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Journal of Chromatography B, 810 (2004) 173–186
JOURNAL OF CHROMATOGRAPHY B
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Normal phase liquid chromatography–electrospray ionization tandem mass spectrometry analysis of phospholipid molecular species in blood mononuclear cells: application to cystic fibrosis

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Received 16 February 2004; accepted 1 July 2004

Abstract

The use of HPLC coupled on-line with a mass spectrometer is a very powerful tool in order to analyze intact PLs molecular species (PMS) without the need of derivatization, thus decreasing the risk of artifacts formation. A normal-phase HPLC–ESI-MS–MS method has been developed in order to study the human blood mononuclear cell PMS composition. This method was applied to characterize PMS from seven CF subjects and from seven age-matched healthy subjects. More than 140 phospholipid molecular species from phosphatidylethanolamine (PE), plasmalogen phosphatidylethanolamine (pPE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and sphingomyelin (Sph) were identified and compared. Differences between the two groups were found in pPE (p16:0/22:6), pPE (p18:0/22:6), PE (16:0/20:4) and PC (16:0/18:2) which were significantly lower in CF subjects and in PC (16:0/16:1) which was significantly higher in CF subjects.
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Keywords: Phospholipids; HPLC; Mass spectrometry; Cystic fibrosis; Blood mononuclear cells

1. Introduction

Since 1962, when Kuo et al. found abnormalities in the fatty acid composition of serum chylomicrons and adipose tissue of children with cystic fibrosis (CF) [1], serum and cell membrane lipids have been widely studied in subjects with CF [2,3]. Many abnormalities were subsequently discovered [4,5] and different oral [6,7] or parenteral [8,9] lipid supplements were administered to CF subjects in order to correct the defects [10,11]. Early studies attributed these abnormalities only to fat malabsorption; afterwards, the existence of an association between the basic defect and abnormal metabolism of essential fatty acids in CF was hypothesized [4,12]. Nowadays, the study of the “fatty acids status” in CF patients is useful to evaluate the biological effects of a lipidic supplement [13,14] and particular indexes, such as the linoleic acid (LA) content and the arachidonic acid/docosahexaenoic acid ratio are also proposed in diagnostics as biochemical markers [14,15]. Phospholipid-bound fatty acids are mainly involved in these abnormalities. Phospholipids (PLs) are the main constituents of biological membranes and have an important role in signal processing and as precursors for many other biologically active molecules, mainly involved in inflammation response.

Current analytical methods allow to evaluate the fatty acids composition of phospholipid classes in plasma and homogenated tissues with high reproducibility. However, relatively little attention has been paid up to now upon the analysis of the molecular species of the different PLs classes. The reason for this is probably that the separation and detection of these compounds have been relatively complicated and time consuming so far.

The preferred methods for evaluation of fatty acids composition of phospholipid classes is the use of thin layer chromatography (TLC) or preparative high performance liquid

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doi:10.1016/j.jchromb.2004.07.001

chromatography (HPLC) for class separation, followed by extraction, transesterification and use of gas chromatography (GC) for detection and quantitation [16]. Normal-phase HPLC coupled to a light scattering detector is widely used also for the separation, identification and quantitation of phospholipid classes but fails to discriminate the presence of different fatty acids in the single class [17].

The use of HPLC coupled on-line with a mass spectrometer is a very useful approach to improve the specificity of phospholipid analysis. It makes it possible to combine the separation power of HPLC with the selective mass spectrometry (MS) detection and enables the structural analysis of intact PLs in a relatively short time. The analysis of intact molecular species of PLs without the need of derivatization decreases the risk of the formation of artifacts, thus the information on the fatty acid composition of the lipid class is preserved [18].

Detection of phospholipid by MS has been performed with several ionization techniques, such as particle beam [19], thermospray [20], discharge assisted thermospray [21], fast atom bombardment (FAB) [22,23], and recently, electrospray ionization (ESI) [24,25]. The last method is widely employed because it offers enhanced sensitivity and the possibility of accomodating chromatographic flow rates up to 1 mL/min.

