in the eluent prevents the secondary interaction of PL with underivatized silanol groups. As a consequence, even molecular species with the same numbers of carbon atoms and double bonds showed baseline separation.

4.2 Detection and quantitation

The detection of PLs can be accomplished by using various apparatus such as refractive index (RI) (Itoh et al 1985), ultraviolet (UV), mass spectrometry (MS) and evaporative light-scattering (ELS) detectors. RI shows a low sensitivity and high degree of variability depending on the solvent. Thus, it is only applied when the isocratic elution is performed. The UV detection can be carried out at 200-220 nm since PLs have no chromophoric groups absorbing light in higher UV or visibile range. UV detector presents high sensitivity but it does not allow the use of mobile phase that absorb in UV range, such as chloroform widely used in NP system. Generally the solvents system used are limited to two systems: acetonitrile–methanol–water (Chen & Kou, 1982; Wang et al, 2003) and n-hexane–isopropanol–water (Yandrasitz et al, 1981; Dugan et al, 1986; Guan et al, 2001; Seppanen-Laakso et al, 2001; Neron et al 2004) on silica gel-based columns. Nevertheless, the quantification is difficult using UV detector due to the response of PL dependent on the unsaturated double bonds present in the acyl groups. Saturated PL yielded lower responses than unsaturated PL. In order to enhance UV absorbance, PL can be derivatized with benzoates, dinitrobenzoates, pentafluorobenzoate and nicotinic acid. Dobson et al (2001) identified the molecular species of PC from soybean, egg yolk and bovine liver after conversion to UV-absorbing diacylglycerol nicotinate derivatives. Anyway, they found the overlapping of several components.

In the last decade, the mass spectrometric detection has become the most popular method of PL molecular species analysis because it can overcome many of the problems reported above and it has strong identification ability, high sensitivity and specificity. The several types of spectrometers and their ionisation sources available for PL analysis were recently overviewed elsewhere (Peterson & Cummings, 2006). Xia & Jemal (2009) evaluated optimal mass spectrometric techniques to monitor the fate of PL during development of bionalytical LC MS/MS method since plasma PL may cause matrix effects. The electrospray ionization (ESI) (Murphy et al 2001; Smith, 2006; Pulfer & Murphy 2003) and matrix-assisted laser desorption ionization (MALDI) (Schiller et al., 2004; Fuchs et al. 2010) are the ionization methods more frequently applied. These are soft ionization methods which allow the ionization of non-volatile and thermolabile compounds. They preserve the information based on the relative position of acyl radicals on the glycerol backbone since a minimal fragmentation of molecules is produced. Compared to other ionization methods ESI is very useful for analyzing polar lipids, because polar lipids such as phospholipids are sensitive to soft ionization without a

derivatization procedure. Furthermore, ESI can be coupled with nano-flow HPLC easily (Bang et al, 2006; Isaac et al, 2003; Ito et al, 2008).Two different approaches can be adopted for analysis of PLs by means of ESI. The direct infusion of crude lipids extracts to the MS instrument was proposed by Benfenati & Reginato (1985), whereas the other approach employs liquid chromatography coupled on line with mass detector (LC-ESI-MS). The latest is more frequently applied compared to direct infusion, since the combination of HPLC and ESI MS reduces the ionisation suppression effect of low abundance PL species generated by the presence of polar impurities (Annesley, 2003; Issac et al 2003; Hermansson et al 2005; Houjou et al, 2005). Positive or negative ES ionization will occur at either the phosphate group or on the head group of PL. Normally, positively charged phospholipids such as PC and Sph appear as ions in the positive mode, whereas PE, PI, PG, PS and PA form ions in negative mode. It is possible to detect PG in the positive ion if ammonium adducts are formed. The quantitative analyses performed by using ESI MS are not straightforward because it is not possible to make the assumption that the intensity of any observed ion is proportional to the concentration of PL in the mixture. MS signals of PL are influenced by different effects related to acyl chain length, the degree of acyl chain unsaturation and concentration. Several authors (Berdeaux et al; Ahn et al 2007) have shown that the ESI-MS response increases with increasing degree of insaturation of the fatty acid side chains whereas it decreases with an increase in chain length when the phospholipids concentration range in the samples is higher than picomolar. Only the picomolar range concentration results, in fact, in a linear response. This non-linearity is more severe in negative ionization rather than positive mode (Zacarias et al, 2002). To overcome the dependence of ESI signal to the PL structures, in order to quantify the species, an internal standard chemically similar to the sample of interest can be used. A deuterated standard of each PL molecular species should ideally be used (Koc et al, 2002; Enjalbal et al., 2004). However, deuterated standards are very expensive and not always available for each PL molecular species (several hundreds of different phospholipid molecular species are present in biological samples considering all the subclasses and individual molecular species within a class). Moreover, even if an isotopic internal standard is applied, the scaling response implies a nonlinear relationship between intensity and concentration. The larger the discrepancy in intensity between the internal standard and the analyte of interest is correlated with a greater error in estimating the concentration when a linear approximation is used. Thus, the other possible way is to use a synthetic PL standard that has a low abundance in the sample, since PL species of the same class show similar ESI MS peak intensities. The standard should have a molecular weight outside of the molecular weight envelope that encompasses the naturally occurring molecular species. Pacetti et al. (2004) achieved the quantification of seven PC molecular species from the serum of cystic fibrosis subjects using ESI MS in the negative scan mode with 1,2-diundecanoyl-sn-glycero-phosphocholine as the internal standard. They showed that the use of mass spectrometry as detector allowed a low limit of quantification (12 ppm of PC). Han and Gross (2005) used 15:0-15:0 diacyl PS and 14:1-14:1 PC. When is sufficient to observe differences in phospholipid composition among different samples, a semi-quantitative approach can be also performed. The relative abundance of individual molecular species within a phospholipid class can be calculated from the single ion current responses. This strategy is widely applied to address a specific biochemical issue or to characterize the phospholipid composition in foods.

Otherwise to the ESI MS detection, for quantification purposes the use of an Evaporative Light Scattering Detector (ELSD) resulted more fast and easy. It allows the non-selective determination of all the non volatile components in the polar lipid extract and a precise

quantification of the PL classes, when a calibration curve is calculated for each phospholipid class. It is very sensitive and it can quantitate nanomolar amounts of PL. ELSD usually gives a stable flat baseline. Its response is independent of the number of double bonds in the molecules. The ELSD baseline signal is scarcely affected by solvent changes (Stith et al, 2000; Becart et al, 1990) and thus allows the use of gradient elution needed to manage complex separations. Differently, the ELSD response can be affected by parameters of the nebulization step and particularly the mobile phase composition that affects the size of the droplets (Godoy Ramos et al 2008). **Fig. 2** shows how the evaporation temperature affects the ELSD response: the temperature increase is correlated to a change of the baseline profile.
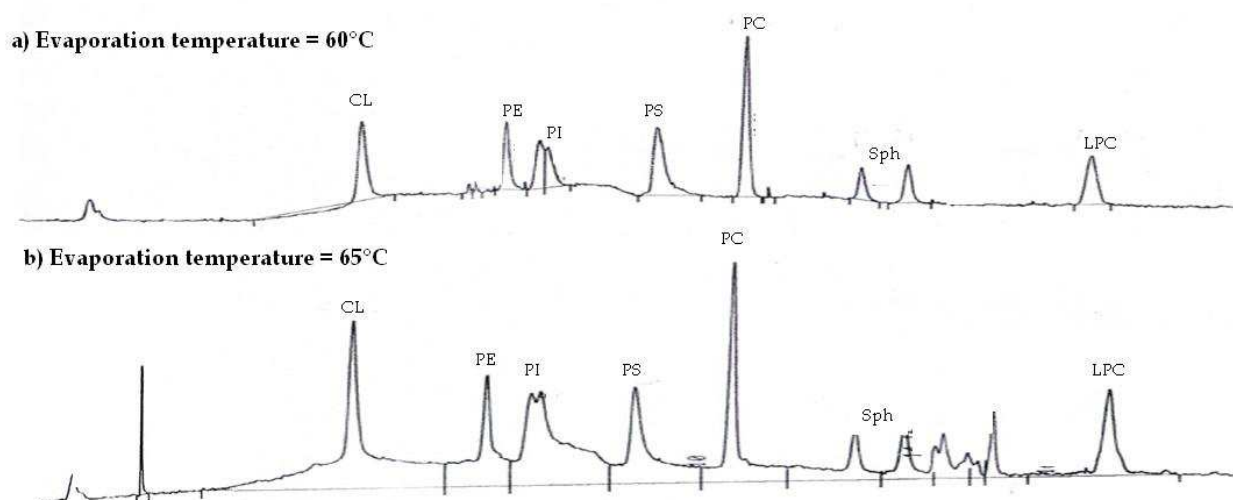
Fig. 2. NP-HPLC- ELSD trace of a standard PL mixture (CL,PE,PI,PS,PC, Sph, LPC). The gas flow (from air compressor) of the ELSD was 7.5 L/min and the vaporization temperature was set: a) at 60 °C b) 65 °C.

The effect of the different mobile phase composition on variation of baseline is reported in figure 3.

