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HIGHLY INFORMATIVE MULTICLASS PROFILING OF LIPIDS BY ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY – LOW RESOLUTION (QUADRUPOLE) MASS SPECTROMETRY BY USING ELECTROSPRAY IONIZATION AND ATMOSPHERIC PRESSURE CHEMICAL IONIZATION INTERFACES

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KEYWORDS

Lipids ; Lipidomics ; Lipid fragmentation ; Atmospheric pressure chemical ionization ; Low resolution mass spectrometry ; Human plasma

ABSTRACT

A simple, fast, and versatile method, using an ultra-high performance liquid chromatography system coupled with a low resolution (single quadrupole) mass spectrometer was optimized to perform multiclass lipid profiling of human plasma. Particular attention was made to develop a method suitable for both electrospray ionization and atmospheric pressure chemical ionization interfaces (sequentially in positive- and negative-ion mode), without any modification of the chromatographic conditions (mobile phase, flow-rate, gradient, etc.).

Emphasis was given to the extrapolation of the structural information based on the fragmentation pattern obtained using atmospheric pressure chemical ionization interface, under each different ionization condition, highlighting the complementary information obtained using the electrospray ionization interface, of support for related molecule ions identification. Furthermore, mass spectra of phosphatidylserine and phosphatidylinositol obtained using the atmospheric pressure chemical ionization interface are reported and discussed for the first time.

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1. Introduction

Lipidomics is a branch of metabolomics that investigates in detail the structures, functions and dynamic changes of lipids in cells, tissues or body fluids, often to correlate them to the health status of the human organism. In fact, some lipids can be involved in many diseases, such as cancer, diabetes, and cardiovascular disorders [1]. Cellular lipids are a complex of biological metabolites and can be divided into eight main categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, prenols, saccharolipids, and polyketides [2]. They play multiple and critical roles, for instance, non-polar lipids such as triacylglycerols (TAGs) are important energy storage components, amphiphilic glycerophospholipids (GPs) are the main building blocks of biological membranes, and some polar lipids act as signalling molecules between organelles or cells [3].

The high structural diversity in lipid classes present in biological samples makes their characterization a very challenging task. Chromatographic techniques, and in particular liquid chromatography hyphenated to mass spectrometry (LC–MS) provides several advantages over direct infusion techniques (e.g. shotgun MS). The main advantages are: (I) more reliable identification of individual lipid species, even at trace levels; (II) separation of isomers and isobars; and (III) reduced ion-suppression effects [4], [5], [6]. Different LC configurations can be used for lipid analysis in general. Silver ion LC and non-aqueous reversed-phase (NARP)-LC are usually used to analyze non-polar lipids or single class of lipids, while normal phase (NP), RP, and hydrophilic interaction chromatography (HILIC) are preferred for complex lipid mixtures [7]. However, RP-LC is the separation mechanism of choice within the lipidomics field. RP-LC exploits hydrophobic interactions that depends generally on the equivalent carbon number (ECN) related to the carbon-chain length (CN) and the number of double bonds (DB) (ECN = CN-2DB) [7]. Therefore, lipid species containing longer acyl chains are eluted from the LC column later than shorter chain lipids, and saturated acyl structures are eluted later than polyunsaturated analogs. Different MS systems can be used coupled to the LC system. The choice is based on the goal of the study, mainly whether untargeted or targeted investigation is carried out [8], [9]. According to a recent review on LC–MS in lipidomics, 80% of the applications relied on an MS capable of full mass spectra acquisition [7]. Time-of-flight (ToF) based techniques dominate the scene with about 40% of the total applications, followed by quadrupole linear ion trap (QLIT), triple quadrupole (QqQ), and ion trap; while the single quadrupole (qMS) is employed only in the 2% of the applications [7]. Regarding the MS ionization mode used in lipidomics, the most popular is electrospray ionization (ESI), which is mainly employed for the identification of polar lipids (e.g. phospholipids), while atmospheric pressure chemical ionization (APCI), used in a much smaller number of applications, is preferred for non-polar lipids (e.g. TAGs and cholesterol esters, CEs), but it has been successfully used for polar lipids in few applications [10], [11], [12], [13]. APCI, differently from ESI, produces some degree of fragmentation useful for structural characterization [14]. In 1997, Byrdwell and Borchman [10] reported LC-APCI-MS characterization of sphingolipids (SLs) and other phospholipids (PLs) of human eye lens membrane extract. A year later, Byrdwell [11] extended the previous LC–MS system by employing two MS detectors, namely a qMS and a QqQ MS, equipped with APCI and ESI interfaces, respectively. Several classes of compounds, such as sphingomyelin (SM), glycerophospholipid, and plasmalogen molecular species in different biological samples were analysed, pinpointing the complementarity of the two interfaces. Cai & Sayge in 2006 [12] and successively Imbert et al. in 2012 [13] compared API interfaces in lipids and lipidomics analysis, in term of sensitivity.

The aim of this work was to develop a simple, fast, and versatile ultra-high performance LC (UHPLC) method suitable for both ESI and APCI interfaces, without any modification of the chromatographic conditions (mobile phase, flow, injection volume, gradient, etc.), coupled with a low resolution (LR)MS (quadrupole). The proposed method was tested for untargeted lipid profile characterization of human plasma. Furthermore, the capability of qMS to work in positive- and negative-ion mode sequentially was exploited to increase the information acquired in a single run. An extensive explanation of the mass patterns obtained under each different ionization condition is provided, highlighting the complementary information obtained and the presence of important diagnostic fragments.

2. Materials and methods

2.1. SAMPLE, STANDARD COMPOUNDS, AND REAGENTS

A plasma sample was supplied by a clinical laboratory located in Messina (Italy). The fatty acid (FA) standard compounds used were lauric acid (La: C12:0), tridecanoic acid (C13:0), myristic acid (M: C14:0), pentadecanoic acid (Pd: C15:0), palmitic acid (P: C16:0), palmitoleic acid (Po: C16:1), stearic acid (S: C18:0), oleic acid (O: C18:1n9), linoleic acid (L: C18:2n6), linolenic acid (Ln: C18:3n3), and arachidonic acid (Ar: C20:4n6). TAG standards used were tripentadecanoin (C15:0C15:0C15:0), tripalmitolein (C16:1C16:1C16:1), triheptadecanoin (C17:0C17:0C17:0), triolein (C18:1C18:1C18:1), trilinolein (C18:2C18:2C18:2), trilinolenin (C18:3C18:3C18:3), triarachidonin (C20:4C20:4C20:4), trieicosapentaenoin (C20:5C20:5C20:5), and tridocosahexaenoin (C22:6C22:6C22:6). Sterol standards used were cholesterol (Chol), cholesteryl palmitate (Chol-C16:0), cholesteryl stearate (Chol-C18:0), cholesteryl oleate (Chol-C18:1) and cholesteryl linoleate (Chol-C18:2). All standards were obtained from Larodan (Solna, Sweden). L-α-phospatidyl-l-serine (PS) from Glycine max (soybean), L-α-phosphatidylethanolamine (PE) from Glycine max (soybean), L-α-phosphatidylcholine (PC) from egg yolk and sphyngomyelin (SM) from chicken egg yolk, L-α-phosphatidylinositol from Glycine max (soybean) and L-α-lysophosphatidylcholine (LPC) from Glycine max (soybean) were purchased from Sigma-Aldrich (Milan, Italy). Methanol (MeOH), chloroform ($CHCl_3$) and water employed for the extraction procedure were obtained from Sigma-Aldrich (Milan, Italy). For LC–MS analyses, the solvents (all LC–MS grade) isopropanol (IPA) and MeOH were supplied by Sigma-Aldrich (Milan, Italy), acetonitrile (ACN) and H_2O by Panreac Quimica S.L.U. (Barcelona, Spain). Powdered anhydrous Na_2SO_4 and formic acid (HCOOH) were purchased from Sigma-Aldrich (Milan, Italy); ammonium formate (NH_4COOH) was obtained from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany).

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Stock solutions of individual lipids, namely FAs, PCs, LPCs, SMs, PSs, PEs, Chol, CEs, and TAGs, were prepared in MeOH/IPA (1:1 v/v), in the 5–25 g/L concentration range. Prior to LC injection (1 µL) each stock solution was diluted at 50 mg/L.

2.2. SAMPLE PREPARATION

The lipid fraction was extracted according to a modified Folch method [15]. Briefly, 1 mL of plasma was placed in a Pyrex tube with 9 mL of a chloroform/methanol (2:1 v/v) mixture, extracted for three times and centrifuged for ca. 20 min at 3000 rpm. Five hundred µL of water were added to promote phase separation. The lower lipid-containing organic phase was gathered, dried with anhydrous Na_2SO_4, filtered on filter paper, and then dried with a rotating evaporator. Final extract was dissolved in 1.0 mL of IPA/MeOH (1:1 v/v) and 1.0 µL of solution injected into the LC–MS.

2.3. INSTRUMENTATION AND ANALYTICAL CONDITIONS

The analyses were performed on a Shimadzu Ultra High Performance Liquid Chromatograph-Nexera system (Shimadzu, Milan, Italy), including a CBM-20A controller, two LC-30 AD dual-plunger parallel-flow pumps, a DGU-20A3R degasser, a CTO-20AC column oven and a SIL-30AC autosampler. The UHPLC system was coupled with an LCMS-2020 quadrupole mass spectrometer equipped with both ESI and APCI interfaces (Shimadzu). MS data acquisition was performed by the Shimadzu LabSolution software (ver. 5.60 SP2).

Chromatographic separation was achieved on a Titan C18, 100 × 2.1 mm I.D. with particle size of 1.9 µm (Supelco, Bellefonte, PA). The injection volume was 1 µL, mobile phase consisted of water with 20 mM ammonium formate (solvent A) and IPA/ACN/H2O with 0.1% formic acid (60:36:4, v/v) (solvent B) and the linear gradient profile was as follows: 0 min, 80% B, 6 min, 100% B (hold for 16 min). Starting conditions were achieved in 0.01 min and the column was re-equilibrated for 3 min, resulting in a total run time of 25 min. Flow-rate was 0.4 mL/min; oven temperature was 40 °C. MS acquisition was performed using alternatively ESI and APCI, in positive (+) and negative (−) ionization modes. For the sake of clarity and brevity the interface and ionization mode will be indicated as follows: ESI(+), ESI(−), APCI(+), APCI(−). The samples were analyzed in full scan mode and under selected-ion monitoring (SIM) acquisition modes sequentially. Full-scan LC–MS chromatograms were obtained by scanning *m*/*z* 350–1250, with a scan speed of 5000 amu/s and an event time of 0.2 s, in positive mode both for APCI and ESI, and from *m*/*z* 150–1250 with a scan speed of 6000 amu/s and an event time of 0.2 s, in negative mode for APCI and ESI. Sequentially, about 30 selected ions (reported in Table S1), which represent the most common FAs in plasma samples [16], were acquired in SIM(−) mode to obtain a more accurate identification.