HPLC–ESI-MS has been successfully applied for the analysis of individual molecular species in human blood [26], human gastric juice [27], mice synaptic plasma membrane [28], rat glial tumor C-6 cells [29] and atlantic salmon head kidney [30].

This paper presents a HPLC–ESI-MS method for the analysis of PLs in human peripheral blood mononuclear cell (PBMC). This procedure was used to determine individual molecular species of phosphatidylethanolamine (PE), plasmalogen phosphatidylethanolamine (pPE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and sphingomyelin (Sph) in PBMC, and to compare the results obtained from adolescents–adults CF subjects with respect to normal subjects.

2. Experimental

2.1. Materials

Chloroform and methanol were HPLC grade from Lab-Scan (Dublin, Irland), ammonia solution (30%) pro-analysis grade was from Carlo Erba (Milano, Italy). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1,2-dipalmitoyl-*sn*-glycero-phosphoserine (DPPS), bovine liver phosphatidylinositol ammonium salt (PI, i.e. a mixture of different species), *N*-palmitoyl-D-sphingomyelin (PSph), egg yolk lysophosphatidylcholine (LPC, i.e. a mixture of different species), 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (PDPC) and 1,2-diundecanoyl-*sn*-glycero-phosphocholine (DUPC) were purchased from Sigma Chemicals Co. (St. Louis, MO).

All other chemicals, with noted exceptions, were obtained from Sigma Chemicals Co. (St. Louis, MO). Cell preparation tubes, Vacutainer CPT, were from Becton Dickinson (Franklin Lakes, NJ).

2.2. Subjects and sample preparation

Seven CF patients and seven age-matched normal subjects were recruited from the Cystic Fibrosis Center, G. Salesi Children's Hospital of Ancona (Italy). The informed consent was obtained from all the subjects and the approval for the study was obtained from the "Tribunale dei Diritti del Malato" of Ancona.

CF subjects characteristics are reported in Table 1. Subjects receiving insulin, corticosteroids and other drugs affecting fat mass were excluded, as well as patients consuming fish oil or any ω3 supplements before the experimental period.

Blood samples were collected into cell preparation tubes (CPT) with sodium citrate and blood separation media composed of a thixotropic polyester gel and a Ficoll Hypaque solution. Isolation of PBMC was obtained as described by others [31], using phosphate buffer saline solution (PBS) 0.01 M, pH = 7.4, as a washing solution. Samples were centrifuged, within 2 h of blood collection, at room temperature in a horizontal rotor for 25 min at 1650 × *g*. The mononuclear cell layer was transferred into a conical centrifuge tube with cap. Cells were suspended in PBS and centrifuged for 15 min at 300 × *g*. The cell pellet was resuspended in PBS and centrifuged for 10 min at 300 × *g*. The cell pellet was freeze-dried, homogenized and extracted with chloroform/methanol (2:1 (v/v)). The extract was centrifuged and the supernatant was purified according to the method of Folch et al. [32]. The purified extract was dried under nitrogen and stored in 0.5 mL tetrahydrofuran (THF) at −20 °C. Prior to analysis the samples were redissolved in 250 μL of chloroform/methanol/water (5:5:1 (v/v)), of which 5 μL was injected into the HPLC–MS chromatographic system.

2.3. HPLC conditions

The HPLC system consisted of a degassing unit (Gastorr GT-103), a ternary gradient module (Jasco LG-980-02), and

Table 1
Age, gender and status of pancreas disease of the subjects

Subjects characteristics	CF	Normal
n	7	7
Mean age (years ± S.D.)	19.8 ± 9.5	18.6 ± 9.0
Adolescents: 12–18 years (*n*)	3	3
Adults: >18 years (*n*)	4	4
Male to female ratio	2/5	4/3
P_{ins}[a] to P_{suff}[b] ratio	5/2	

[a] P_{ins}: subjects with pancreas insufficiency.
[b] P_{suff}: subjects with pancreas sufficiency.