In the literature, several papers reported the quantification of PL with ELSD (Rodríguez-Alcalá & Fontecha, 2010; Seri et al, 2010; Wang et al, 2009; Rombaut et al 2007). Yan et al 2010 have successfully quantified PL extracted from human, porcine, and bovine erythrocyte ghost membranes using isocratic elution on a silica column coupled to an ELS detector. The detection limits for PS, PE, PC and Sph were 50, 50, 80 and 150 ng, respectively. Caboni et al. (2000) determined the content of phospholipids and lysophospholipids in Grana Cheese samples at different ripening stages. Narváez-Rivas and coworker (2001) developed a HPLC/ELSD method that allowed the quantification of cardiolipin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and sphingomyelin in subcutaneous fat from Iberian pig. The limit of quantification were below 0.03 and 0.05 mg kg^{-1} for each phospholipid class. The performance of charged aerosol detection (CAD) was compared to ELSD for the NP-HPLC analysis of *Leishmania* membrane PL (phosphatidic acid, PG, cardiolipin, PI, PE, PS, lysoPE, PC, Sph and lysoPC) classes. The accuracy of the methods ranged from 62.8 to 115.8% and from 58.4 to 110.5% for ELSD and CAD, respectively. With HPLC-ELSD the limits of detection (LODs) were between 71 and 1195 ng and the limits of quantification (LOQs) were
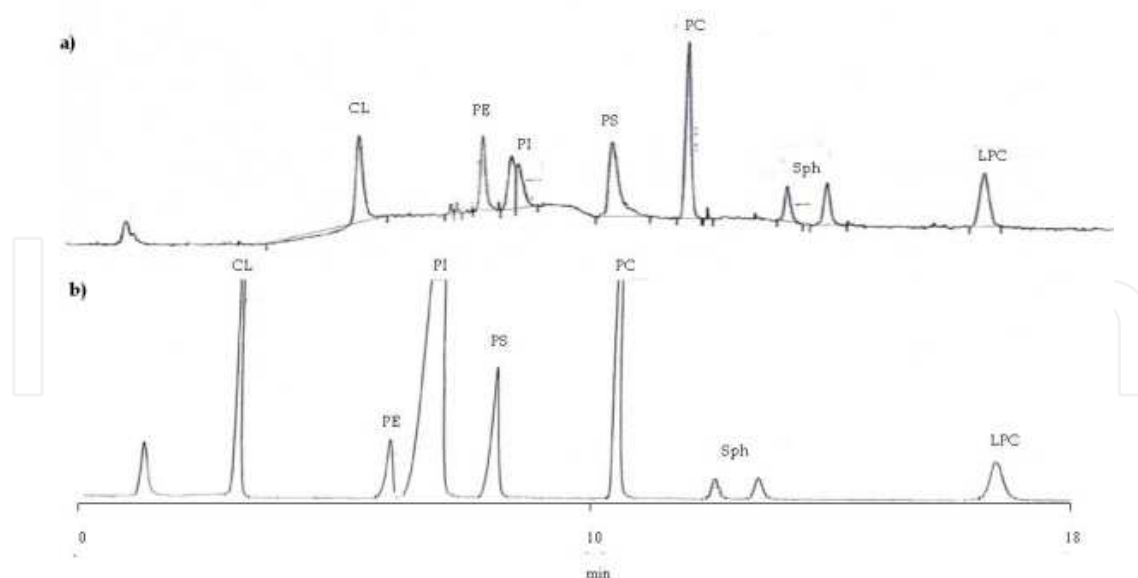
Fig. 3. NP-HPLC-ELSD trace of PL mixture obtained using a silica column (3μ, 150 mm x 4.6 mm) as stationary phase and a) a gradient of solvent A [CHCl₃/ CH₃OH /NH₄OH (30%) 70:25:1, v/v] and solvent B [CHCl₃/CH₃OH/H₂O/NH₄OH (30%) 60:34:5.5:0.5, v/v], b) a gradient of A [CHCl₃/ CH₃OH /NH₄OH (30%) 60:40:1, v/v] and solvent B [CHCl₃/CH₃OH/H₂O/NH₄OH (30%) 60:34:5.5:0.5, v/v]. The gas flow of the ELSD was 7.5 L/min and the evaporation temperature was 60 °C.

between 215 and 3622 ng. With HPLC-CAD, the LODs were between 15 and 249 ng whereas the limits of quantification (LOQs) were between 45 and 707 ng (Godoy Ramos et al, 2008). Considering that the ELS detector is really useful for qualitative analysis and that ESI MS detector is really indispensable to discriminate the PL molecular species, some authors suggest the use of the strategy where HPLC apparatus is coupled on-line with both detection systems. Boselli and co-workers (2008) proposed an analytical method for the simultaneous quantification of phospholipid classes and identification of phospholipid molecular species within each class in raw and cooked pork meat. The NP-HPLC apparatus was coupled on-line with two detection systems: an ion trap equipped with electrospray ionization for tandem mass spectrometry and an ELSD. Donato et al (2011) performed the characterization and quantification of phospholipid fraction in cow's and donkey's milk simultaneously coupling HPLC system with hybrid ion trap-time of flight (IT-TOF) mass analyzer and an ELS detector.

5. ES ionization and tandem mass spectrometry (MS/MS)

The ES ionization generates lipid ions by adding a proton $[M+H]^+$, by adding a cation, such as the sodium $[M+Na]^+$ ions or by removing a proton, $[M-H]^-$. Chloride $[M-Cl]^-$ and formiate $[M-HCOO]^-$ adduct ions or multiply-charged ions such as $[M+2H]^{2+}$, are also easily formed. The formation of ion species is strictly affected by the concentration of Na^+ and the pH of the solution, with which the ESI events take place. Dimeric molecular species are often formed during ionization if the PL concentration is high.

The variable formation of adducts and the multiplicity of PL molecular species present in the samples considerably complicate molecular species assignments from such complex spectra. Thus, often, assigning definite molecular identities to individual ion peaks is not

possible with single MS. This problem becomes especially important for isobaric molecular species of the same phospholipid class, which have identical mass but differ in combination of acyl chains, or for the identification of oxidized PL. In these cases, experiments of tandem mass spectrometry (MS/MS) are needed as additional approach (Cui & Thomas, 2009; Postle et al., 2007). In MS/MS experiments, the precursor ion created in the first MS undergoes a further fragmentation either by collision-activated dissociation (CAD) or spontaneous dissociation. Larsen et al. (2001) compared the product ion achieved using CAD with an ion-trap mass spectrometry (ITMS) with those obtained with a triple quadrupole. In the fragmentation upon CAD in an ion-trap the main product ions were the *sn*-1 and *sn*-2 lyso-phospholipids whereas CAD in triple quadrupole gave primarily the *sn*-1 and *sn*-2 carboxylate anions. Hsu & Turk (2009) describe the mechanisms underlying the fragmentation processes under low-energy CAD with tandem quadrupole mass spectrometry and with multiple-stage ion-trap mass spectrometry of PL in various ions forms generated by ESI in negative and positive-ion modes ([M−H]$^-$, and [M−2H+Alk]$^-$; [M+H]$^+$, [M+Alk]$^+$, [M−H+2Alk]$^+$; Alk = Na, Li). They noted that the fragment ions leading to structural characterization arising from CAD of the [M+H]$^+$ ions are often of low abundance, and thus are less useful for structural identification. In contrast, fragment ions arising from alkali adduct ions, in particular, the [M+Li]$^+$ ions, as well as from the [M−H]$^-$ ions are abundant and are suitable for unambiguous structural identification. Moreover, for many PL CID of [M+H]$^+$ reveals information about the polar headgroup, while collision induced dissociation of the negative [M−H]$^-$ provides information about the fatty acyl chain.

The discussion of product ions spectra achieved using CAD with ITMS of different PL ions generated by ESI (positive and negative) is reported below.

5.1 Cardiolipin (CL)

The approach using multiple-stage ITMS methods for the characterization of CL as its positive and negative ions was widely studied by several authors. Hsu and co-workers described the characterization of CL molecular species as their [M-H]$^-$, [M-2H]$^{2-}$ and [M-2H+Na]$^-$ ions generated by ESI in negative ions mode (Hsu et al 2005; Hsu & Turk 2006a) and as their sodiated adducts ([M-2H+3Na]$^+$, [M-H+2Na]$^+$, [M+Na]$^+$) formed by positive ES ionization (Hsu & Turk 2006b).

The MS^2-spectra of the [M - H]$^-$ and of the [M - 2H]$^{2-}$ ions contain two sets of prominent fragment ions that comprise a phosphatidic acid (fatty acid/fatty acid-PA), a dehydrated phosphatidylglycerol, and a anion formed by phosphatidic acid and tricyclic glycerophosphate ester (phosphatidic acid + 136). The substantial differences in the abundance of the two distinct phosphatidic anions observed in the MS^2-spectra of the [M - H]$^-$ and [M - 2H]$^{2-}$ and ions lead to the assignment of the phosphatidyl moieties attached to the 1' or 3' position of central glycerol. The differences in the intensity of carboxylate anions reflecting the fatty acyl substituent shown, exclusively, in the MS^2-spectra of the [M - 2H]$^{2-}$ provide information to identify the fatty acyl substituents and their position in the glycerol backbone: the carboxylate anion containing the fatty acyl substituent at *sn*-1 (or *sn*-1') is less abundant than the corresponding ion having that at *sn*-2 (or *sn*-2'). Anyway, the abundance of these two carboxylate ions are reversed in MS^3-spectra of the [M - H]$^-$ ions.

The MS^2-spectra of all sodiated adducts (positive and negative) showed two prominent fragment ion pairs that consist of the phosphatidyl moieties attached to the 1'- and 3'- position of the central glycerol, respectively, resulting from the differential losses of the diacylglycerol moieties containing A and B glycerol, respectively.

The identities of the fatty acyl substituents and their positions on the glycerol backbones (glycerol A and B) are deduced from further degradation (MS^3 experiments) of the above ion pairs that give the fragment ions reflecting the fatty acid substituents at the *sn*-1 (or *sn*-1′) and *sn*-2 (or *sn*-2′) positions. The ions that arise from losses of the fatty acid substituents at *sn*-1 and *sn*-1′, respectively, are prominent.

Different MS^2 spectra of the monosodiated adducts ([M+Na]$^+$) were obtained by Boselli et al. (2008). They identified the pork meat CL molecular species and the CAD fragmentation of the *m/z* 1471.6, i.e. [M+Na]$^+$ (C$_{18:1}$/C$_{18:3}$)/(C$_{18:2}$/C$_{18:2}$)-CL or (C$_{18:2}$/C$_{18:2}$)/(C$_{18:2}$/C$_{18:2}$)-CL, which resulted the preponderant species in both raw and cooked meat and produced only two complementary fragments due to the cleavage at the phosphatidylglyceridic ester, as reported in **Fig. 4**.