ESI parameters were as follows: nebulizing gas (N_2) flow rate: 2 L/min; drying gas (N_2) flow: 15 L/min; detector voltage: 1.5 kV; interface voltage: 4.5 kV; desolvation line (DL) temperature: 250 °C; heat block temperature: 200 °C.

APCI parameters were as follows: nebulizing gas (N2) flow rate: 3 L/min; drying gas (N2) flow: 15 L/min; detector voltage: 1.5 kV; interface voltage: 4.5 kV; interface temperature: 450 °C; DL temperature: 250 °C; heat block temperature: 200 °C.

A mixture of three lipid standards, namely FA-C13:0, TAG-C15:0C15:0C15:0, and TAG-C17:0C17:0C17:0, were injected six times at two level of concentration (10 and 25 mg/L). Retention time and area repeatabilities were assessed. The following ions were monitored for each compound using the APCI interface: 213 *m*/*z* for FA-C13:0 in SIM(−) mode, and 523 and 579 *m*/*z* in EIC(+) mode for TAG-C15:0C15:0C15:0 and TAG-C17:0C17:0C17:0, respectively.

3. Results and discussion

ESI–MS has arguably become the most employed method for lipid analysis, especially for polar lipids. ESI–MS normally produces no fragmentation, unless specific setup is used [11]; therefore HRMS, MS/MS or MS^n experiments are often necessary for complete structural elucidation. MS/MS experiments (over HR) allow identification of isobaric species, through a different fragmentation related to the specific structure.

On the other hand, APCI-MS can produce a low protonated (or deprotonated) molecule ion intensity for some compounds, thus losing important information that was provided by ESI. For instance, some phospholipids produce such small abundances of protonated or deprotonated molecule under APCI conditions, that information on molecular weight provided by ESI can be invaluable. Thus, combining the complementary information provided by ESI and APCI interfaces, an in-depth characterization of lipids can be carried out, even without the necessity of very expensive MS analysers.

An RP-UHPLC method, suitable to be performed using both ESI and APCI interfaces coupled to a LRMS (quadrupole), is herein proposed. The capability to work sequentially in both (+) and (−) ion modes was exploited for a more comprehensive characterization.

3.1. RP-UHPLC-LRMS METHOD

When a large number of samples is analyzed, as in clinical cohorts, the total run time for each analysis plays an important role. Therefore, a compromise is needed to maximize the sample throughput and the lipidome coverage, while maintaining high quality MS information. A UHPLC system, equipped with a column having sub–2 µm particles, allows to reduce the analysis time, without loss of resolution under optimized conditions. In RP-LC the amount of water in the mobile phase is critical, in particular at the beginning of the gradient, affecting the chromatographic resolution of polar lipids. A higher concentration of water significantly improves not only the elution profile, but also the signal-to-noise ratio [17], [18]. Different organic solvents (MeOH or ACN) in combination with water were tested to optimize the UHPLC method, but a rather long separation time (>30 min) was required to elute TAGs and CEs. Better results were achieved using water containing ammonium formate (20 mM) as solvent A and a mixture of IPA/ACN/water (water with 20 mM of ammonium formate) (60:36:4 v/v/v) containing formic acid 0.1% as solvent B. The relatively low pH, obtained by using formic acid, allowed to minimize the tailing of FAs caused by the interaction of the ionized carboxyl function with free silanol sites on the LC column packing. The chromatographic pattern obtained partially fitted to the well-known model where the retention of lipids increases proportionally to their ECN [17], [18], [19], [20], [21], [22]. Some exceptions from this rule can be observed for lipids containing the combination of highly polyunsaturated and saturated fatty acyls, which can be retained more strongly and elute in higher ECN groups [21], [22]. The chromatographic LC method proposed lasts 20 min and it can be used in combination with both ESI and APCI interfaces, without requiring any adjustment. In such a way, two chromatographic profiles, perfectly equivalent in terms of retention times is obtained, from which complementary MS information can be extrapolated for a comprehensive and detailed characterization.

Retention time and area repeatability (as coefficient of variation, CV%) was assessed using three standards, namely FA-C13:0, TAG-C15:0C15:0C15:0, and TAG-C17:0C17:0C17:0. For all the compounds tested a CV% < 0.5% and <0.8% were obtained for retention times and area, respectively.

3.2. STANDARD LIPIDS ANALYSED BY UHPLC-LRMS

All standard lipids reported in Section 2.1 were analyzed and identified using the RP-UHPLC–MS method proposed, coupled with both ESI and APCI interfaces and acquired alternatingly in (+) and (−) modes. The retention times, ECN values and CN and DB (CN:DB) of the 54 standards are reported in Table S2. In general, ESI–MS using mobile phase modifiers (i.e. ammonium formate) is more sensitive than APCI-MS, but typical adducts generated are less stable and either non-linear or with dramatically reduced linear dynamic range [12]. However, the fragmentation produced by the APCI interface in a single MS event can provide similar information as ESI–MS/MS to elucidate the lipid structure [10], [11], [14].

In the following sections, mass spectra of the standard lipids analyzed are discussed in detail, divided into two main classes (polar and non-polar) and subclasses (FAs, TAGs, etc.). APCI mass spectra are reported and discussed in more detail, since the mass spectra obtained by ESI have been widely investigated and reported in previous works [12], [19], [20], [23], [24], [25], [26], [27], [28]. Table 1 summarizes the main ions present in the mass spectra of the different lipid classes (along with their relative intensities calculated on the standards) and herein discussed in the text. The fragment mechanisms reported in this manuscript are hypothesized, unless supported by previous works involving tandem MS, high-resolution accurate mass measurements and/or the use of deuterium labeled standard for fragments investigation. No investigation about regioisomeric species was carried out.

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***Table 1**. All ions generated from the analysis of lipid standards by APCI interace.*

LIPID$_{CN:DB}$	**APCI(+)**	**% int. Average (min/max)**	**APCI(−)**	**% int. Average (min/max)**
FFA	–		$[M-H]^-$	100 (100/100)
(FA$_{18:2}$; FA$_{16:0}$; FA$_{18:1}$; FA$_{18:0}$)			$[M+HCOO]^- = [M+45]^-$	50 (40/60)
LPC	$[M+H-C_5H_{13}N]^+ = [M+H-87]^+$	100 (100/100)	$[M-C_5H_{12}N-H_2O]^- = [M-104]^-$	100 (100/100)
(LPC$_{18:2}$; LPC$_{16:0}$; LPC$_{18:1}$; LPC$_{18:0}$)	$[M+H-C_4H_{11}N]^+ = [M+H-73]^+$	77 (73/80)	$[M-CH_3]^- = [M-15]^-$	30 (15/45)
	$[M+H-C_4H_{11}N-H_2O]^+ = [M+H-91]^+$	75 (65/85)	$[M-C_5H_{12}N]^- = [M-86]^-$	n.q. (n.d/10)
	$[M+H]^+$	60 (10/85)	$[M-R_{sn-1/2}COOH-C_5H_{12}N]^- = [M-FA_{sn-1/2}-86]^-$	n.d.
	$[M+H-CH_2-H_2O]^+ = [M+H-32]^+$	57 (40/74)	$[M-(R_{sn-1/2} = C{=}O)-C_5H_{12}N]^- = [M-Ketene_{sn-1/2}-86]^-$	n.d.
	$[M+H-(CH_2)_3]^+ = [M+H-42]^+$	50 (20/65)	$[R_{sn-1/2}COOH-H]^- = [FA_{sn-1/2}-H]^-$	n.d.
	$[M+H-C_5H_{13}N-H_2O]^+ = [M+H-105]^+$	52 (49/58)		
	$[M+H-C_3H_9N]^+ = [M+H-59]^+$	35 (25/45)		
	$[M+H-C_3H_9N-H_2O]^+ = [M+H-77]^+$	23 (18/27)		
	$[M+H-CH_2]^+ = [M+H-14]^+$	n.q. (n.d./12)		
PC	$[M+H-C_5H_{14}NO_4P]^+ = [M+H-183]^+$	90 (60/100)	$[M-CH_3]^- = [M-15]^-$	100 (100/100)
(PC$_{34:2}$; PC$_{34:1}$; PC$_{36:2}$; PC$_{38:4}$)	$[M+H]^+$	71(60/86)	$[M-C_4H_{10}N]^- = [M-72]^-$	76 (46/87)
	$[M+H-CH_2]^+ = [M+H-14]^+$	57 (36/100)	$[M-H-C_3H_9N]^- = [M-60]^-$	13 (7/21)
	$[M+K-CH_2]^+ = [M+K-14]^+$	42 (39/47)	$[M-C_5H_{12}N]^- = [M-86]^-$	7 (4/9)
	$[M+H-(CH_2)_3]^+[M+H-42]^+$	40 (27/56)	$[M-R_{sn-1/2}COOH-C_5H_{12}N]^- = [M-FA_{sn-1/2}-86]^-$	n.q.
	$[M+Na-C_3H_9N]^+=[M+Na-59]^+$	18 (12/30)	$[M-(R_{sn-1/2} = C{=}O)-C_5H_{12}N]^- = [M-Ketene_{sn-1/2}-86]^-$	n.q.
	$[M+H-C_3H_9N]^+ = [M+H-59]^+$	14 (8/22)	$[R_{sn-1/2}COOH-H]^- = [FA_{sn-1/2}-H]^-$	n.q.
	$[M+Na-CH_2]^+ = [M+Na-14]^+$	12 (4/24)		
	$[M+H-C_5H_{13}N]^+ = [M+H-87]^+$	n.d.		
	$[M+H-R_{sn-1/2}COOH]^+ = [M+H-FA_{sn-1/2}]^+$	n.d.		.
SPC	$[M+H-C_5H_{14}NO_4P]^+ = [M+H-183]^+$	100 (100/100)	$[M-C_5H_{12}NO_3 + HCOO]^- = [M-120]^+$	100 (100/100)
(SPC$_{34:1}$; SPC$_{34:0}$; SPC$_{36:1}$)	$[M+H-C_5H_{12}NO_3P]^+ = [M+H-165]^+$	16 (12/28)	$[M-CH_3]^- = [M-15]^-$	55 (36/84)
	$[M+H-C_3H_9N-H_2O]^+ = [M+H-77]^+$	16 (11/24)	$[M-C_4H_{10}N]^- = [M-72]^-$	45 (30/60)
	$[M+H]^+$	10 (8/13)	$[M-C_5H_{12}N-H_2O]^- = [M-104]^-$	27 (19/35)
	$[M+H-C_3H_9N]^+ = [M+H-59]^+$	9 (6/12)	$[M-H-C_3H_9N]^- = [M-60]^-$	22 (18/25)
	$[M+H-CH_2]^+ = [M+H-14]^+$	6 (5/7)	$[M+HCOO]^- = [M+45]^-$	13 (7/23)
	$[M+H-CH_2-H_2O]^+ = [M+H-32]^+$	n.q. (n.d./10)	$[M-C_5H_{12}N]^- = [M-86]^-$	11 (8/15)
	$[M+H-C_5H_{13}N]^+ = =[M+H-87]^+$	n.d.		