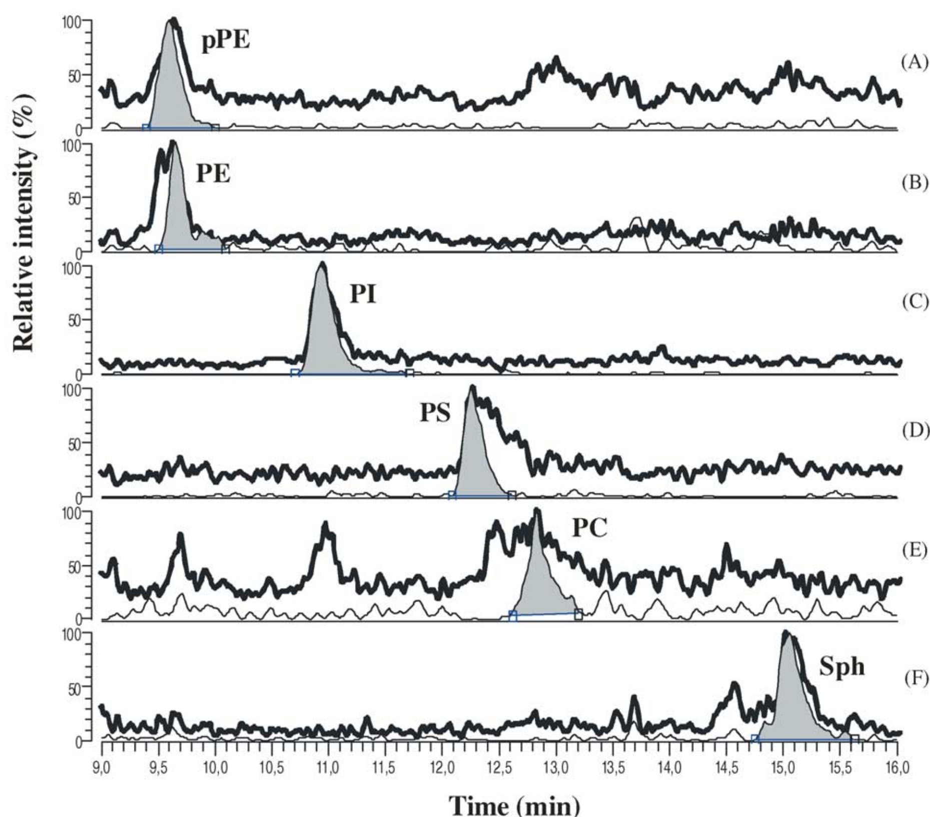

Fig. 1. Negative-ion HPLC–ESI-MS analysis of blood mononuclear cell phospholipid with the MS operating in the scanning mode. (A) pPE; (B) PE; (C) PI; (D) PS; (E) PC; (F) Sph. The integrated area for each of the most representative species is also shown (grey color). The most representative species displayed are: (A) *m/z* 750.5, i.e. pPE (p18:0/20:4); (B) *m/z* 766.5, i.e. PE (18:0/20:4); (C) *m/z* 885.5, i.e. PI (18:0/20:4); (D) *m/z* 810.5, i.e. PS (18:0/20:4); (E) *m/z* 794.5, i.e. PC (16:0/18:1); (F) *m/z* 737.5, i.e. Sph (16:0).