These results point out that only the MS^2-spectra of the [M-2H]$^{2-}$ is sufficient to confirm structural assignment of CL since it contains complementary information. Conversely, the MS^2-spectra of the all CL sodiated adducts are not suitable to elucidate the composition of the isobaric species; thus, the experiments of third order (MS^3) would be needed.
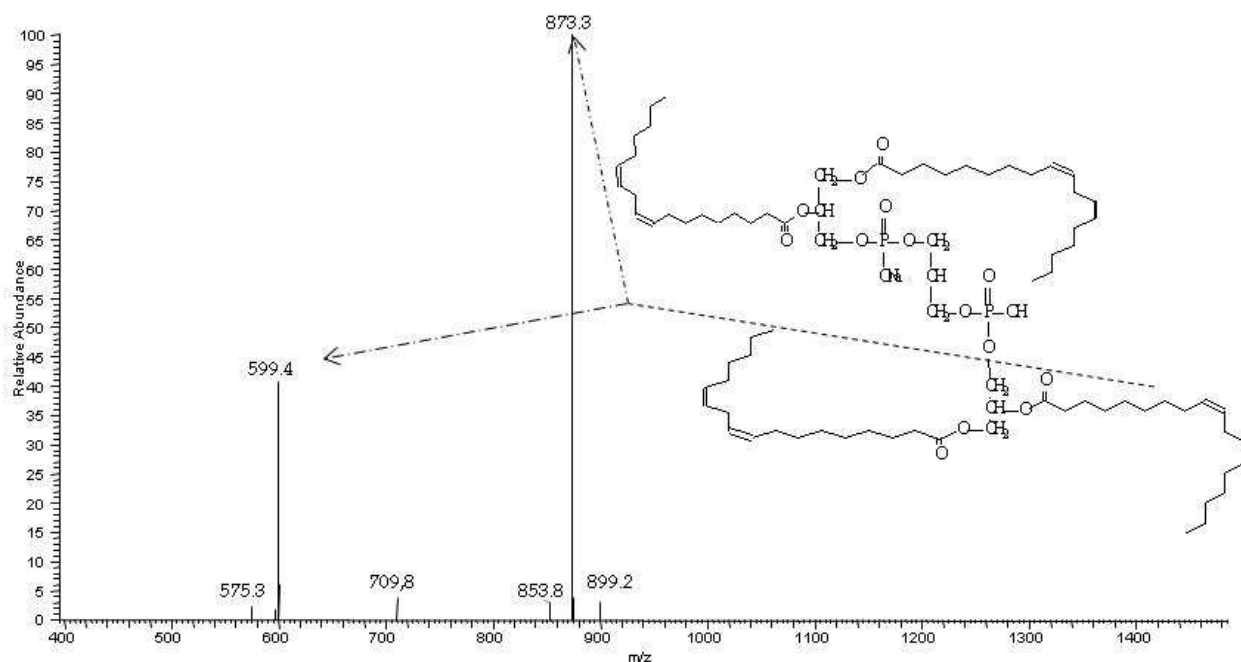
Fig. 4. Positive MS^2-spectra of the [M+Na]$^+$ of CL(C$_{18:1}$)/(C$_{18:3}$)/(C$_{18:2}$)/(C$_{18:2}$) or (C$_{18:2}$)/(C$_{18:2}$)/(C$_{18:2}$)/(C$_{18:2}$) at m/z= 1471.6

5.2 Phosphatidic acid (PA)

When being subjected to ESI in negative-ion mode, phosphatidic acid yields a [M-H]$^-$ ion. Its product ion spectra is really suitable to identify the PA species since it shows fragments arising from the loss of both the fatty acyl substituents. In **Fig. 5** is reported a product ion spectrum of *m/z*= 697.5, i.e PA (C$_{18:1}$/C$_{18:2}$) as [M-H]$^-$ ion which was detected in the almond of the avocado (*Persea americana* Mill) fruit. The spectrum is dominated by the fragments at *m/z* = 415.1 and *m/z* = 417.3 arising from neutral loss of water followed by loss of oleyl moieties (R$_{C18:1}$CO) and linoleyl moieties (R$_{C18:2}$CO), respectively. The fragment ions at *m/z* = 279.3 and *m/z* = 281.3, corresponding to the carboxylate anions, are also visible.
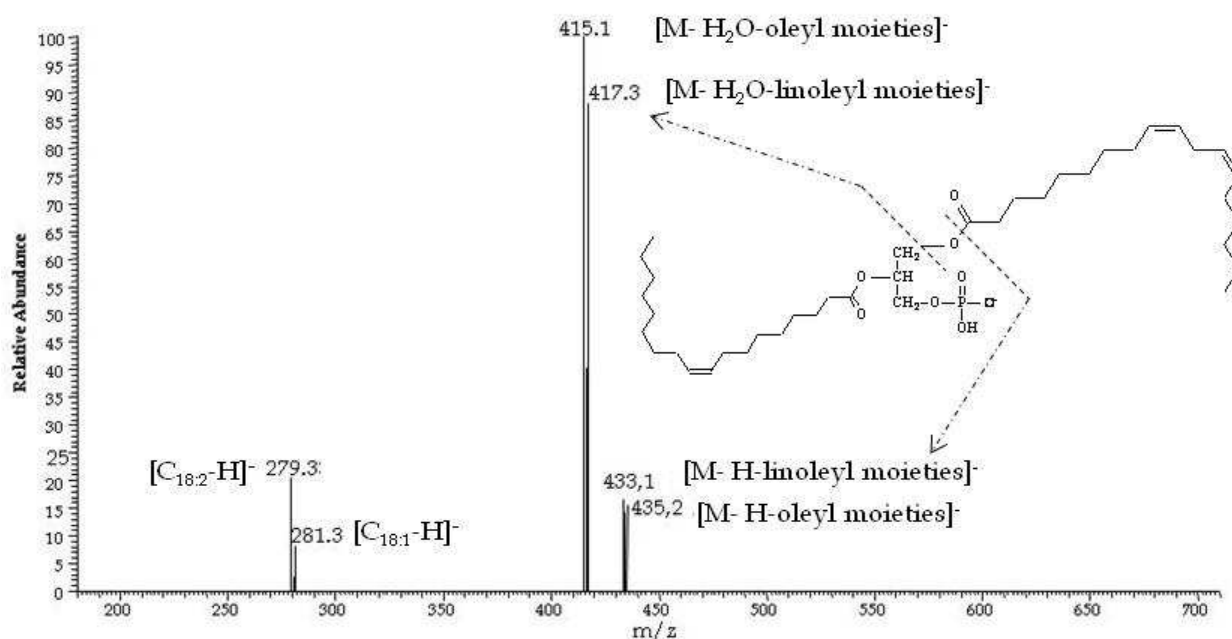
Fig. 5. Negative MS²-spectra of the [M-H]⁻ of PA ($C_{18:2}/C_{18:1}$) at *m/z* 697.5

These observation are consisting with the results obtained by Hsu and Turk (2009) which described the fragmentation processes of [M-H]⁻, [M+H]⁺, [M+Li]⁺ and [M-H+2Li]⁺ ions of various phospholipids, including PA. Moreover, they found that the sensitivity observed for PA as the [M+Li]⁺ and [M−H+2Li]⁺ adduct ions are significantly poorer than those observed as the [M−H]⁻ ions in negative-ion mode. In addition, the fragment ions produced from CAD of the [M+H]⁺ and of the [M+Li]⁺ ions are less useful for structural identification than the fragment ions [M-H+2Li]⁺ and [M-H]⁻. In fact, the MS²-spectra of [M+H]⁺ and of [M+Li]⁺ do not contain the fragments peculiar of individual molecular species, such as fragments having fatty acid moieties. Differently, [M-H+2Li]⁺ and [M−H]− ions produce MS²-spectra with fragments arising from the losses of the fatty acyl substituents at the *sn*-1 and *sn*-2 positions. In addition, due to the preferential loss of the fatty acid in *sn*-1 position, the fragments containing the fatty acid in *sn*-1 position become more abundant than those containing a fatty acid in *sn*-2 position. For this reasons, characterization of PA as dilithiated adduct and deprotonated ions affords the identification of the fatty acid substituents and their location on the glycerol backbone.

Concerning the ES positive-ion mode ionization, in addition to lithium and protonated adducts, PA can form ammonium adducts. **Fig. 6** shows a positive ESI-MS² product ion spectra of [M+NH₄]⁺ ions at *m/z*=720.5, i.e. PA ($C_{18:0}/C_{18:1}$) detected in avocado fruit (Pacetti et al 2007).
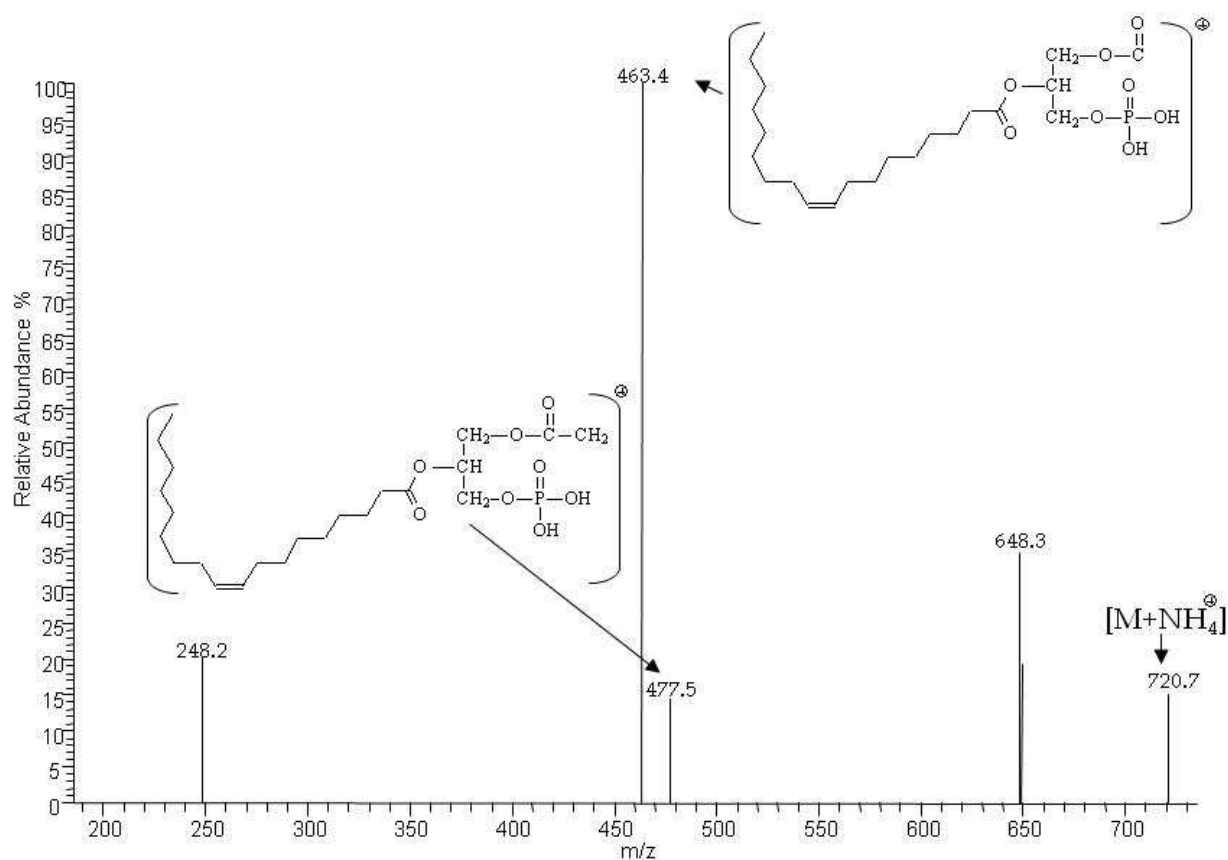
Fig. 6. Positive MS^2-spectra of the [M+NH$_4$]+ of PA (C$_{18:0}$/C$_{18:1}$) at *m/z* 720.5.