LIPID$_{CN:DB}$	**APCI(+)**	**% int. Average (min/max)**	**APCI(−)**	**% int. Average (min/max)**
PE	$[M+H-C_2H_8NO_4P]^+ = [M+H-141]^+$	95 (80/100)	$[M-H]^-$	100 (100/100)
(PE$_{36:5}$; PE$_{36:4}$; PE$_{34:2}$; PE$_{34:1}$)	$[M+H]^+$	94 (85/100)	$[M-H+Na+HCOO]^- = [M+77]^-$	12 (6/22)
	$[M+H+Na+C_3H_8O]^+ = [M+H-83]^+$	52 (47/62)	$[M-H-R_{1/2}COOH-C_2H_6N]^- = [M-H-FA_{1/2}-44]^-$	n.q.
	$[M+Na]^+$	37 (16/55)	$[M-H-R_{sn-1/2}COOH]^- = [M-H-FA_{sn-1/2}]^-$	n.q.
	$[M+H-R_{sn-1/2}COOH]^+ = [M+H-FA_{sn-1/2}]^+$	n.q.	$[M-H-(R_{sn1/2}=C=O)]^- = [M-H-Ketene_{sn-1/2}]^-$	n.q.
			$[M-H-(R_{sn1/2}=C=O)-C_2H_6N]^- = [M-H-Ketene_{sn-1/2}-44]^-$	n.d.
			$[R_{1/2}COOH-H]^- = [FA_{sn-1/2}-H]^-$	n.d.
PS	$[M+H-C_3H_8NO_6P]^+ = [M+H-185]^+$	100 (100/100)	$[M-H-C_3H_5NO_2]^- = [M-H-87]^-$	100 (100/100)
(PS$_{36:5}$; PS$_{36:4}$; PS$_{36:3}$; PS$_{34:2}$)	$[M+H]^+$	55 (50/61)	$[M-H]^-$	43 (41/46)
	$[M+H+C_3H_8O+Na-C_3H_5NO_2]^+ = [M+H-4]^+$	52 (40/75)	$[M-H-NH_2]^- = [M-17]^-$	38 (32/45)
	$[M+NH_4-C_3H_5NO_2]^+ = [M+NH_4-87]^+$	48 (47/50)	$[M-R_{sn-1/2}COOH]^- = [M-H-FA_{sn-1/2}]^-$	n.q.
	$[M+H+Na+C_3H_8O]^+ = [M+H-83]^+$	37 (32/45)	$[M-R_{sn-1/2}COOH-C_3H_5NO_2]^- = [M-FA_{sn-1/2}-87]^-$	n.q.
	$[M+NH_4-C_3H_7NO_5P]^+ = [M+NH_4-167]^+$	23 (18/26)	$[M-(R_{sn1/2}=C=O)-C_3H_5NO_2]^- = [M-Ketene_{sn-1/2}-87]^-$	n.q.
	$[M+H-C_3H_5NO_2]^+ = [M+H-87]^+$	n.q. (n.d./12)	$[M-(R_{sn1/2}=C=O)]^- = [M-H-Ketene_{sn-1/2}]^-$	n.d.
	$[M+H-R_{sn-1/2}COOH]^+ = [M+H-FA_{sn-1/2}]^+$	n.d.		
PI	$[M+NH_4-C_6H_{11}O_8P]^+ = [M+NH_4-242]^+$	100 (100/100)	$[M-H]^-$	100 (100/100)
(PI$_{34:3}$; PI$_{36:4}$; PI$_{34:2}$; PI$_{36:2}$)	$[M+H-C_6H_{13}O_9P]^+ = [M+H-260]^+$	72 (64/83)	$[C_6H_9O_7P]^- = [241]^-$	35 (20/50)
	$[M+H+C_3H_8O+Na]^+ = [M+H-83]^+$	63 (57/65)	$[C_6H_{11}O_8P]^- = [259]^-$	20 (10/25)
	$[M+H-C_6H_{11}O_8P]^+ = [M+H-242]^+$	36 (20/44)	$[C_6H_7O_8P]^- = [273]^-$	10 (7/13)
	$[M+NH_4]^+$	20 (15/25)	$[C_6H_7O_6P]^- = [223]^-$	n.q.
	$[M+Na]^+$	15 (7/20)	$[M-R_{sn-1/2}COOH-C_6H_{10}O_5]^- = [M-FA_{sn-1/2}-162]^-$	n.q.
	$[M+H]^+$	n.q. (n.d/10)	$[M-R_{sn-1/2}COOH]^- = [M-FA_{sn-1/2}]^-$	n.q.
	$[M+H-R_{sn-1/2}COOH]^+ = [M+H-FA_{sn-1/2}]^+$	n.q	$[M-(R_{sn1/2}=C=O)]^- = [M-Ketene_{sn-1/2}]^-$	n.d.
			$[M-(R_{sn-1/2}=C=O)-C_6H_{10}O_5]^- = [M-Ketene_{sn-1/2}-162]^-$	n.d.
Chol	$[M+H-H_2O]^+ = [C_{27}H_{44}+H]^+ = [369]^+$	100 (100/100)	–	
(free cholesterol)				

LIPID$_{CN:DB}$	**APCI(+)**	**% int. Average (min/max)**	**APCI(−)**	**% int. Average (min/max)**
CE	$[M+H\text{-}FA]^+ = [C_{27}H_{44}+H]^+ = [369]^+$	100 (100/100)	$[M\text{-}H\text{-}C_{27}H_{45}]^- = [FA\text{-}H]^-$	100 (100/100)
(CE$_{18:2}$; CE$_{18:1}$; CE$_{16:0}$; CE$_{18:0}$)			$[M+HCOO\text{-}C_{27}H_{45}]^- = [FA+HCOO]^- = [FA+45]^-$	40 (27/58)
TAG	$[M+H]^+$	n.q	$[R_{sn\text{-}1/2/3}COOH\text{-}H]^- = [FA_{sn\text{-}1/2/3}\text{-}H]^-$	n.q.
	$[M\text{-}R_{1/2/3}COOH]^+ = [M+H\text{-}FA_{1/2/3}]^+$	n.q.	$[R_{sn\text{-}1/2/3}COOH+HCOO]^- = [FA_{sn\text{-}1/2/3} + 45]^{--}$	n.q.
			$[M−H]^-$	n.q.

n.q: Ion intensity non-quantifiable, where the ion intensity can depend by the combination of different factors (e.g. number of unsaturations, carbon chain lenght, and/or position of FA on glycerol backbone). **n.d**: Ion aspected but non detected.

3.2.1. POLAR STANDARDS LIPIDS

3.2.1.1 Fatty acid (FAs)

FAs can be analysed in both (+) and (−) ion modes with API interfaces, although the ionization of choice in our case is always the negative one (both in scan and SIM mode). The base peak was the de-protonated molecule ion $[M−H]^-$, followed, in some cases, by formate adduct $[M+HCOO]^- = [M+45]^-$ ion, while no fragmentation at all was observed. The SIM(−) values used to detect FA are reported in Table S1.

3.2.1.2. Phosphatydilcholine (PC) and lysophosphatydilcholine (LPC)

All the combination, both ESI and APCI under positive and negative modes were applied. The use of ESI(+) leads to the protonated molecule, $[M+H]^+$, as the base peak, followed by the sodium adduct, $[M+Na]^+$. No fragmentation was observed. The use of ESI(−) generally gave mass spectra containing different PC molecule-related ions. However, if formic acid (HCOOH) is used as mobile phase additive (as in the proposed method), the PC species form an adduct $[M+45]^-$ ion as base peak, as also observed by other authors [25], [27].

By using the APCI(+) interface, the main ions were the fragments $[M+H−C_5H_{14}NO_4P]^+ = [M+H−183]^+$ (loss of the polar head group, $C_5H_{14}NO_4P$) and $[M+H−CH_2]^+ = [M+H−14]^+$ [11], [12], [13], [27], [29]. The protonated molecule ion $[M+H]^+$ is also present. Other characteristic fragments were, $[M+H−N(CH_3)_3]^+ = [M+H−59]^+$ and a particular fragment ion $[M+H−42]^+$ that was tentatively identified as $[M+H−(CH_2)_3]^+$. The latter fragment can be confounding since it can lead to a misinterpretation of the lipid class. The loss of three methyl groups of choline and substitution with H^+ ions convert a choline to an ethanolamine (i.e. $[PC\_34:2 + H−(CH_2)_3]^+ = [PE\_34:2+H]^+$ in term of *m*/*z*). This misinterpretation can be resolved considering the ions generated by APCI(−), where the presence of the $[M−H]^-$ ion is needed for confirming that it belongs to the PE class (i.e. $[PE\_34:2−H]^-$, see also Section 3.2.1.4).