a pump module (Jasco PU-980). The lipids were separated on a Hypersil Si (150 mm × 4.6 mm i.d., 3 μm particle size) column (Phenomenex, Torrance, USA). The mobile phase A was $CHCl_3$/MeOH/NH_4OH (30%) (80:19.5:0.5 (v/v)), and the mobile phase B was $CHCl_3$/MeOH/H_2O/NH_4OH (30%) (60:34:5.5:0.5 (v/v)). Separation was obtained by using a gradient starting at 100% mobile phase A, decreasing to 0% A in 10 min, held at 0% A for 15 min and then back to 100% A in 5 min. The total chromatographic run time was 35 min. The flow rate was 0.8 mL/min and the column was maintained at room temperature (25 °C). Silica column have earlier been reported to have a short life using mobile phases containing NH_4OH, however, probably due to the very low concentration of ammonia and to the periodic replacement of the guard column, we did not observe any relevant change in the column performance even after 200 injections.

2.4. Mass spectrometry

The HPLC system was coupled on-line to an LCQ ion-trap mass spectrometer (Finnigan, San José, CA, USA) equipped with an electrospray ionization source. The HPLC effluent was splitted and 0.3 mL/min entered the MS through a steel ionization needle set at 5.0 kV and a heated capillary set to 200 °C. The sheath gas flow was approximately 90 arbitrary units. The ion source and the ion optic parameters were optimized with respect to the negative and positive molecular related ions of phospholipid standards. The molecular mass peaks from the HPLC effluent were detected by using negative ion full-scan ESI-MS. Mass resolution was 0.6 Da at half peak height and isolation width ±0.3 Da. MS^2 experiments were carried out with a relative collision energy of 30–40%.

2.5. Statistical analysis

Results are expressed as mean ± standard deviation (S.D.). Data were analyzed with Student's *t*-test for normally distributed data and with Mann–Whitney *U*-test when data were not normally distributed. Statistical analysis was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). Significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Separation of phospholipid classes

Since mass spectra of different PLs often overlap, it is important to achieve class separation of the PLs. The separation