The spectrum displayed as major fragment the ion at *m/z*= 463.4 resulting from the loss of the hydrocarbon chain of stearic acid at the carbon C1. Also the ion at *m/z*= 477.5 arising from the chain rupture at the carbon C2 is weakly visible.

5.3 Phosphatidylcholine (PC)

ESI tandem mass spectrometry analysis of PC molecular species can be somewhat complicated because the same molecular species can generate a complex population of ion adducts. The structure of PC is characterized by the presence of a quaternary nitrogen atom having positive charge and by the presence of a negative charge of the phosphate group. Thus, PC is always ionized, independently of the pH. All PC species yield abundant pseudomolecular ions in positive ionization studies, most likely because the phosphate group readily accepts a proton during ESI ([M+H]$^+$). Since ESI solvents contain alkali metal ions, such as Na, K or Li, abundant alkyl adducts species ([M+Na]$^+$, [M+K]$^+$ or [M+Li]$^+$) are observed. The fragment ions arising from the [M+Li]$^+$ adduct ions of PC lipids are abundant and structural identification and distinction among isomers can be achieved (Hsu et al., 1998). In contrast, product-ion spectra from the [M+Na]$^+$ or [M+K]$^+$ ions yield little information about the fatty acids substituent (Hsu and Turk, 2009). Detailed studies of the product-ion spectra arising from the [M+H]$^+$ ions were reported by several authors, such as Pacetti and coworker (2006), Hsu and Turk (2009) and Murphy and Axelsen (2011). The product ion spectra obtained by Pacetti resulted different from the spectra presented by other authors. In detail, according to what reported by Hsu and Turk and by Murphy and Axelsen, the product-ion spectra arising from the [M+H]$^+$ ions is dominated by the *m/z* = 184

ion, representing a phosphocholine ion and by the [M+H]+ ion. Thus, the ions related to the structural information are of low abundance or absent. Differently, the CAD spectra of [M+H]+ showed by Pacetti (**Fig. 7**) are richer in diagnostic fragment ions. The most abundant fragment ion corresponding to the loss of trimethylamine [M-N(CH$_3$)$_3$]+ and the fragments resulting from the loss of one fatty acid [M+H−R$_1$CHCOO]+, from the loss of an acyl group [M+H−R$_1$CHCO]+ and from the loss of phosphocholine are clearly detectable (the abundance is higher than 50%).
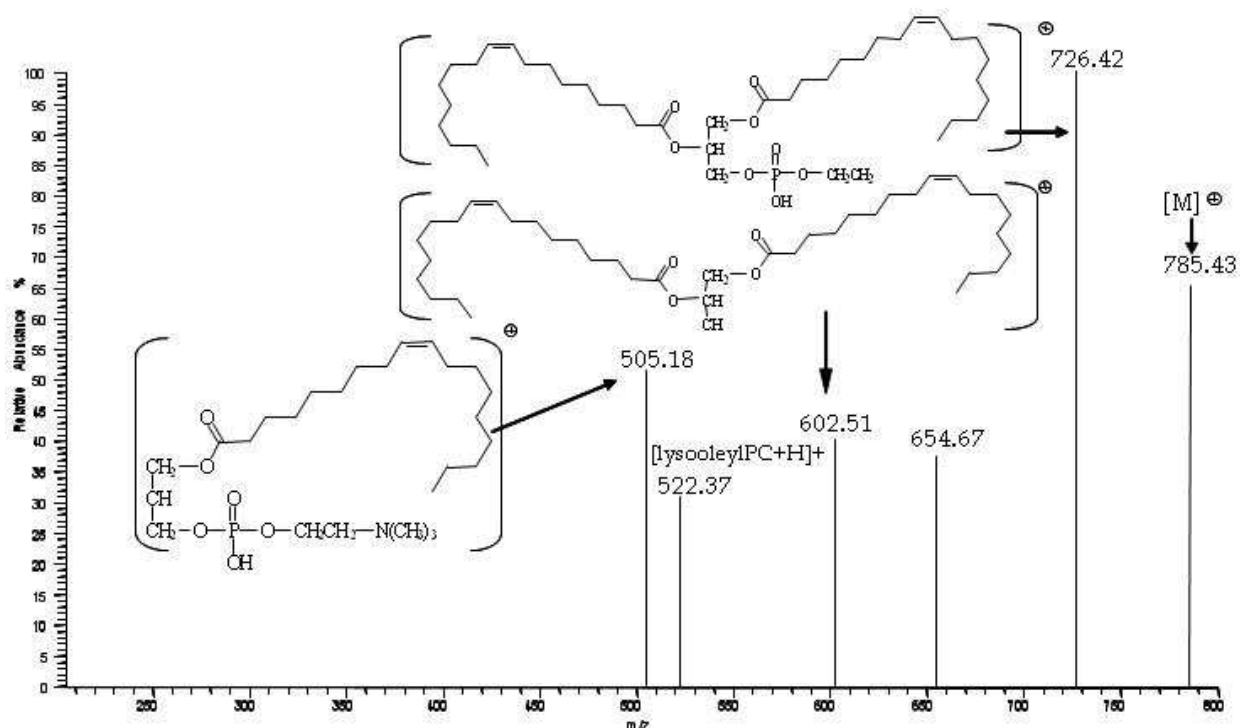
Fig. 7. Positive MS^2-spectra of the [M+H]+ of PC (C$_{18:1}$/C$_{18:1}$) at m/z 786.5.

Studies on the fragmentation of various PC including deuterium-labeled analogs indicated the formation of the ion at m/z 184 which involves the participation of mainly the α-hydrogen of the fatty acyl at sn-2 (Hsu & Turk, 2003). The studies also revealed that the α-hydrogen of the fatty acyl at sn-2 is more labile than that at sn-1, resulting in the more favorable formation of the [M+H−R$_2$CH CO]+ ion than the [M+H−R$_1$CH CO]+ ion, arising from the loss of the fatty acyl substituents at sn-2 and at sn-1 as ketenes, respectively. Therefore, the position of the fatty acyl moieties on the glycerol backbone can be assigned.

The product-ion spectrum from the [M+Li]+ adduct ions show a fragment arising from the loss of trimethylamine moiety as major fragment. The two fragments yielded from the loss of fatty acyl groups are present as minor fragments and have different abundance. According to Hsu and Turk (2009), the α-hydrogen at the fatty acyl chain is involved in the elimination of the adjacent fatty acyl substituent as an acid. Because the α-hydrogens of the fatty acyl at sn-2 are more labile than those at sn-1, preferential loss of R$_1$CO$_2$H to yield the [M+Li−R$_1$CO$_2$H]+ ion over the loss of R$_2$CO$_2$H that yields [M+Li−R$_2$CO$_2$H]+ is observed. The different abundance can be used to distinguish PC positional isomers.

Electrospray ionization also leads to the formation of negative ions derived from PC lipids in spite of the quaternary nitrogen atom with its permanent positive charge. Under negative

ESI condition, PC generate [M-15]⁻ ions, resulting from the demethylation of the choline moiety and the negative ions by forming adducts with anions, such as chloride [M+35]⁻, formiate [M+45]⁻ and acetate [M+59]⁻. When ESI is performed using a spectrometer with high orifice potentials, the [M-15]⁻ ions becomes the most important since the high potential leads also to the demethylation of the anion adducts. The MS² spectra of [M-15]⁻ and [M+59]⁻ ions show anion fragments which are more suitable for identification of the fatty acid substituents and location of their position on the glycerol backbone compared to the product ion spectra obtained from [M+35]⁻ and from [M+45]⁻ ions. In detail, the product-ion spectra obtained from [M-15]⁻ ion are characterized by the presence of both carboxylate anions [R₁COO]⁻ and [R₂COO]⁻. The latest fragment results more abundant than [R₁COO]⁻. Anyway, this discrepancy of abundance is not so visible when the PC molecular species contain a long-chain fatty acid linked to the *sn*-2 position (Berdeaux et al 2010).

Kerwin, Tuininga, & Ericsson (1994) described the formation of an ion cluster with acetate [M+59]⁻ and the subsequent decomposition of that ion species during collisional activation to yield the [M-15]⁻ ion that corresponds to the loss of methyl acetate, as well as ions characteristic of the fatty acyl group esterified at the *sn*-1 and *sn*-2 positions.

Pacetti et al. 2005 observed that the product ion spectra obtained from [M+35]⁻ and from [M+45]⁻ ions contain only the [M−CH₃−HCOO]⁻ anion fragment; thus, the negative MS²-spectrum of the [M+NH₄]⁺ of PC (C₁₆:₀/C₁₈:₁) at *m/z* 804.4 contains the fragment at 744.4 m/z.

5.4 Lysophosphatidylcholine (LPC)

The behavior of LPC molecular species under ESI is equal to those shown by PC. LPC molecular species can be inferred from their protonated molecules [M + H]⁺ when positive ESI is performed. Negative ESI yields the demethylated ion ([M–CH₃]⁻). The presence of metal or anions in the ESI system generates the adduct ions, such as ([M + Na]⁺, [M + Li]⁺, [M+HCOO]⁻, ([M+ Cl]⁻). As displayed for PC, the product ion mass spectra related to the [M+H]⁺ ion result more informative than all the other ions.

An example of MS² spectra yielded by the [M+H]⁺ ion at *m/z*= 568.3, i.e *sn*-lyso-docosahexaenoic-phosphatidylcholine [LPC (C₂₂:₆)], is reported in **Fig. 8**. The spectrum displays two major fragments. One at m/z= 550.2 given by the loss of one water molecule [M-H₂O]⁺ and the other at *m/z* = 183.9 corresponding to the polar headgroup.

Differently, the product ion spectrum from the ion at *m/z* = 612.3, corresponding to LPC C₂₂:₆ as a [M+HCOO]⁻ ion, displays the unique fragment arising from demethylation of choline [M-CH₃]⁺) with 552.1 *m/z*.

5.5 Phosphatidylethanolamine (PE)

PE can form positive and negative ions. In the ESI mass spectra, [M+H]⁺ ions are usually detected when working in positive mode, and [M−H]⁻ ions are displayed in negative mode.