When subjected to APCI(−), PC showed no ions from the intact molecule, such as a deprotonated molecule ion. The ion $[M−H]^-$ was reported only once by Qiu et al. [28]. PC does not contain any acid protons and the authors did not discuss about this remarkable phenomenon. Instead, PC forms mainly a set of four ion species: $[M{-}CH_3]^- = [M{-}15]^-$ (loss of methyl group), $[M{-}H{-}C_3H_9N]^- = [M{-}60]^-$ (loss of trimethylamine group), $[M{-}C_5H_{12}N]^- = [M{-}86]^-$ (loss of choline residue), and $[M{-}C_4H_{10}N]^- = [M{-}72]^-$ fragment ion. Some of these fragments generated by APCI(−) have been observed before, but never considered [12], [28], [29]. Additional characteristic fragments, such as $[M{-}R_{sn\text{-}1/2}COOH{-}C_5H_{12}N]^- = [M{-}FA_{sn\text{-}1/2}\text{-}86]^-$ (loss of the fatty acyl substituents as FA and the choline residue), $[M{-}(R_{sn\text{-}1/2} = C = O){-}C_5H_{12}N]^- = [M\text{-}Ketene_{sn\text{-}1/2}\text{-}86]^-$ (loss of the fatty acyl substituent as a ketene and the choline residue) and $[R_{sn\text{-}1/2}COOH{-}H]^- = [FA_{sn\text{-}1/2}{-}H]^-$ (fatty carboxylate anions) were observed. Mass spectra of PC-16:0_18:2 from egg yolk, in both APCI(+) and (−) are reported in Figure 1, while in Figure 2 the hypothesized structures related to a fragmentation of the choline group are shown.

LPC in ESI(+) led to the protonated molecule ion $[M+H]^+$ as base peak, while in the ESI(−) showed the adduct $[M+HCOO]^-$ and the fragment $[M{-}CH_3]^- = [M{-}15]^-$. APCI(+) generated $[M+H{-}C_5H_{13}N]^+ = [M+H{-}87]^+$ (loss of the choline residue) as base peak ion, followed by $[M+H{-}C_4H_{11}N]^+ = [M+H{-}73]^+$, $[M+H{-}C_4H_{11}N{-}H_2O]^+ = [M+H{-}91]^+$, the protonated molecule ($[M+H]^+$), $[M+H{-}CH_2{-}H_2O]^+ = [M+H{-}32]^+$, $[M+H{-}(CH_2)_3]^+ = [M+H{-}42]^+$, $[M+H{-}C_5H_{13}N{-}H_2O]^+ = [M+H{-}105]^+$ (loss of the $C_5H_{13}N$ choline residue + loss of H_2O from the glycerol backbone), $[M+H{-}C_3H_9N]^+ = [M+H{-}59]^+$, $[M+H{-}C_3H_9N{-}H_2O]^+ = [M+H{-}77]^+$, and $[M+H{-}CH_2]^+ = [M+H{-}14]^+$ ions. Moreover, APCI(+) generates a multitude of additional fragment-ions, some of which are difficult to identify. Important structural information was obtained in APCI(−). The $[M\text{-}C_5H_{12}N\text{-}H_2O]^- = [M{-}104]^-$ ion (loss of $C_5H_{12}N$ choline residue + loss of H_2O from the glycerol backbone) was the main ion, followed by $[M{-}CH_3]^- = [M{-}15]^-$, and $[M{-}C_4H_{10}N]^- = [M{-}72]^-$, the same fragment-ion found for each choline-related lipid (see Figure 2b). $[R_{sn\text{-}1/2}COOH\text{-}H]^- = [FA_{sn\text{-}1/2}{-}H]^-$ ions may also be present.

***Figure 1**. Full scan mass spectra of PC-34:2 standard (PC-16:0_18:2) by APCI(+) (a) and APCI(−) (b).*

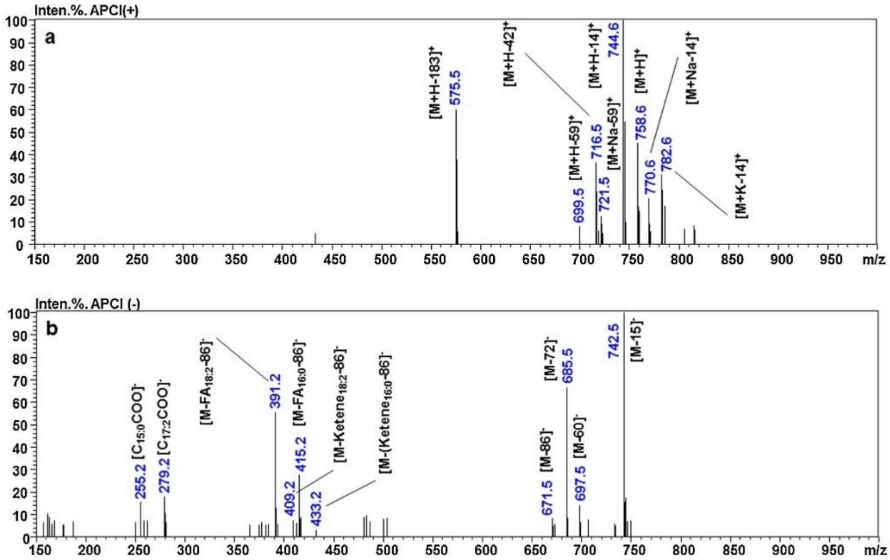
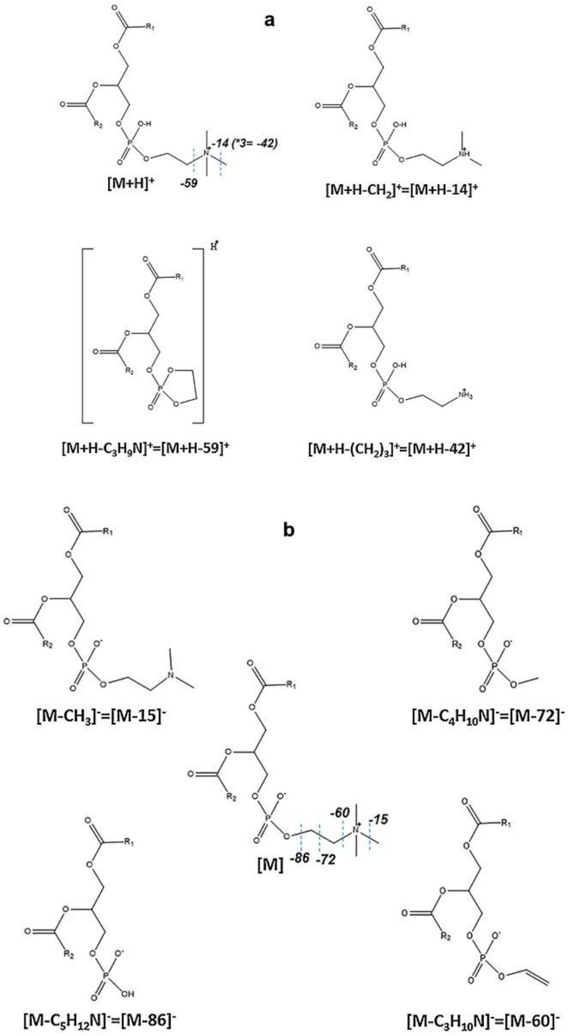


***Figure 2**. Hypothesized structures related to a fragmentation of choline group in PC lipid class by APCI(+) (a) and APCI(−) (b).*



3.2.1.3. Sphingophsphocholine (SPC) [sphingomyelin (SM) and dihydrosphingomyelin (DSM)]

The base peaks in the mass spectra of SPCs, analyzed using ESI(+), were the protonated molecule ion, $[M+H]^+$, and the sodium adduct, $[M+Na]^+$. With ESI(−) the $[M+HCOO]^-$ ion was the major adduct observed. Poor structural information was obtained from these fragments, even when ESI–MS/MS was employed, as reported by other research groups [29]. APCI(+) due to more extensive in-source fragmentation, gave mainly ceramide (Cer)-like product ions, $[M+H-C_5H_{14}NO_4P]^+ = [M+H-183]^+$ (loss of the polar head group $C_5H_{14}NO_4P$, corresponding to a $[Cer-H_2O]^+$ ion), but also $[M+H]^+$ and $[M+H-C_5H_{12}NO_3P]^+ = [M+H-165]^+$ (corresponding to a $[Cer+H]^+$ ion) were present. Other characteristic fragments were $[M+H-C_3H_9N-H_2O]^+ = [M+H-59-18]^+ = [M+H-77]^+$, $[M+H-CH_2]^+ = [M+H-14]^+$, $[M+H-C_3H_9N]^+ = [M+H-59]^+$, and $[M+H-CH_2-H_2O]^+ = [M+H-14-18]^+ = [M+H-32]^+$. All these fragment ions were reported as "tentatively assigned" by Karlsson and co-workers [27], [29]. APCI(+) has been already reported to be more useful than ESI(+) for structural characterization of these compounds [10], [11], [29], [30], [31], while very little information about SPCs' fragments, obtained using APCI(−) mode have been reported. The same set of four ion species, obtained from PCs were observed, plus two additional diagnostic fragments, namely $[M-104]^-$ and $[M-120]^-$. The first fragment, observed also in LPC class, was most probably generated from the simultaneous loss of a choline residue (86 *m/z*) and H_2O (18 *m/z*) from the sphingosine backbone ($[M-C_5H_{12}N-H_2O]^- = [M-86-18]^-$), while the latter fragment was the predominant ion in the SPCs class (Table 1). This fragment was initially confused with a $[Cer+HCOO]^-$ formate adduct ion (loss of the $C_5H_{12}NO_3P$ residue from polar head group, corresponding to ceramide, + formate adduct), but this form would require a not usual scission of the bond between the oxygen next to the serine backbone and the phosphor. Moreover, the instability of proton-shifted imidic acid tautomer could not lead the formation of the formate adduct, especially in a such a high amount. This fragment could be obtained from several other precursors, which justify the abundance of this fragment. Either the formate adduct $[M+45]^-$ ($[(M+HCOO)-H_2O-OCH_2CH_2N(CH_3)_3-HCOO+H]^-$) or other fragments as $[M-15]^-$ ($[(M-CH_3)-H_2O-C_4H_{10}NO+H]^-$) could undergo loss of the 3-hydroxyl group by dehydration (18 *m/z*) combined with cleavage of the phosphocholine moiety between the phosphorus and the oxygen attached to the ethane linkage to the quaternary amine (the phosphorus picks up a hydrogen to give a remaining HPO_3) to form $[M-120]^-$. The identity of the fragment, here tentatively assigned, can readily be distinguished if high-resolution accurate mass (HRAM) MS is used.