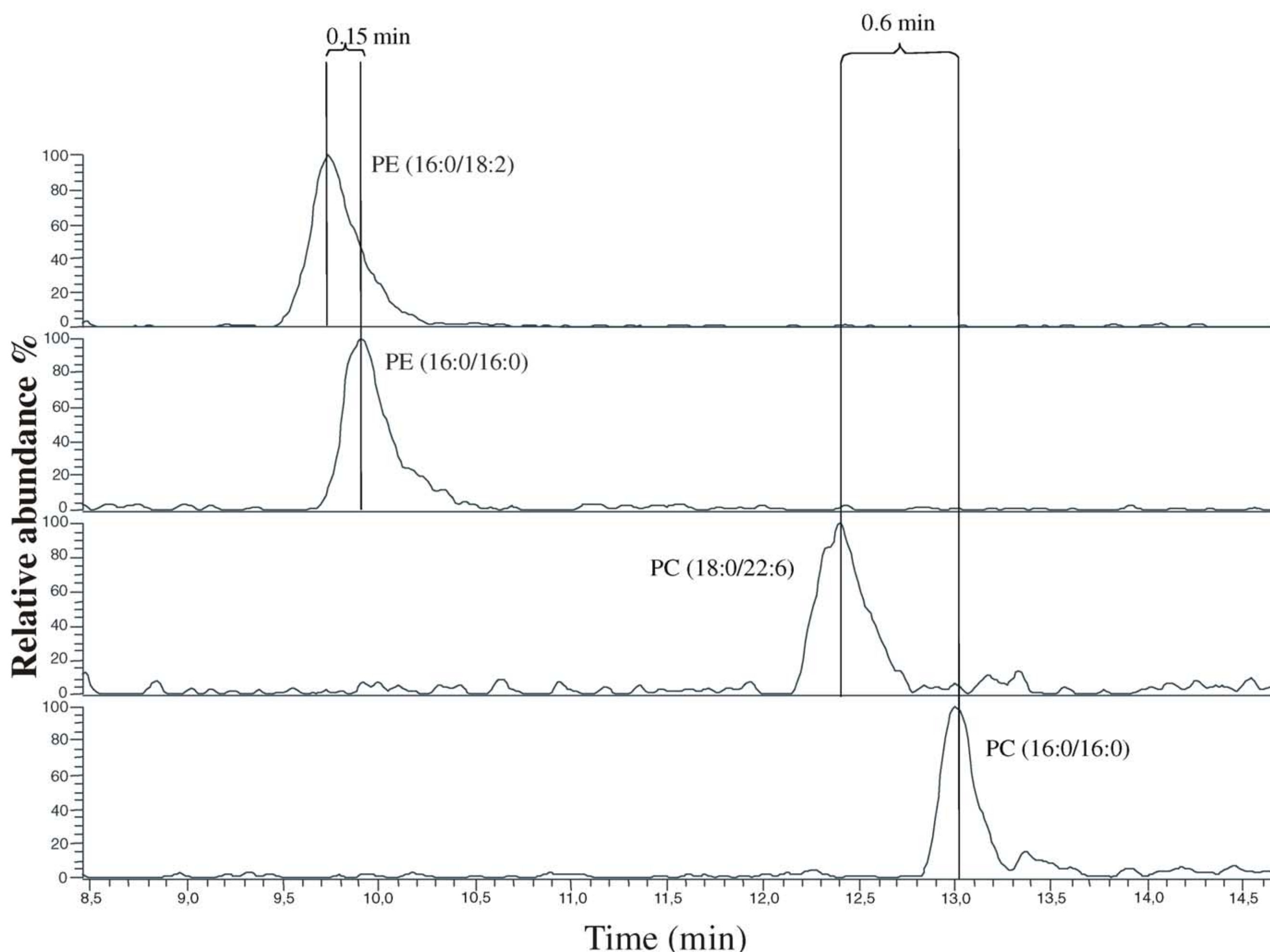

Fig. 2. Minor separation between phospholipid molecular species within a given class obtained with standard PL. (Left) Separation between PE (16:0/18:2) and PE (16:0/16:0). (Right) Separation between PC (18:0/22:6) and PC (16:0/16:0).

of six main phospholipid classes from human PBMC extract was obtained as shown in Fig. 1. The figure shows the overlap between the chromatogram (bold line) obtained selecting the sum of individual species of phospholipid classes reported in Tables 3–7, and the chromatogram (thin line) obtained selecting the most representative species for each class. The retention times for the different classes increased in the following order: pPE and PE, PI, PS, PC and Sph. PE coeluted with plasmalogen PE (pPE), and PC partially coeluted with PS, but the on-line MS detection allows selective mass fragment analysis and identification of the different species. Traces of lyso-phosphatidylcholine (LPC) were detected in the tail of the chromatograms, in the time range comprised between 17 and 20 min. We observed that the amount of LPC increased when samples were stored for a few days at 4 °C in the injection solution, probably due to hydrolysis of PC. Therefore, the analysis of LPC species has not been performed.

As reported in Fig. 2, the injection of a phospholipid standard mixture constituted of 1-palmitoyl-2-linoleoyl-phosphatidylethanolammine, dipalmitoyl-phosphatidylethanolamine, stearoyl-docosahexaenoyl-phosphatidylcholine and dipalmitoyl-phosphatidylcholine (approximately 0.10 mg/mL of each) showed that some minor separation among different species within a given class was also obtained, but the difference in retention times among species was generally less than the difference in retention time among PL classes. It was found that the elution order of the molecular species within each class depends mainly on the length of the fatty acid chains. Thus, the shorter was the fatty acid chain, the longer was the retention time of phospholipid molecular species. Hence, the chromatographic retention times can be used to identify each class and, most importantly, the system can be used to separate isobaric species from different phospholipid classes.