The product ion spectra arising from the [M+H]⁺ are often simple and therefore less applicable, although achievable, for structure characterization. They displayed a most abundant fragment resulting from the loss of the polar headgroup ([M-NH₃(CH₂)₂OPO₃H]⁺ = [M+H−141]⁺) and a low intensity fragment resulting from the loss of one acyl group ([M-R₁CO]⁺). For example, the product-ion spectrum of the [M+H]⁺ ion of PE (C₁₆:₀/C₂₂:₆) at *m/z* 764.5 (**Fig. 9**) is dominated by the *m/z* 623.4 ([M+H−141]⁺) ion. The fragments at *m/z* 526.3 and at *m/z* 386.2, corresponding to the loss of palmitoyl acyl moieties and the simultaneous loss of both polar head group and palmitoyl acyl group, respectively, are also visible. Their
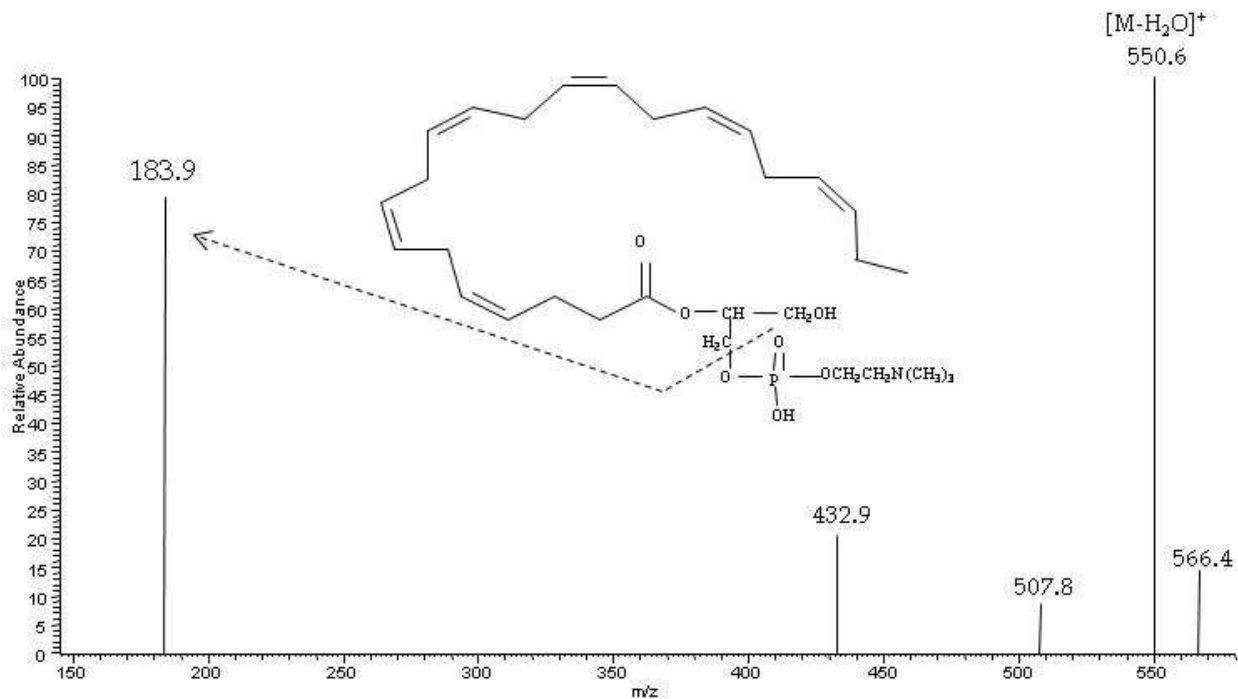
Fig. 8. Positive MS²-spectrum of the [M+H]⁺ of LPC (C$_{22:6}$) at *m/z* 568.3.

intensity are low as well as those of the fragment at 454.1 *m/z* arising from the loss of docohexanoyl acyl moieties. According to Hsu & Turk (2000), the fragmentation process leading to the [M+H−141]⁺ ion also involves the participation of the α-hydrogen of the fatty acyl substituent, mainly at *sn*-2. In addition, the observation of [M+H−141]⁺ rather than a protonated phosphoethanolamine at *m/z* 142 indicates that the phosphoethanolamine (having a zwitterion ion form) is less competitive for proton. For this reason, the response of PE in positive mode is lower than negative mode.
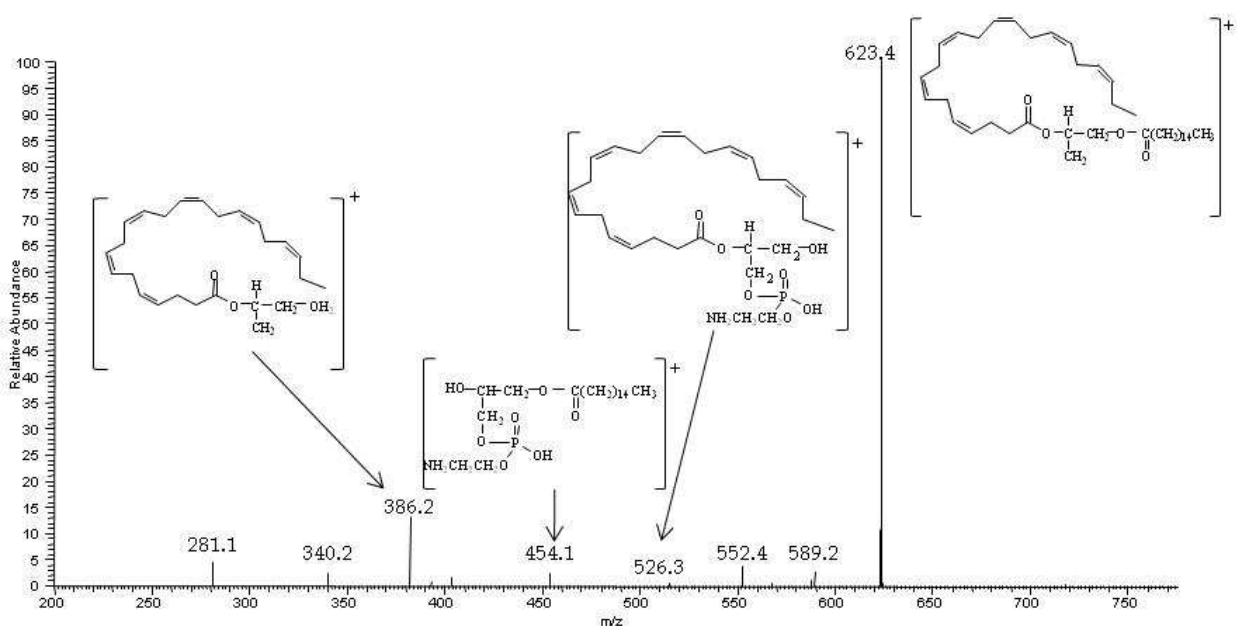
Fig. 9. Positive MS²-spectra of the [M+H]⁺ of PE (C$_{16:0}$/C$_{22:6}$) at *m/z* 764.5.

The performance of ESI working in positive mode can be enhanced by inducing the formation of lithium adducts. When ionized in the presence of lithium, PE forms both monolithiated ($[M+Li]^+$) and dilithiated ($[M-H+2Li]^+$) adduct ions. The intensity of the $[M+Li]^+$ and the $[M-H+2Li]^+$ ions are dependent on the concentration of the lithium ion (Hsu & Turk, 2009). The fragmentation of these ions produces the major fragment related to the loss of 147 Da (lithiated polar head) and the minor fragments arising from the loss of aziridine ($[M-CH_2CH_2NH]^+ = [M-43]^+$) and from the loss of one of the fatty acid substituents ($[M-R_1COOH+2Li]^+$ and $[M-R_2COOH+2Li]^+$).

The fragmentation pathway of $[M-H]^-$ leads to the CAD spectra which displayed as major fragments one or two ions resulting from fragmentation at the ester bond ($[LPE-H]^-$) and one or two fatty acid carboxylate anion fragments. As reported elsewhere (Hsu & Turk, 2009; Murphy & Axelsen, 2011), the carboxylate anion and the $[LPE-H]^-$ fragments derived from the fragmentation at the *sn*-2 position are prominent. In Fig. 10, the CAD spectrum of m/z = 762.5, i.e. PE ($C_{16:0}/C_{22:6}$) as $[M-H]^-$ ion, shows a minor carboxylate anion at m/z= 255.2 ($[C16:0-H]^-$), a major carboxylate anion fragment at m/z= 327.12 ($[C22:6-H]^-$), a minor $[LPE-H]^-$ fragment at 524.2 and a major $[LPE-H]^-$ at 452.2, due to the loss of the acyl moieties of the docosahexaenoic acid.
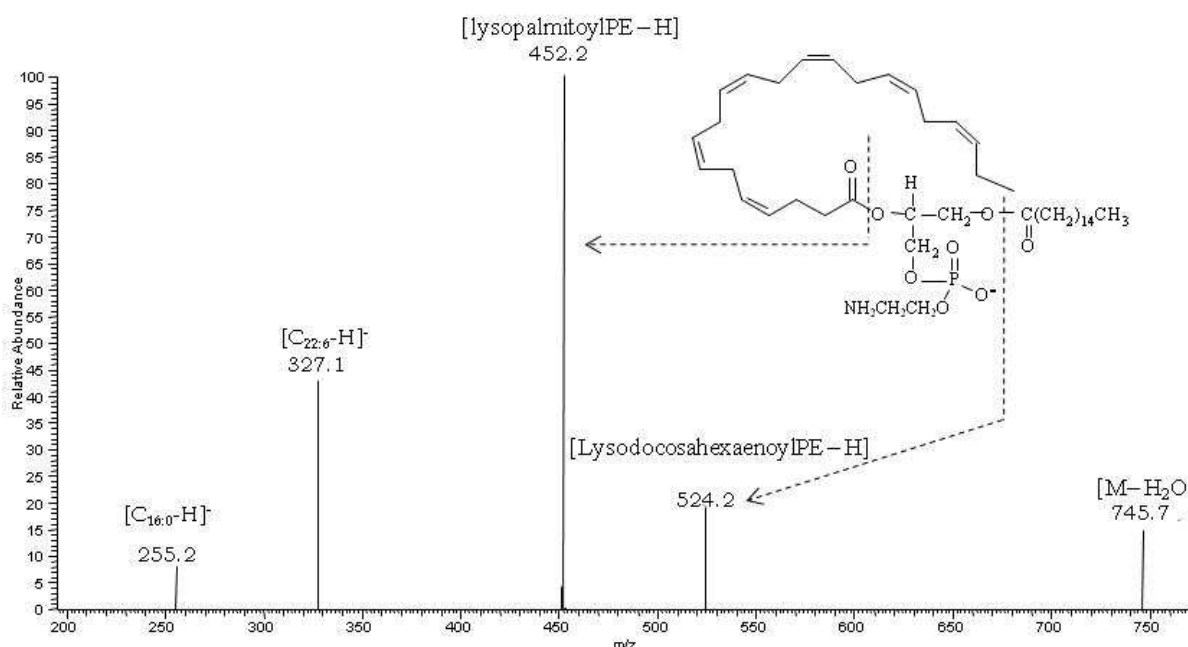

Fig. 10. Negative MS²-spectra of the $[M-H]^-$ of PE ($C_{16:0}/C_{22:6}$) at m/z 762.5.