DSMs, much less common than SMs, differ from SMs only in the absence of 4,5-unsaturation in the sphingosine backbone. A single quadrupole MS does not provide information on the long-chain based part and on the fatty acid part used to differentiate DSM from SM [10], [30]. To perform such a discrimination a tandem MS analysis is needed [10], [11], [29], [30], [31]. For these reason, regard to these sub-classes, the generic SPC nomenclature was used. SMs and DSM show exactly the same fragmentation under APCI-MS [10]. For instance, the SPC-36:1, it could be SM-d18:1/18:0 or DSM-d18:0/18:1.

In Figure 3, mass spectra obtained using APCI(+) and (−) of the SPC-34:0 standard (in this case the SPC sub-class is a DSM, because no unsaturation is present) are shown. The hypothesized structures related to a fragmentation of the choline group are reported (Figure 4).

***Figure 3**. Full scan mass spectra of SM-34:0 standard by APCI(+) (a) and APCI(−) (b).*

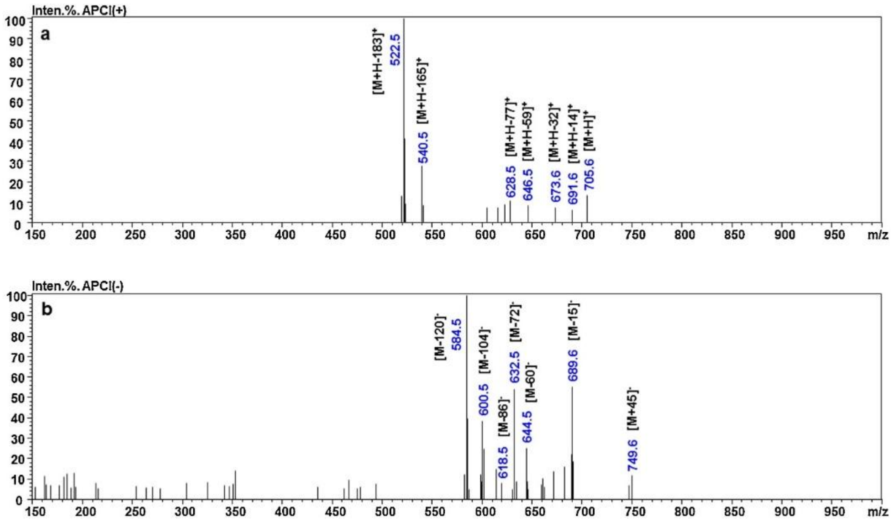
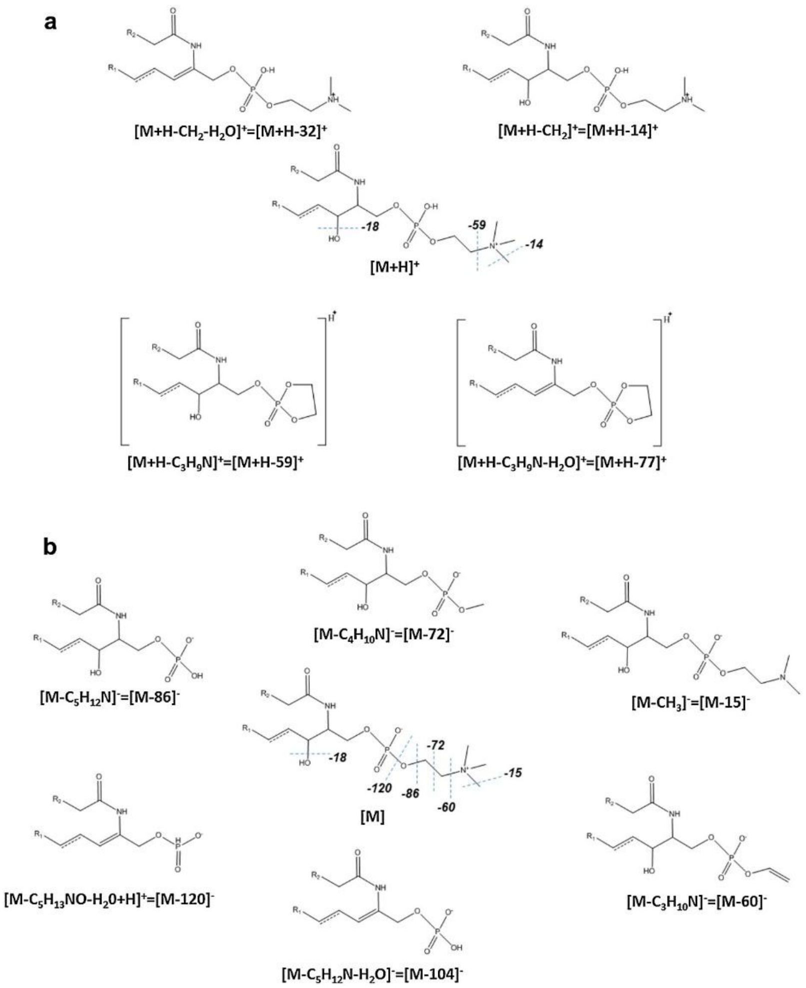


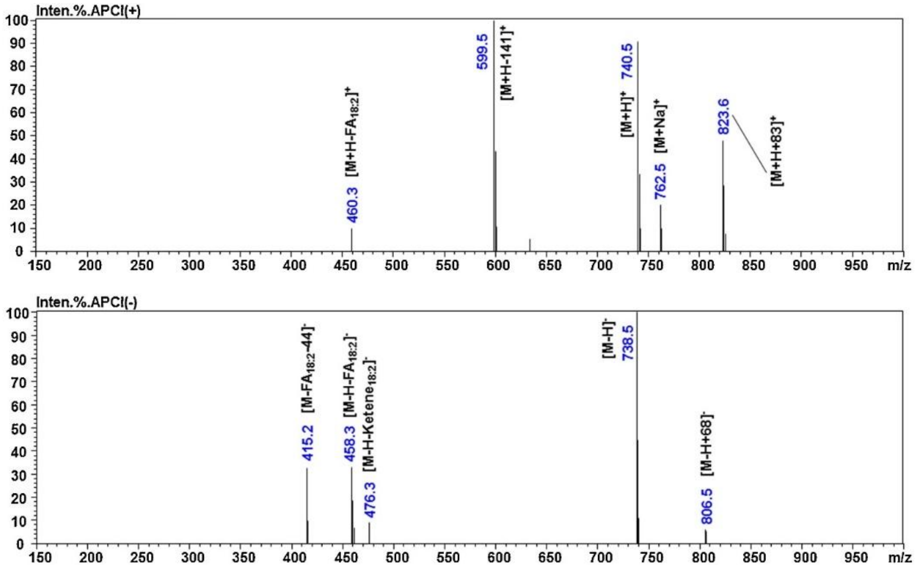
***Figure 4**. Hypothesized structures related to a fragmentation of the choline residue in SPC lipid class by APCI(+) (a) and APCI(−) (b). The 4,5-trans double bond is indicated as a dotted line, since can be present, as in the case of SM, or absent as for DSM.*



3.2.1.4. Phosphatidylethanolamine (PE)

The main molecule-related ions of PE by using ESI(+) were $[M+H]^+$ and its sodium adduct $[M+Na]^+$. PE was the only phospholipid where $[M+H−C_2H_8NO_4P]^+ = [M+H−141]^+$ ion (loss of the polar head group) was observed in ESI(+). ESI(−) generated mainly the $[M−H]^−$ ion and no adducts was observed. By using APCI(+), the main ions were $[M+H−C_2H_8NO_4P]^+ = [M+H−141]^+$ and the protonated molecule $[M+H]^+$, followed by sodium adduct $[M+Na]^+$ and a proposed adduct $[M+H+83]^+$ ion that was tentatively assigned as $[M+H+Na+C_3H_8O]^+$ (C_3H_8O: isopropanol). The latter adduct-ion (also generated from PS and PI under APCI(+) mode) was reported by Fiehn [32] as possible adduct-solvent-contaminant-ion observed by API-HRMS. In APCI(−), $[M−H]^−$ ion was the base peak for each PE investigated, followed by a $[M−H+Na+HCOO]^− = [M−H+Na + 45]^− = [M−H+68]^−$. Other fragments, as $[M\text{-}H\text{-}R_{sn\text{-}1/2}COOH]^- = [M\text{-}H\text{-}FA_{sn\text{-}1/2}]^-$ (loss of fatty acid), $[M\text{-}H\text{-}(R_{sn1/2} = C{=}O)]^- = [M\text{-}H\text{-}Ketene_{sn\text{-}1/2}]^-$ (loss of the fatty acyl substituent as ketenes) and $[M −R_{sn\text{-}1/2}COOH−C_2H_6N]^− = [M\text{-} FA_{sn\text{-}1/2}\text{-}44]^−$ (loss of the fatty acyl substituents + ethanolamine residue) ions were also observed. These fragments have been reported by Hsu and Turk by ESI–MS/MS [33], [34]. In Figure 5, mass spectra of PE-C34:6 (PE-C18:2_C18:2) obtained by using both APCI(+) and APCI(−), are shown.