3.2. Species characterization of phospholipid classes from blood mononuclear cells

The phospholipid species of PE, pPE, PI, and PS from PBMC extracts were detected as $[M - H]^-$ ions using negative ion scan. The PC and Sph classes, having a choline group at the polar head, were detected as chlorine adducts

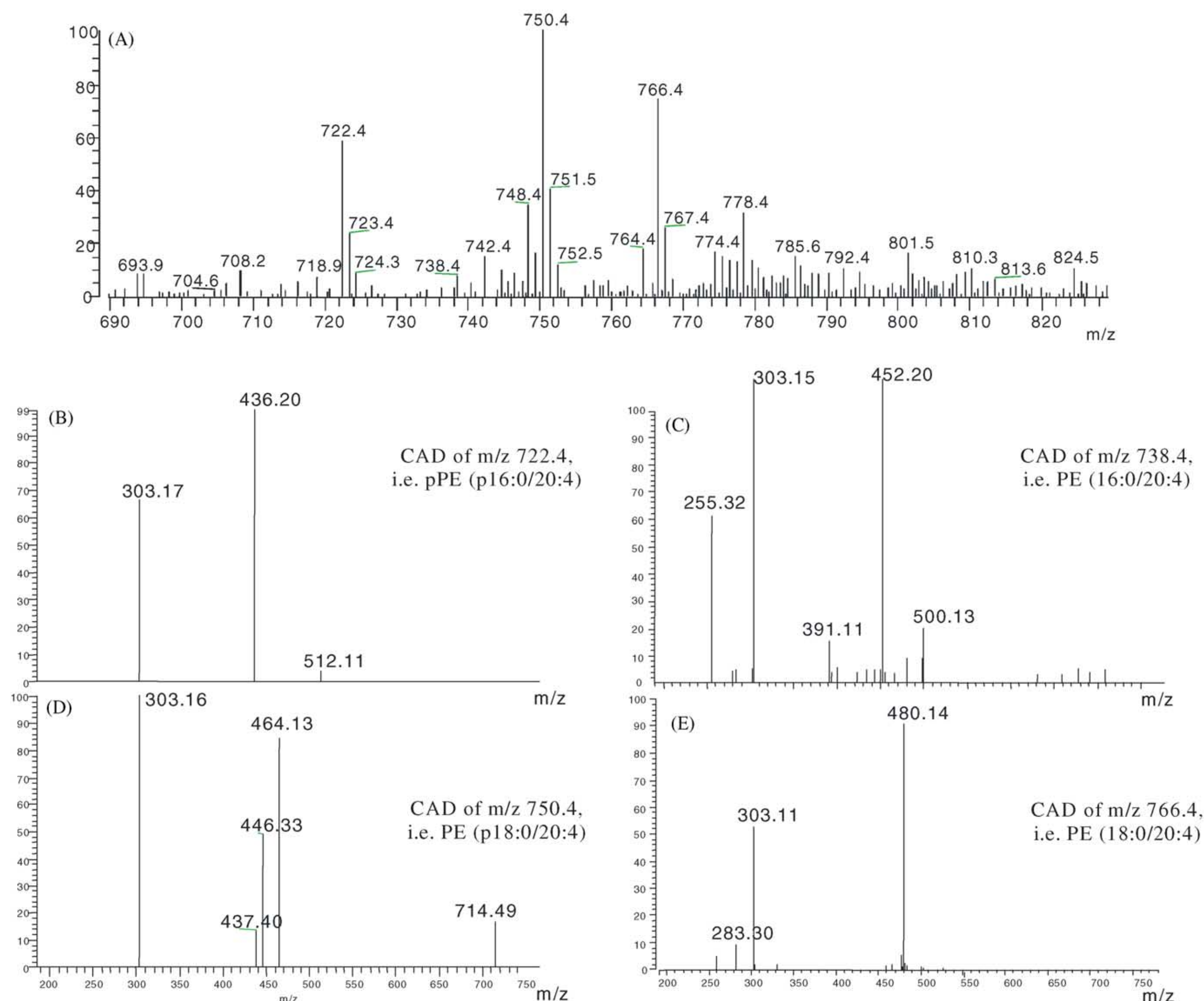

Fig. 3. Negative-ion HPLC–ESI-MS analysis of PE and pPE molecular species from blood mononuclear cells. (A) Mass spectrum of PE and pPE (from 9.0 to 10.0 min of the classes as shown in Fig. 1A and B); (B) CAD of *m/z* 722.39, i.e. pPE (p16:0/20:4); (C) CAD of *m/z* 738.48, i.e. PE (16:0/20:4); (D) CAD of *m/z* 750.44, i.e. pPE (p18:0/20:4); (E) CAD of *m/z* 766.41, i.e. PE (18:0/20:4).

$[M + Cl]^-$. However, in the mass spectra of PC and Sph, other minor intensity ions were observed at $[M - 15]^-$ due to loss of CH_3 from the choline group. The molecular mass peaks obtained from PE and pPE are reported in Fig. 3A, those from PI and PS in Figs. 4A and 5A, respectively, and those from PC and Sph in Fig. 6A and B. The mass spectra were acquired under peaks obtained from the reconstructed negative ion chromatogram in the time range expected for the elution of each class molecular species.

To perform species determination, tandem mass spectra obtained from collisionally activated dissociation (CAD) of blood mononuclear cell phospholipid molecular ions was performed and compared with those obtained from known standards. The identification of species was obtained by using the fragments listed in Table 2, setting the ion-trap in the mass dependent scan mode.

3.3. PE and pPE species

CAD spectra of PE molecular species displayed as major fragments one or two ions resulting from the fragmentation at the ester bond ($[LPE–H]^-$) and one or two fatty acid carboxylate anion fragments. As reported elsewhere [33] and confirmed by MS^2 analysis of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine standard solution, the carboxylate anion and the $[LPE–H]^-$ fragments derived from the fragmentation at the *sn*-2 position were prominent. The fragmentations of the molecular ions *m/z* 738.4 and 766.4 obtained from MS spectra of PE from PBMC extracts, are reported in Fig. 3C and E. In Fig. 3C, the CAD spectrum of *m/z* 738.4 ion showed a minor carboxylate anion fragment at *m/z* 255.3 (C, 16:0), a major carboxylate anion fragment at *m/z* 303.3 (C, 20:4), a minor $[LPE–H]^-$ fragment at 500.1 and a