5.6 Phosphatidylethanolamine plasmalogen (pPE)

Phosphatidylethanolamine plasmalogen (pPE) differs from PE because it has a vinyl ether substituent at the *sn*-1 position instead of a fatty acyl moiety.

The use of tandem mass spectrometry to study the positive ion mode collision-induced dissociation of various pPE present in the membrane of human neutrophils and in pork meat was performed by Zemski Berry et al (2004) and by Boselli et al (2008), respectively. They found that the prominent ion detected in positive ion electrospray mass spectrum corresponded to $[M+H]^+$ and showed a product ion spectrum resulting in fragments characteristic of *sn*-1 substituent, in a peculiar fragment of *sn*-2 substituent and in a fragment

ion deriving from the loss of the polar head group ([M-NH$_3$(CH$_2$)$_2$OPO$_3$H]$^+$). As an example, the CAD of m/z= 700.4, i.e pPE [M+H]$^+$(p16:0/C$_{18:2}$) (**Fig. 11**) resulted in a major fragment at m/z 364.1, containing the phosphatidylethanolamine head group linked with alky-1'-enyl moieties of sn-1 substituent, in a minor fragment at m/z 559.5, resulting from the loss of the polar headgroup ([M-NH$_3$(CH$_2$)$_2$OPO$_3$H]$^+$ and in a fragment at m/z 337.1, corresponding to the loss of both polar head and 1-O-alky-1'-enyl groups. In contrast, as seen for MS^2 mass spectra of all PE [M+H]$^+$ ions, the fragment resulting from the loss of the polar headgroup is present as minor fragment in the product ion spectrum of pPE [M+H]$^+$. For this reason, the quantification of pPE in a lipid mixture by using constant neutral loss of 141 Da is not suitable as it is for quantification of PE.
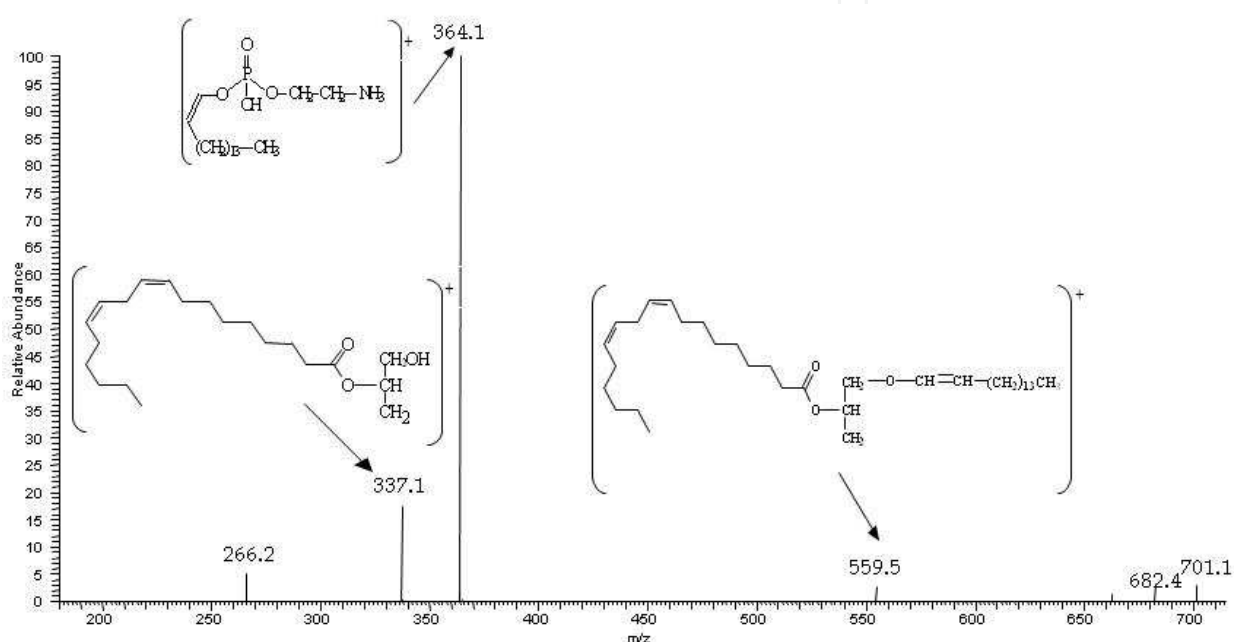

Fig. 11. Positive MS^2-spectra of the [M+H]$^+$ pPE (p16:0/C$_{20:4}$) at m/z 700.4.

The mechanism of formation of the predominant fragment ion at m/z 364.1 was proposed by Zemski Berry & Murphy (2004). It involves the oxygen atom at the *sn*-1 position attacking the phosphorous atom, which results in the formation of a new O-P bond and concomitant abstraction of the hydrogen from C-2 of the glycerol backbone to form a double bond between C-1 and C-2 of the glycerol structure. Since this fragment is characteristic of the hexadecyl vinyl ether group linked in *sn*-1 position, it is possible to detect all the pPE molecular species containing this substituent by using a precursor ion scan at m/z= 364 or all pPE species with an octadecyl vinyl ether by a precursor ion scan of m/z = 392.

The characterization of pPE molecular species by means of ESI working in negative ion-mode is reported by Malavolta et al (2004) and by Hsu & Turk 2009. Both groups observed the [M-H]$^-$ ions in negative mode but the product ion spectra obtained by the two authors are different. In detail, Hsu and Turk reported that the CAD spectra of pPE is dominated by the carboxylate anion identifying the fatty acyl moiety of the molecule. Differently, Malavolta et al. showed that the tandem mass spectrum was similar to that obtained with PE. The spectrum has an anion generated from the loss of the fatty acyl moiety ([M-RCO-H]$^-$) as predominat fragment and a carboxylate anion fragment ([RCOO]$^-$) with lower

abundance. The fragmentation of the vinyl-ether bond did not occur. The CAD of $m/z=$ 746.4, i.e pPE [M-H]⁻ (p16:0/$C_{22:6}$) according to Malavolta et al. is reported in **Fig. 12.**
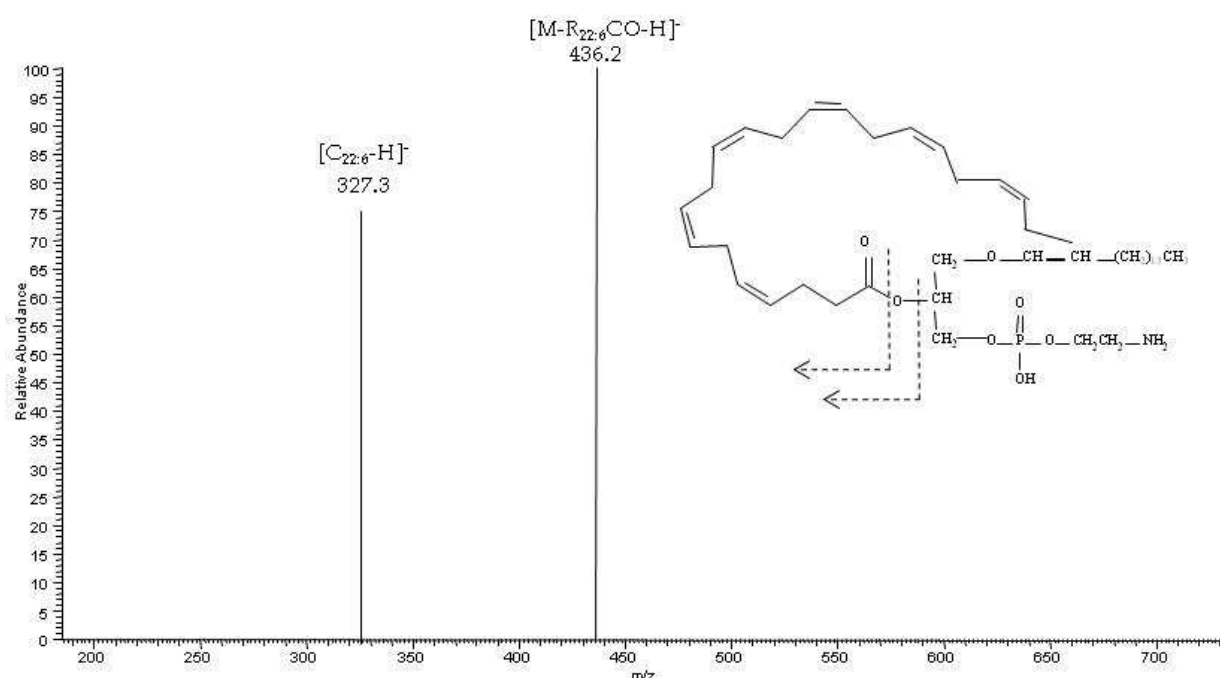

Fig. 12. Negative MS^2-spectra of the [M-H]⁻ of pPE (p16:0/$C_{22:6}$) at m/z 746.4.

5.7 Phosphatidylinositol (PI)

PI can be characterized by positive and negative ions. However, negative ions are more abundant and informative because PI contains inositol as a headgroup, which has intrinsic negative charge.

Molecular ions produced by ESI in negative mode corresponded to [M-H]⁻. Collisional activation of PI negative molecular ion species [M-H]⁻ was reviewed by several authors (Hsu & Turk, 2009; Pacetti et al., 2005; Malavolta et al., 2004). They observed that the MS^2 - spectrum of [M-H]⁻ PI ions is rich of several informative ions, such as carboxylate anions and fragments arising from fragmentation at the ester bond. An example of CAD of m/z 909.4, i.e. PI ($C_{18:0}$/$C_{22:6}$) is shown in **Fig. 13**. The spectrum displayed abundant ions resulting from the fragmentation at the ester bond (m/z 599.2, [lysoPI–H]⁻) followed by neutral loss of H_2O (m/z 581.4, [LPI–H–H_2O]⁻) and inositol (m/z 419.2, [LPI–H–H_2O–$C_6H_{10}O_5$]⁻). The latest fragment is found for both fatty acids ([LysostearoylPI–H_2O–$C_6H_{10}O_5$]⁻ and [lysodocosahexaenoyl PI–H_2O–$C_6H_{10}O_5$]⁻). Only one fatty acid carboxylate anion was detected ([$C_{18:0}$–H]⁻). These patterns are strongly useful for PI species confirmation.