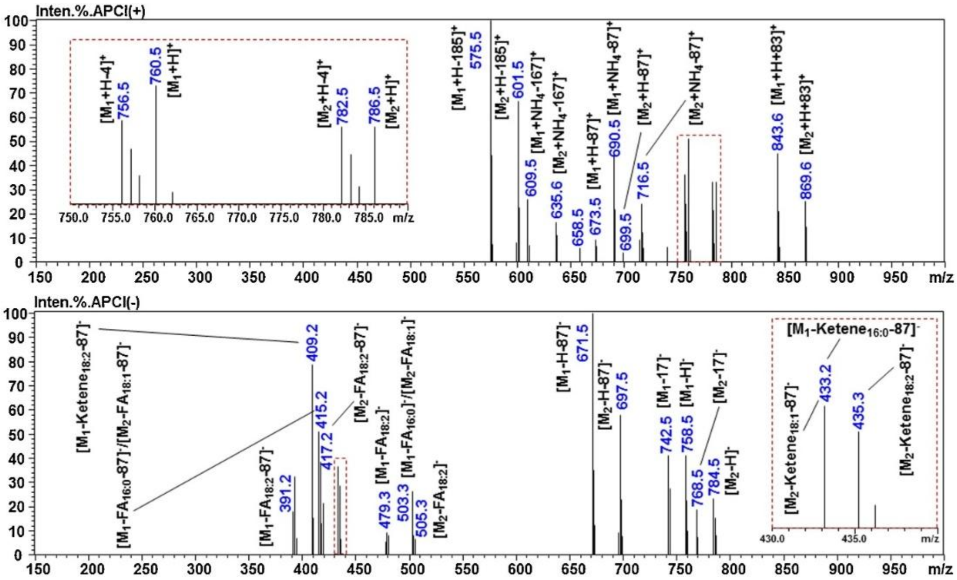
***Figure 5**. Full scan mass spectra of PE-36:4 standard from Glycine max (soybean) (PE-18:2_18:2) by APCI(+) (a) and APCI(−) (b).*



3.2.1.5. Phosphatidylserine (PS)

In ESI(+), the major related molecule ions were $[M+H]^+$ and sodium adduct $[M+Na]^+$. When subjected to ESI(−), PS generated mainly $[M-H]^-$ ion. APCI(+) generated $[M+H-C_3H_8NO_6P]^+ = [M+H-185]^+$ (loss of the polar head group, corresponding to a $[DAG\text{-}H_2O]^+$) as the base peak followed by $[M+H]^+$ ion. As PE, PS in APCI(+) showed the formation of $[M+H+Na+C_3H_8O]^+ = [M+H+83]^+$ adduct ion. Furthermore, a series of related serine fragmentation ions from the loss of the serine residue (87 *m/z*) were observed: $[M+H-C_3H_5NO_2]^+ = [M+H-87]^+$, $[M+NH_4\text{-}C_3H_5NO_2]^+ = [M+NH_4-87]^+$, and $[M+H+Na+C_3H_8O\text{-}C_3H_5NO_2]^+ = [M+H+23+60-87]^+ = [M+H-4]^+$. This inusual $[M+H\text{-}4]^+$ fragment ion under APCI(+) was tentatively assigned as the loss of the serine residue (87 *m/z*) from the adduct-solvent-ion $[M+H+83]^+$ [32], although HRMS and/or MS/MS experiments are needed for an unambiguous confirmation. $[M+NH_4-C_3H_7NO_5P]^+ = [M+NH_4\text{-}167]^+$ ion (loss of $C_3H_6NO_5P$ the polar head group residue) was also observed. Even if APCI(+) was very informative, APCI(−) gave more structural information in this class of phospholipid and resulted in spectra very similar to those obtainable from ESI–MS/MS(−) [35]. $[M\text{-}H\text{-}C_3H_5NO_2]^- = [M\text{-}H\text{-}87]^-$ ion (neutral loss of the serine group) was the base peak, followed by $[M-H]^-$ and an undefined $[M-17]^-$ ions. The latter ion was tentatively attributed as $[M-H-NH_2]^-$ ion (loss of amino group). $[M-(R_{sn1/2}=C{=}O)\text{-}C_3H_5NO_2]^- = [M-Ketene_{sn\text{-}1/2}\text{-}87]^-$ (neutral loss of the serine group and the acyl ketene), $[M-R_{sn\text{-}1/2}COOH\text{-}C_3H_5NO_2]^- = [M\text{-}FA_{sn\text{-}1/2}\text{-}87]^-$ (neutral losses of serine group and the FA) and, $[M-R_{sn\text{-}1/2}COOH]^- = [M-FA_{sn\text{-}1/2}]^-$ (loss of FA) ions were present. Figure 6 shows the APCI(+/−) mass spectra of two co-eluted PSs (PS-C16:0_C18:2; PS-C18:2_C18:1), where all fragment ions aforementioned are present.

Figure 6*. Full scan mass spectra of co-eluted PS-34:2 (M1) and PS-36:3 (M2) standards from Glycine max (soybean) (M1: PS-16:0_18:2; M2: PS-18:1_18:2) by APCI(+) (a) and APCI(−) (b).*



3.2.1.6. Phosphatidylinositol (PI)

In ESI(+), $[M+Na]^+$ and, in low intensity, the protonated $[M+H]^+$ ions were observed, while ESI(−) formed an abundant $[M-H]^-$ ions of PI species, followed by $[M+45]^-$. In APCI(+) a series of related molecule ions as $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, and the same $[M+H+C_3H_8O+Na]^+ = [M+H+83]^+$ adduct ion observed in the PE and PS LC–MS analysis, was observed. The base peak was $[M+NH_4-C_6H_{11}O_8P]^+ = [M+ NH_4-242]^+ = [M-224]^+$, corresponding to a $[DAG+NH_4]^+$ ion (where 242 corresponds to the loss of the $C_6H_{11}O_8P$ polar head group residue of the monophosphate inositol), followed by $[M+H-C_6H_{13}O_9P]^+ = [M+H-260]^+$ and the $[M+H-C_6H_{11}O_8P]^+ = [M+H-242]^+$ ions, corresponding to a $[DAG+H]^+$ ions (loss of the polar head residue). $[M-R_{sn-1/2}COOH]^+ = [M-FA_{sn-1/2}]^+$ can also be observed. APCI(−) led to the formation of a different ions related to the phosphoinositol molecule: mainly 241 *m/z* $[C_6H_{10}O_8P]^-$, 259 *m/z* $[C_6H_{12}O_9P]^-$ (corresponding to the inositol monophosphate), 273 *m/z* $[C_7H_{14}O_9P]^-$, and 223 *m/z* $[C_6H_8O_7P]^-$. Under APCI(−) conditions, deprotonated molecule ion $[M-H]^-$ (as base peak), followed by $[M-H-R_{sn-1/2}COOH-C_6H_{10}O_5]^- = [M-H-FA_{sn-1/2}-162]^-$ (or at the same *m/z* value $[M-H-R_{sn-1/2}C=O-C_6H_{12}O_6]^- = [M-H-Ketene_{sn-1/2}-180]^-$) were observed. The detailed mechanism for the formation of these fragment ions have been studied and reported by Hsu and Turk [36].

3.2.2. NON-POLAR LIPIDS

3.2.2.1. Triacylglycerol (TAG)

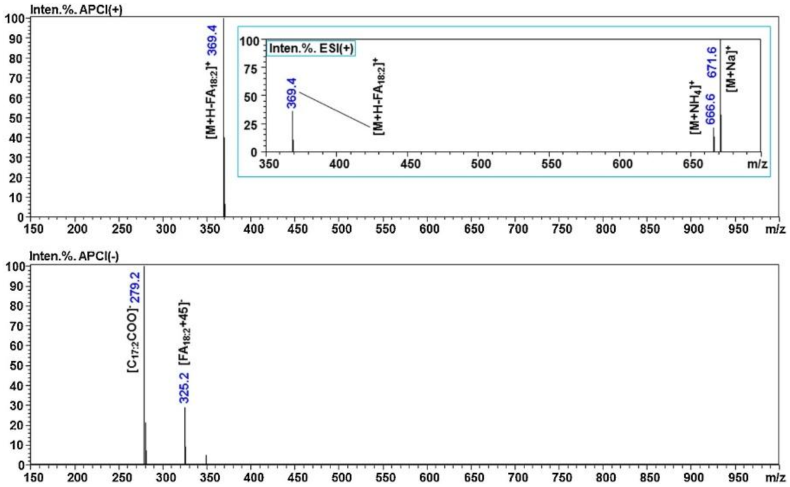
By using ESI(+) a series of adduct ions $[M+X]^+$ ($X = NH_4^+$; Na^+) were generated, but less or no fragmentation was observed. No information was obtained by ESI(−). APCI(+) is the most applied TAGs ionization mode and the fragmentation pattern is widely known [14], [37], [38], [39], [40].

Briefly, APCI(+) was able to produce protonated molecule ion $[M+H]^+$ and fragments (corresponding to $[DAG\text{-}H_2O]^+$ ion) generated for the loss a fatty acid forming a $[M+H\text{-}R_{sn\text{-}1/2/3}COOH]^+ = [M+H\text{-}FA_{sn\text{-}1/2/3}]^+$ ion. In APCI(−) the base peak was represented by the loss of a fatty acids from TAG, but in general poor fragmentation was observed. Only the ESI(+) and APCI(+) were considered for further identification.

3.2.2.2. Cholesterol (Chol) and cholesterol-ester (CE)

ESI(+) generated adduct molecule ions $[M+X]^+$ ($X = NH_4^+$, Na^+) and fragment ions $[M+H\text{-}RCOOH]^+ = [M+H\text{-}FA]^+$ for the CEs, and $[M+H\text{-}H_2O]^+$ for the free cholesterol (Chol); while APCI(+) generated $[M+H\text{-}H_2O]^+$ and $[M+H\text{-}FA]^+$ as base peak, for Chol and CEs, respectively, giving in both cases 369 *m/z*. Using only APCI(+) it would have been not possible to determine any structure information from different CEs, due to the absence of fragments related to the loss of the fatty acid bonded on the cholesterol backbone. Therefore, identification could rely only on the chromatographic behaviour related to the ECN values and the use of pure standards. APCI(−) generated the fragment deriving by the loss of a fatty acid from CE $[M{-}H\text{-}C_{27}H_{45}]^- = [FA\text{-}H]^-$, followed by the respective formate adduct $[M+HCOO\text{-}C_{27}H_{45}]^- = [FA+HCOO]^- = [FA+45]^-$. Thus, combining the information obtained by APCI spectra acquired using both (+) and (−) mode, the complete identification of CEs was possible. A comparison of ESI and APCI mass spectra of a CE standard (Chol-C18:2) is shown in Figure 7.