Molecular ions observed in the positive ion-mode were consistent with [M+H]⁺ and [M+Na]⁺. The latest resulted the most abundant ion. Anyway the spectra are complicated by the concomitant presence of both protonated and sodiated adducts. Differently, when the HPLC eluent contains the ammonium ion, the [M+NH_4]⁺ ion can be formed and it results the only ion yielded (Pacetti et al., 2006). Moreover, PI also forms dilithiated adduct [M-H+2Li]⁺ ions upon ESI in presence of concentrated Li⁺ (Hsu & Turk, 2009). Tandem mass spectrometry of the PI [M+H]⁺ ion was studied in detail by Murphy and coworkers (2011). Its product ion spectrum showed a major fragment arising from the neutral loss of inositol
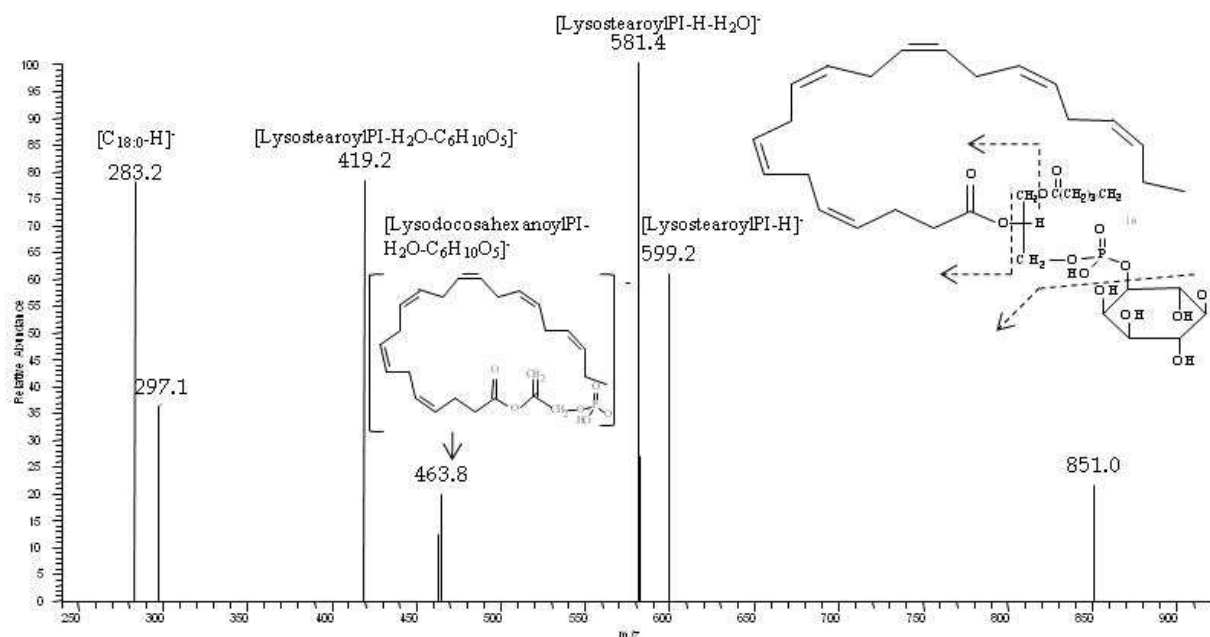
Fig. 13. Negative MS^2-spectra of the [M-H]$^-$ PI (C$_{18:0}$/C$_{22:6}$) at m/z 909.4.

which corresponds to the PA species [M-259]$^+$. Peculiar fragments containing fatty acid moieties are absent. Also the MS^2-spectra of [M-H+2Li]$^+$ did not produce characteristic fragments. It gave rise to a prominent [(LiO)(HO)PO(OX)+Li]$^+$ (where X=inositol$-$H$_2$O) at m/z = 255. The lithiated diacylglycerol ion was of low abundance. On the contrary, the CAD of [M+NH$_4$]$^+$ yielded a tipical fragment obtained from the loss of one fatty acid. The fragmentation of the ion [M+NH$_4$]$^+$ PI (C$_{16:0}$/C$_{18:2}$) at m/z 852.4 is reported in **Fig. 14**. The spectrum is constituted of two fragments: the molecular ion ([M]$^+$, m/z 833.6) and the lyso-linoleylPI ion (m/z 577.4, [M-R$_{18:2}$CO-H$_2$O]$^+$). The molecular ion is the most abundant fragment.
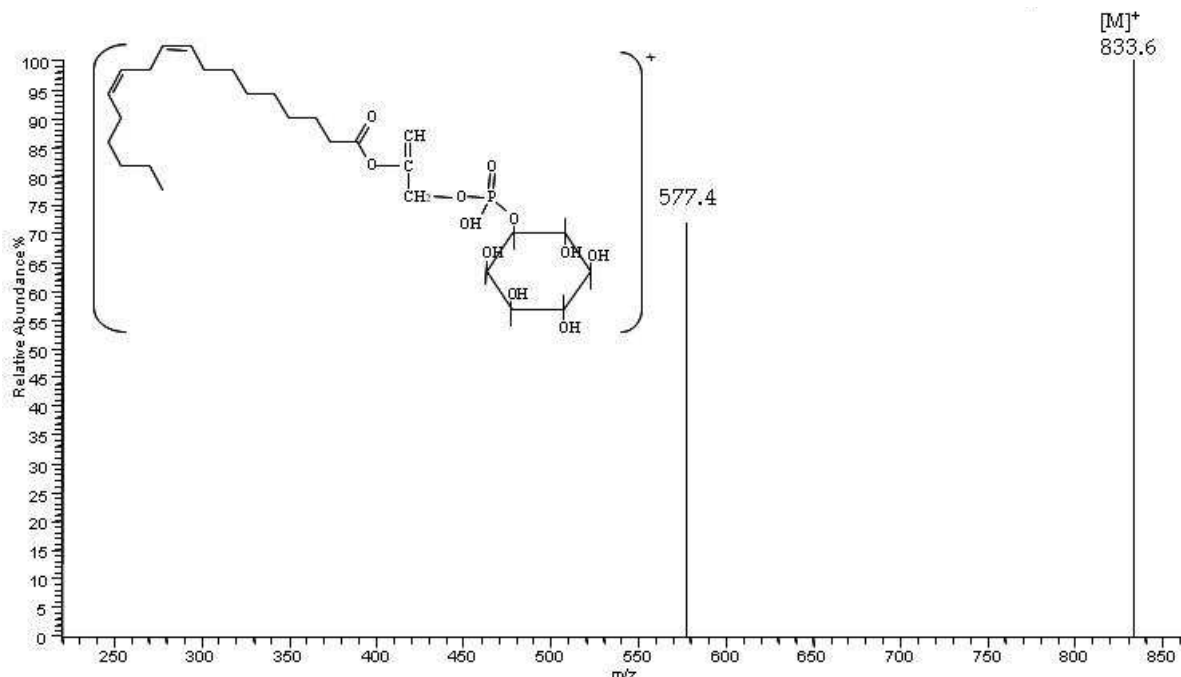

Fig. 14. Positive MS^2-spectrum of the [M-NH$_4$]$^+$ PI (C$_{16:0}$/C$_{18:2}$) at m/z 852.4.

5.8 Phosphatidylserine (PS)

The characterization of PS molecular species by means of ESI ion trap MS yields a positive-ion in form $[M+H]^+$ and negative ions with $[M-H]^-$.

The tandem mass spectrum of the $[M-H]^-$ ion contains complete structural information and offers the utmost sensitivity for structural determination. In contrast, product-ion spectra from the $[M+H]^+$ species are rather simple and are less useful for structural characterization. This statement can be confirmed by comparing the product MS^2 spectra of $[M-H]^-$ ion of PS ($C_{18:0}/C_{22:6}$) at m/z = 834.4 (**Fig 15a**) with MS^2 spectra of $[M+H]^+$ of the same PS species at m/z 836.5 (**Fig 15b**) detected in the lipid fraction of marine fish. The CAD of $[M-H]^-$ produces an abundant ion resulting from the loss of the head group (m/z 747.4, $[PA-H]^-$), two ions resulting from the fragmentation at the ester bond followed by the loss of the serine group $[LPA-H]^-$ and small carboxylate ion fragments. Thus, the two carboxylate anion fragments at m/z 283.5 and m/z 327.3 and the two $[LPA-H]^-$ ions at 419.4 and at 463.1 identify PS ($C_{18:0}/C_{22:6}$) as the parent ion. Differently, the CAD of $[M+H]^+$ at m/z = 836.5 is dominated by m/z 651.4 ($[M+H-(HO)_2P(O)O-serine]^+$) arising from loss of the phosphoserine moiety. Only one of the acylium ions at m/z = 329.1 reflecting the fatty acyl sostituents ($C_{22:6}$) is present; anyway, its signal is low.

In the presence of alkali ions, PS molecular species in the form of $[M+Alk]^+$ and $[M-H+2Alk]^+$ (Alk = Li, Na) can also be observed, attributable to the fact that PS possesses two anionic charge sites of which one can attach to an Alk^+ (Hsu & Turk, 2005). Ions informative for structural characterization of PS are of low abundance in the MS^2 spectra of alkali adduct ions. The MS^2-spectrum of the $[M+Alk]^+$ ion contains a unique ion corresponding to the internal loss of a phosphate group probably via the fragmentation processes involving rearrangement steps. The $[M-H+2Alk]^+$ ion of PS yields a major $[M-H+2Alk-87]^+$ ion, which is equivalent to an alkali adduct ion of a monoalkali salt of PA and gives rise to a greater abundance of $[M-H+2Alk-87-R_1CO_2H]^+$ than $[M-H+2Alk-87-R_2CO_2H]^+$.

5.9 Sphingomyelin (Sph)

ESI of sphingomyelin follows closely that of PC and LPC. The presence of the quaternary nitrogen atom, with permanent positive charge, dominates the ESI behaviour of this molecule. Thus, the Sph generates more abundant ions in the positive ion mode, than the negative ion mode. Moreover, in negative ion mode, Sph can be analyzed only if the addition of an anionic reagent (acetate or formiate) in the spray solvent is performed.

In detail, (+)-ESI yields a protonated ion $[M+H]^+$ and a lithium adduct $[M+Li]^+$, whereas, in (-)-ESI, Sph is detected as chlorine $[M+Cl]^-$, formiate $[M+HCOO]^-$ and acetate $[M+CH_3COO]^-$ adducts.