Figure 7. *Full scan mass spectra of CE-18:2 standard by using APCI(+) (a) and APCI(−) (b). The ESI(+) spectrum of the same CE-18:2 standard is reported as insert in Figure 7a.*



3.3. LIPIDOMICS ANALYSIS OF PLASMA SAMPLE BY RP-UHPLC-LRMS

The blood lipidome is a complicated mixture of different lipid classes, which vary in FAs composition [17]. Most of the FAs are bound as esters, with only small amounts occurring as non-esterified fatty acids. Retention times and pattern fragmentations of each lipid standard, in (+) and (−) ion mode, by using both interfaces, were considered for accurate lipid identification in plasma sample.

The lipid extract from human plasma was analysed by using the described UHPLC-LRMS method. The sample was injected six times and CV% of retention time and area (calculated on the most intense fragment) was evaluated for a series of lipid species present in the plasma sample, namely FA-18:2, Chol, PC-18:1_18:2, CE-18:2, and TAG-C18:1_C18_1_C18_1. CV% was <0.5% and <10.0% for retention time and area, respectively. Total ion current chromatograms acquired using both APCI(+) and APCI(−) are shown in Figure 8. A list of all the identified lipids is reported in Table S3.

As largely discussed, APCI interface allowed the formation of diagnostic fragment ions for all classes of lipid in both (+) and (−) ion modes (except for FAs where the only [M−H]− was observed by using both interfaces). Few additional examples are here discussed to further support the identification potentiality of the proposed approach in a real-world sample. On the top of Figure 9, a mass scan spectrum at 11.85 min of RP-UHPLC-ESI(+)-MS analysis of plasma sample is reported. It is evident that coelution of several TAGs with the same ECN = 46 occured. In fact, four different pairs of *m*/*z* $[M+X]^+$ (where $X = NH_4^+$; Na^+) values, namely 822.8/827.7 (C48:1), 848.8/853.7 (C50:2), 874.9/879.7 (C52:3), 900.8/905.8 (C54:4) were present. Based on the presence of these ions, according to the combination of most common FAs present in human plasma sample into glycerol backbone [40], 31 different TAGs could be hypothesized (on the right of Figure 9), not considering the occurrence of positional isomers. On the bottom of Figure 9, the same mass scan spectrum at 11.86 min obtained using the APCI(+) interface is reported. The presence of fragment $[M+H\text{-}R_{sn\text{-}1/2/3}COOH]^+ = [M+H\text{-}FA_{sn\text{-}1/2/3}]^+$ ions (corresponding to different $[DAG_{(s)}\text{-}H_2O]^+$ species) and the combination with their protonated molecules, $[M+H]^+$ (corresponding to different TAG species), allowed to confirm the identity of only 10 TAGs out of 30 previously hypothesized (marked in red on the right of Figure 9). APCI(+) provided informative spectra, with a protonated molecule and fragment ions, which allowed structural elucidation (bottom of Figure 9).

In (−) mode, less used in this research field, APCI generated important structural information, in particular in cholesterol esters and glycerophospholipids, eliminating some potential ambiguity in identification of possible isobaric fragment ions, generated by positive-ion mode. In Figure 10 the mass spectra by APCI(+/−) of PI compound (PI-18:0_20:4) identified in human plasma is reported. As for other PLs, the use of two polarities (+/−) was needed to obtain unambiguous information on the structure. Although, all ions generated from the analysis of phospholipids standards with APCI interface were previously discussed, only the ions with a ratio greater than or equal to 30% compared to the base peak were considered for the identification (with some exceptions, such as the fragment ions related to the loss of FA and/or FA as ketene, or in the case where all fragment ions were below 30%).

The reported identification of the plasma lipid extract was based on 3 criteria: (i) only ions with a ratio ≥ 30% were considered, unless well-documented in the literature; (ii) the PLs identification was based on both positive and negative ionization modes, and never considering only one polarity; (iii) identification was proposed only when related molecule ions were expressed by ESI–MS. Additional considered criterium was the retention order of lipids on RP column [17], [18], [19], [20], [21], [22].

About 100 lipid species from 10 lipid classes, namely FA, LPC, SPC, PI, PE, PC, DG, Chol, CE, and TAG have been positively identified in the total lipid extract of a human plasma in 25 min analysis time.

The propose method proved to be very powerful. For instance, considering the PL profile, no loss of information (in terms of number of compounds identified) was observed, compared to a multidimensional approach [19]. Moreover, the analysis time was 14 times faster, including re-equilibration time (25 min vs 360 min).

Figure 8. *Total ion current chromatograms of lipid fraction of plasma sample by using both APCI(+) (a) and APCI(−) (b).*

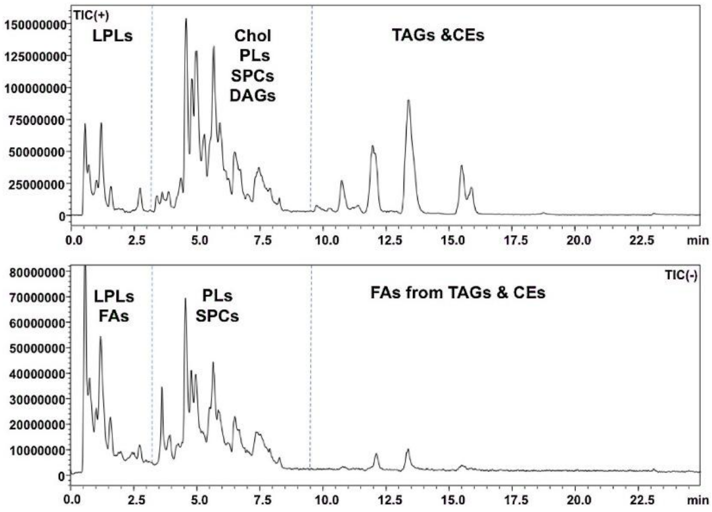


Figure 9. *On the top, a full scan mass spectrum at 11.86 min of RP-UHPLC-ESI-LRMS analysis of human plasma lipid extract. On the bottom, a full scan mass spectrum at the same time analysis by RP-UHPLC-APCI-LRMS of human plasma lipid extract. On the right, the list of 30 different TAGs tentatively identified by ESI(+) and, marked in red, the 10 TAGs confirmed by APCI(+) out of 30 previously hypothesized. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

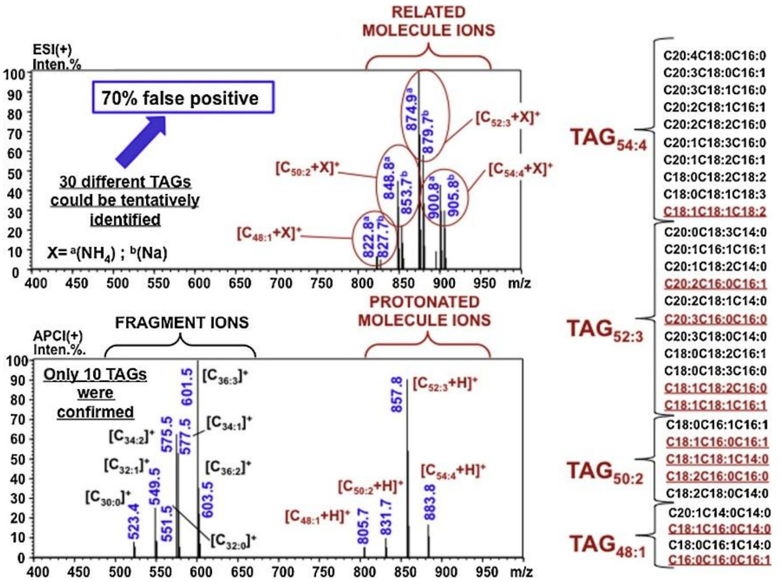
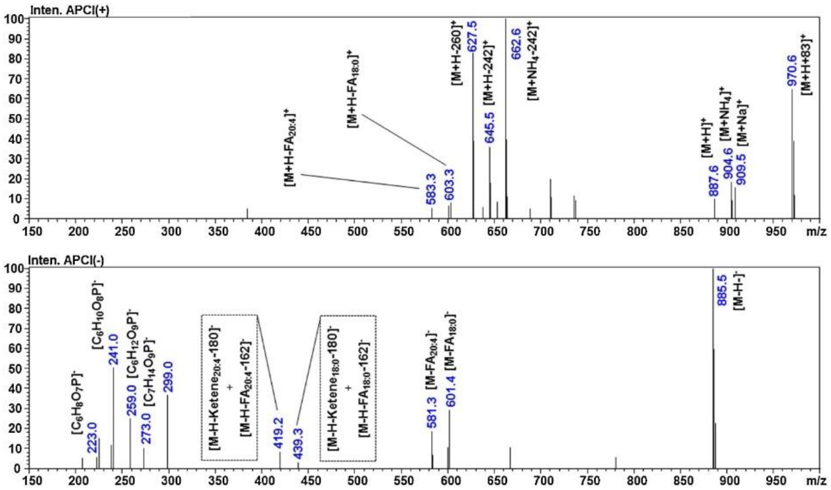


Figure 10*. Full scan mass spectra of PI-38:4 from human plasma lipid extract (PI-18:0_20:4) by APCI(+) (a) and APCI(−) (b) are reported.*



4. Conclusion

The proposed method can be considered a comprehensive platform for lipidomics studies. A UHPLC method using a flow rate compatible with both ESI and APCI interfaces was optimized. Furthermore, no chromatographic conditions modification (i.e. mobile phase composition and/or flow-rate) was required to switch from ESI to APCI. Therefore, two complementary chromatograms, perfectly overlapped, were obtained, providing complementary MS information. Although the sensitivity of APCI-MS is usually less than ESI–MS when a buffer is added in the mobile phase, the structural information to be gleaned from the fragmentation is well worth the tradeoff in sensitivity. On the other hand, ESI is an indispensable complement to APCI, especially when lipids containing oxygen functional group are investigated or for confirming related molecule ions, generally less expressed by APCI. The proposed method can be a valid, simple, and cheaper technique easily reproducible elsewhere for lipidomics studies, which can provide almost similar information compared to more sophisticated and expensive techniques, such as LC-ESI-HRMS, tandem MS, and/or multidimensional LC-ESI–MS.

Moreover, at the best of our knowledge, this is the first time that mass spectra of PS and PI were reported and discussed by using APCI interface.

However, it is important to keep in mind that, unless widely supported by the literature the use of LC-APCI/ESI-LRMS can not provide unequivocal identification of all the fragments observed, for which more powerful techniques are necessary.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.chroma.2017.06.017.

References

[1] Y.A. Hannun, A personal journay with bioactive lipoids, Eur. J. Lipid Sci. 117 (2015) 1814–1821.

[2] E. Fahy, S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill, R.C. Murphy, C.R. Raetz, D.W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van Meer, M.S. VanNieuwenhze, S.H. White, J.L. Witztum, E.A. Dennis, A comprehensive classification system for lipids, J. Lipid Res. 46 (2005) 839–861.

[3] K. Anjani, M. Lhomme, N. Sokolovska, C. Poitou, J.A. Wisnewsky, J.L. Bouillot, P. Lesnik, P. Bedossa, A. Kontush, K. Clement, I. Dugail, J. Tordjman, Circulating phospholipid profiling identifies portal contribution to NASH signature in obesity, J. Hepatol. 62 (2015) 905–912.

[4] G. Astarita, New frontiers for mass spectrometry in lipidomics, part II, LC-GC North Am. 30 (2012) 482–487.

[5] F. Cacciola, P. Donato, M. Beccaria, P. Dugo, L. Mondello, Advances in LC–MS for food analysis, LC-GC Eur. 25 (2012) 15–24.

[6] M. Aguennouz, M. Beccaria, G. Purcaro, M. Oteri, G. Micalizzi, O. Musumesci, A. Ciranni, R.M. Di Giorgio, A. Toscano, P. Dugo, L. Mondello, Analysis of lipid profile in lipid storage myopathy, J. Chromatogr. B 1029 (2016) 157–168.

[7] T. Cajka, O. Fiehn, Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry, Trends Anal. Chem. 61 (2014) 192–206.

[8] I.V. Chernushevich, A.V. Loboda, B.A. Thomson, An introduction to quadrupole-time-of-flight mass spectrometry, J. Mass Spectrom. 36 (2001) 849–865.

[9] M. Holcapek, R. Jirasko, M. Lisa, Recent developments in liquid chromatography-mass spectrometry and related techniques, J. Chromatogr. A 1259 (2012) 3–15.

[10] W.C. Byrdwell, D. Borchman, Liquid chromatography/mass spectrometry characterization of sphingomyelin and dihydrosphingomyelin of human lens membranes, Ophthalmic Res. 29 (1997) 191–206.

[11] W.C. Byrdwell, Dual parallel mass spectrometers for analysis of shingolipid, glycerophospholipid and plasmanogen molecular species, Rapid Commun. Mass Spectrom. 12 (1998) 256–272.

[12] L. Imbert, M. Gaudin, D. Libong, D. Touboul, S. Abreu, P.M. Loiseau, O. Laprevote, P. Chaminade, Comparison of electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization for a lipidomic analysis of Leishmania donovani, J. Chromatogr. A 1242 (2012)75–83.

[13] S.S. Cai, J.A. Sayge, Comparison of atmospheric pressure photoionization atmospheric pressure chemical ionization, and electrospray ionization mass spectrometry for analysis of lipids, Anal. Chem. 78 (2006) 1191–1199.

[14] W.C. Byrdwell, Atmospheric pressure chemical ionization mass spectrometry for analysis of lipids, Lipids 36 (2001) 327–346.

[15] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957)497–509.

[16] O. Quehenberger, A.M. Armando, A.H. Brown, S.B. Milne, D.S. Myers, A.H. Merrill, S. Bandyopadhyay, K.N. Jones, S. Kelly, R.L. Shaner, C.M. Sullards, E. Wang, R.C. Murphy, R.M. Barkley, T.J. Leiker, C.R.H. Raetz, Z. Guan, G.M. Laird, D.A. Six, D.W. Russell, J.G. McDonald, S. Subramaniam, E. Fahy, E.A. Dennis, Lipidomics reveals a remarkable diversity of lipids in human plasma, J. Lipid Res. 51 (2010) 3299–3305.

[17] K. Sandra, A. dos Santos Pereira, G. Vanhoenacker, F. David, P. Sandra, Comprehensive blood plasma lipidomics by liquid chromatography/quadrupole time-of-flight mass spectrometry, J. Chromatogr. A 1217 (2010) 4087–4099.

[18] M. Lisa, E. Cifkova, M. Holkapek, Lipidomic profiling of biological tissues using off-line two-dimensional high-performance liquid chromatography-mass spectrometry, J. Chromatogr. A 1218 (2011) 5146–5156.

[19] P. Dugo, N. Fawzy, F. Cichello, F. Cacciola, P. Donato, L. Mondello, Stop-flow comprehensive two-dimensional liquid chromatography combined with mass spectrometric detection for phospholipid analysis, J. Chromatogr. A 1278 (2013) 46–53.

[20] M. Beccaria, G. Sullini, F. Cacciola, P. Donato, P. Dugo, L. Mondello, High performance characterization of triacylglycerols in milk and milk-related samples by liquid chromatography and mass spectrometry, J. Chromatogr. A1360 (2014) 172–187.

[21] M. Beccaria, R. Costa, G. Sullini, E. Grasso, F. Cacciola, P. Dugo, L. Mondello, Determination of the triacylglycerol fraction in fish oil by comprehensive liquid chromatography techniques with the support of gas chromatography and mass spectrometry data, Anal. Bioanal. Chem. 407 (2015) 5211–5225.

[22] M. Ovčačíková , M. Lísa , E. Cífková , M. Holčapek, Retention behavior of lipids in reversed-phase ultrahigh-performance liquid chromatography–electrospray ionization mass spectrometry, J. Chromatogr. A 1450 (2016) 76–85.

[23] C.A. Lytle, Y.D. Gan, D.C. White, Electrospray ionization/mass spectrometry compatible reversed-phase separation of phospholipids: piperidine as a post column modifier for negative ion detection, J. Microbiol. Methods 41 (2000)227–234.

[24] F.F. Hsu, J. Turk, Electrospray Ionization/tandem quadrupole mass spectrometric studies on phosphatidylcholines: the fragmentation processes, J. Am. Soc. Mass Spectrom. 14 (2003) 352–363.

[25] J.L. Kerwin, A.R. Tuiniga, L.H. Ericsson, Identification of molecular species of glycerophospholipids and sphingomyelin using electrospray mass spectrometry, J. Lipid Res. 35 (1994) 1102–1114.

[26] S. Uran, A. Larsen, P.B. Jacobsen, T. Skotland, Analysis of phospholipid species in human blood using normal-phase liquid chromatography coupled with electrospray ionization ion-trap tandem mass spectrometry, J. Chromatogr. B 758 (2001) 265–275.

[27] A.A. Karlsson, P. Michelsen, A. Larsen, G. Odham, Normal-phase liquid chromatography class separation and species determination of phospholipids utilizing electrospray mass Spectrometry/tandem mass spectrometry, Rapid Commun. Mass Spectrom. 10 (1996) 775–780.

[28] D.F. Qiu, X.Y. Xiao, High-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry of phospholipids in Natronobacterium magadii, Eur. Mass Spectrom. 5 (1999) 151–156.

[29] A.A. Karlsson, P. Michelsen, G. Odham, Molecular species of sphingomyelin: determination by high performance liquid chromatography mass spectrometry with electrospray and high performance liquid chromatography tandem mass spectrometry with atmospheric pressure chemical ionization, J. Mass Spectrom. 11 (1998) 437–449.

[30] W.C. Byrdwell, R.H. Perry, Liquid chromatography with dual parallel mass spectrometry and 31P nuclear magnetic resonance spectroscopy for analysis of sphingomyelin and dihydrosphingomyelin I. Bovine brain and chicken egg yolk, J. Chromatogr. A 1133 (2006) 149–171.

[31] L.H. Couth, M.I. Churchwell, D.R. Doerge, W.H. Tolleson, P.C. Howard, Identification of ceramides in human cells using liquid chromatography with detection by atmospheric pressure chemical ionization-mass spectrometry, Rapid Commun. Mass Spectrom. 11 (1997) 504–512.

[32] Metabolomics Fiehn Lab: http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/, Edited by T. Kind (2010).

[33] F.F. Hsu, J. Turk, Electrospray ionization with low-energy collision allyactivated dissociation tandem mass spectrometry of complex lipids: structural characterization and mechanisms of fragmentation, in: W.C. Byrdwell (Ed.), Modern Methods for Lipid Analysis by Liquid Chromatography/Mass Spectrometry and Related Techniques, AOCS Press, 2005, 2017, pp. 61–178.

[34] F.F. Hsu, J. Turk, Charge-driven and charge-remote fragmentation process in diacyl glycerophosphoethanolamine upon low-energy collisional activation. a mechanism proposal, J. Am. Soc. Mass Spectrom. 11 (2000) 892–899.

[35] F.F. Hsu, J. Turk, Studies on phosphatidylserine by tandem quadrupole and multiple stage quadrupole ion-trap mass spectrometry with electrospray ionization: structural characterization and the fragmentation processes, J. Am. Soc. Mass Spectrom. 16 (2005) 1510–1522.

[36] F.F. Hsu, J. Turk, Characterization of phosphatidylinositol, phosphatidylinositol-4- phosphate andphosphatidylinositol-4,5-bisphosphate by electrospray ionization tandem mass spectrometry: a mechanistic study, J. Am. Soc. Mass Spectrom. 11 (2000) 986–999.

[37] C. Baiocchi, C. Medana, F. Dal Bello, V. Giancotti, R. Aigotti, D. Gastaldi, Analysis of regioisomers of polyunsaturated triacylglycerols in marine matrices by HPLC/HRMS, Food Chem. 166 (2015) 551–560.

[38] T. Řezanka, K. Sigler, The use of atmospheric pressure chemical ionization mass spectrometry with high performance liquid chromatography and other separation techniques for identification of triacylglycerols, Curr. Anal. Chem. 3 (2007) 252–271.

[39] W.C. Byrdwell, APCI-MS in lipid analysis, in: R.O. Adlof (Ed.), Advances in Lipid Methodology, vol. 5, Oily Press Lipid Library Serie, 2003, 2017, pp. 171–253.

[40] S. Salivo, M. Beccaria, G. Sullini, P. Tranchida, P. Dugo, L. Mondello, Analysis of human plasma lipids by using comprehensive two-dimensional gas chromatography with dual detection and with the support of high-resolution time-of-flight mass spectrometry for structural elucidation, J. Sep. Sci. 38 (2015) 267–275.