As reported by Murphy and Axelsen (2011) lithiated-sphingomyelin ions produce $[M-59+Li]^+$ ions representing the neutral loss of $(CH_3)_3N$ and $[M-183+Li]^+$ ions representing the neutral loss of phosphocholine. Differently, CAD of $[M+H]^+$ yields the only fragment at m/z 184 (phosphocholine group). This evidence contrasts with the results obtained by Boselli et al (2008) where the CAD of $[M+H]^+$ at m/z 703.6, i.e. Sph ($C_{16:0}$), yielded fragments deriving from the loss of water (m/z 685.2, $[M+H-H_2O]^+$), the loss of the acyl group (m/z 464.8), the loss of the amidic moiety (m/z 432.2) and a fragment deriving from sphingosin (m/z 295.9) (**Fig. 16**).
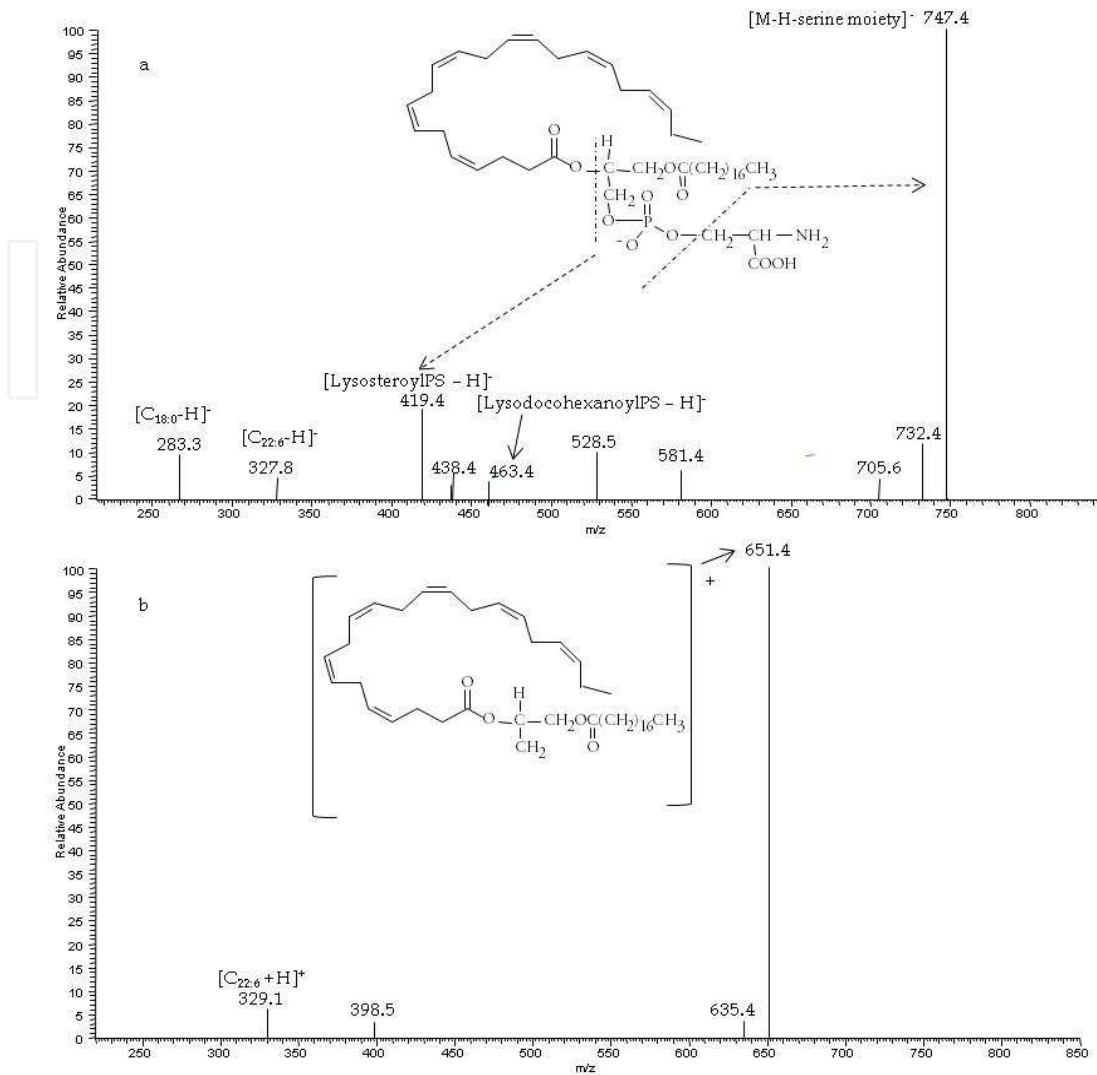
Fig. 15. Positive MS^2 spectrum of the PS (C$_{18:0}$/C$_{22:6}$) ions (a) [M-H]$^-$ ion at m/z 834.4 (b) [M+H]$^+$ ion at m/z 836.5.
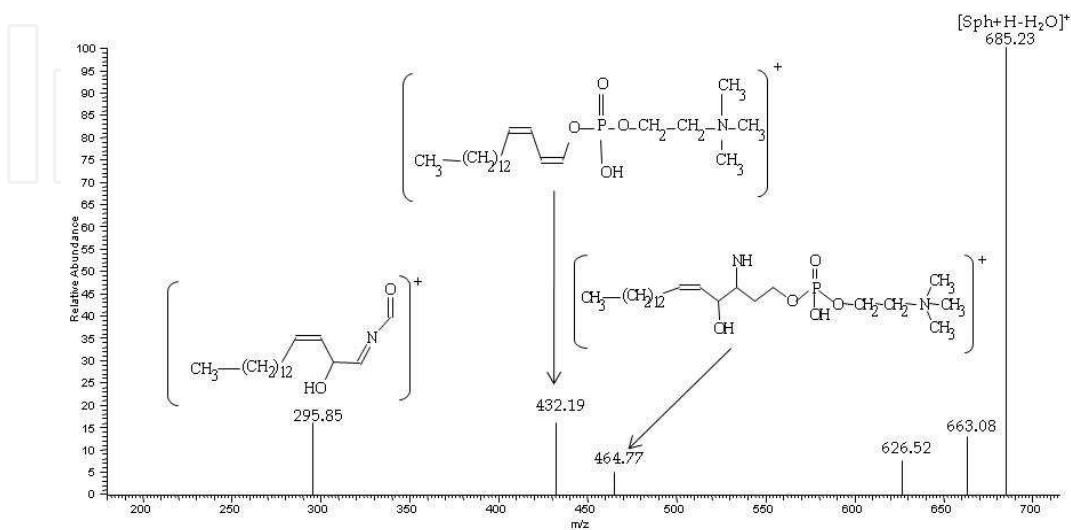

Fig. 16. Positive MS^2-spectra of the [M+H]$^+$ Sph (C$_{16:0}$) at m/z 703.6.

The product ion spectrum of Sph formiate and acetate adducts ions contain exclusively the $[M-CH_3]^-$ ion fragment, due to the loss of the methyl group of choline (Pacetti et al. 2005; Murphy and Axelsen, 2011). The fragmentation of the chlorine adduct produces only the loss of a methyl group, as well. An example of CAD of ion at m/z 747.4, i.e. Sph ($C_{16:0}$) as formiate adduct is reported in **Fig. 17**.
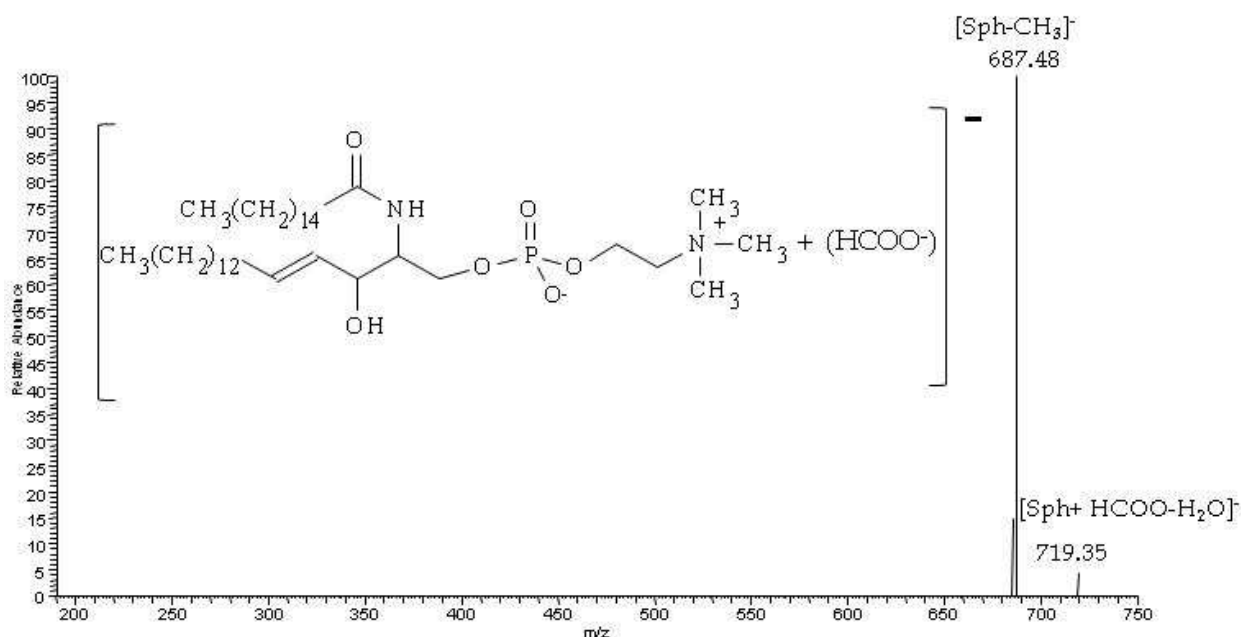

Fig. 17. Negative spectra MS2-spectra of ion at m/z 747.5 corresponding to formiate adducts of Sph containing palmitic acid ($C_{16:0}$).

On the basis of these experimental evidences it is possible to establish that the CID with negative ionization is not useful for an unambiguous identification of Sph isobaric molecular species.

6. Conclusion

HPLC-Tandem Mass Spectrometry needs high-value equipment and trained personnel, however it enables researchers to achieve incomparable results with respect to other non-automated techniques. PL molecular species can be determined either in positive or negative ionization depending on the charge of the polar group. Time and chemical consuming clean-up procedures can be avoided in order to fingerprint the (combination of the couple of) fatty acids bound to the phosphoglyceric bone. In certain cases, however, mass spectrometry of the third order would be needed for the identification of isobaric molecular species.

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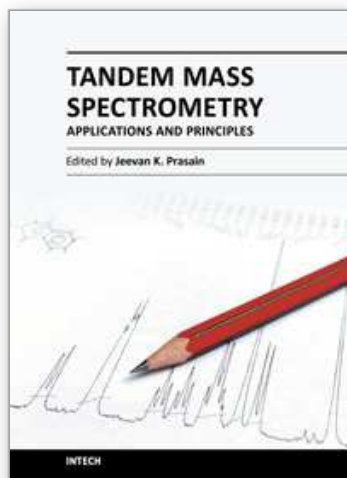
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