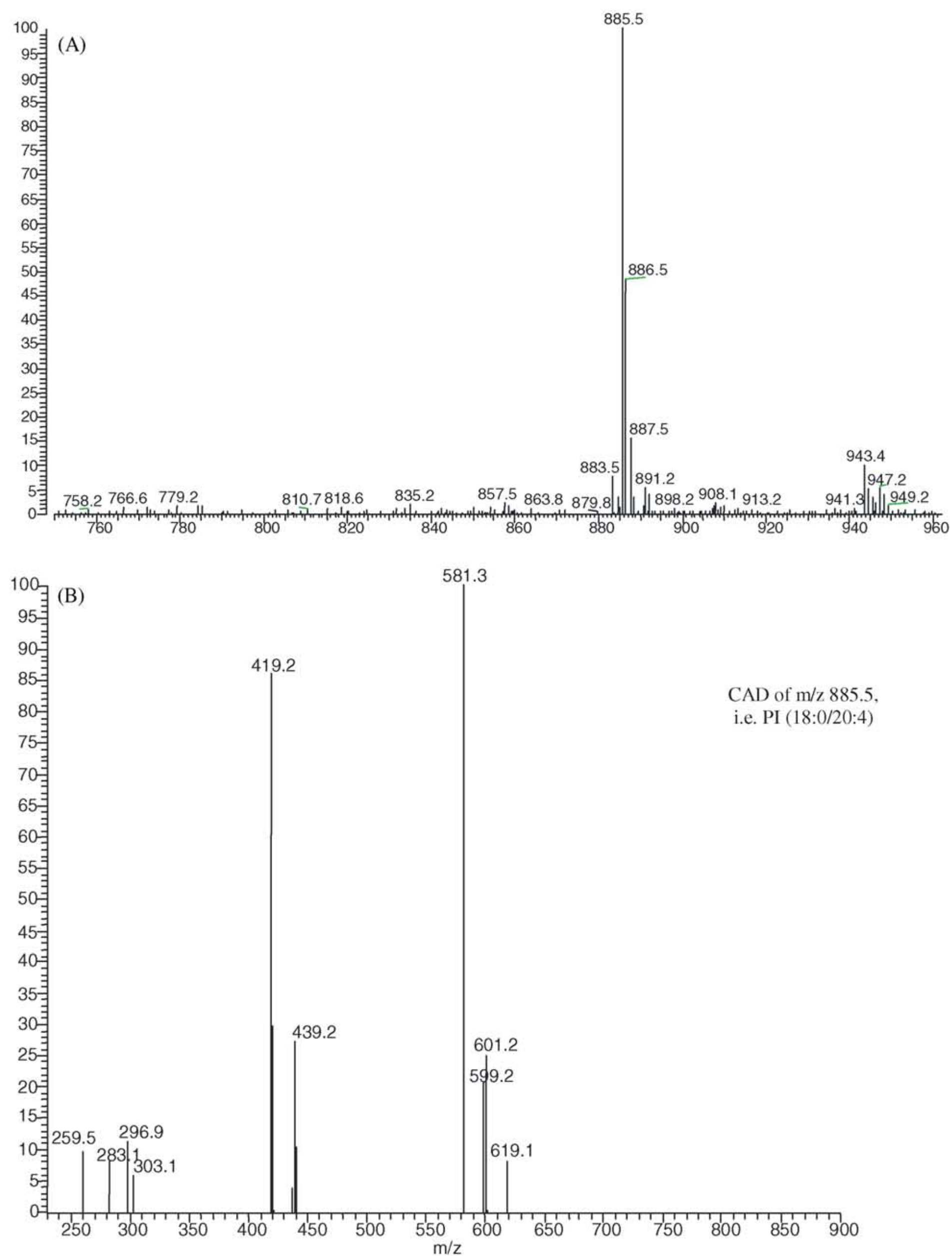

Fig. 4. Negative-ion HPLC–ESI-MS analysis of PI molecular species from blood mononuclear cells. (A) Mass spectrum of PI (from 10.5 to 11.5 min of the classes shown in Fig. 1C). (B) CAD of *m/z* 885.55, i.e. PI (C 18:0/C 20:4).

Table 2
Molecular ions and fragment ions of PLs used in species characterization[a]

PL class	Molecular ions	Main fragments detected by CAD
PE	$[M-H]^-$	$[LPE{-}H]^-$, $[FA{-}H]^-$
pPE	$[M-H]^-$	$[LPE{-}H]^-$, $[FA{-}H]^-$
PI	$[M-H]^-$	$[LPI{-}H{-}H_2O]^-$, $[LPA{-}H{-}H_2O]^-$, $[LPI{-}H]^-$, $[FA{-}H]^-$
PS	$[M-H]^-$	$[PA{-}H]^-$, $[LPA{-}H]^-$, $[FA{-}H]^-$
PC	$[M+35]^-$	$[M{-}CH_3]^-$
Sph	$[M+35]^-$	$[M{-}CH_3]^-$

[a] PE: phosphatidylethanolamine; pPE: plasmalogen phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; Sph: sphyngomyelin; PA: phosphatidic acid; FA: fatty acid; LPA: lyso-phosphatidic acid; LPE: lyso-phosphatidylethanolamine; LPI: lyso-phosphatidylinositol.

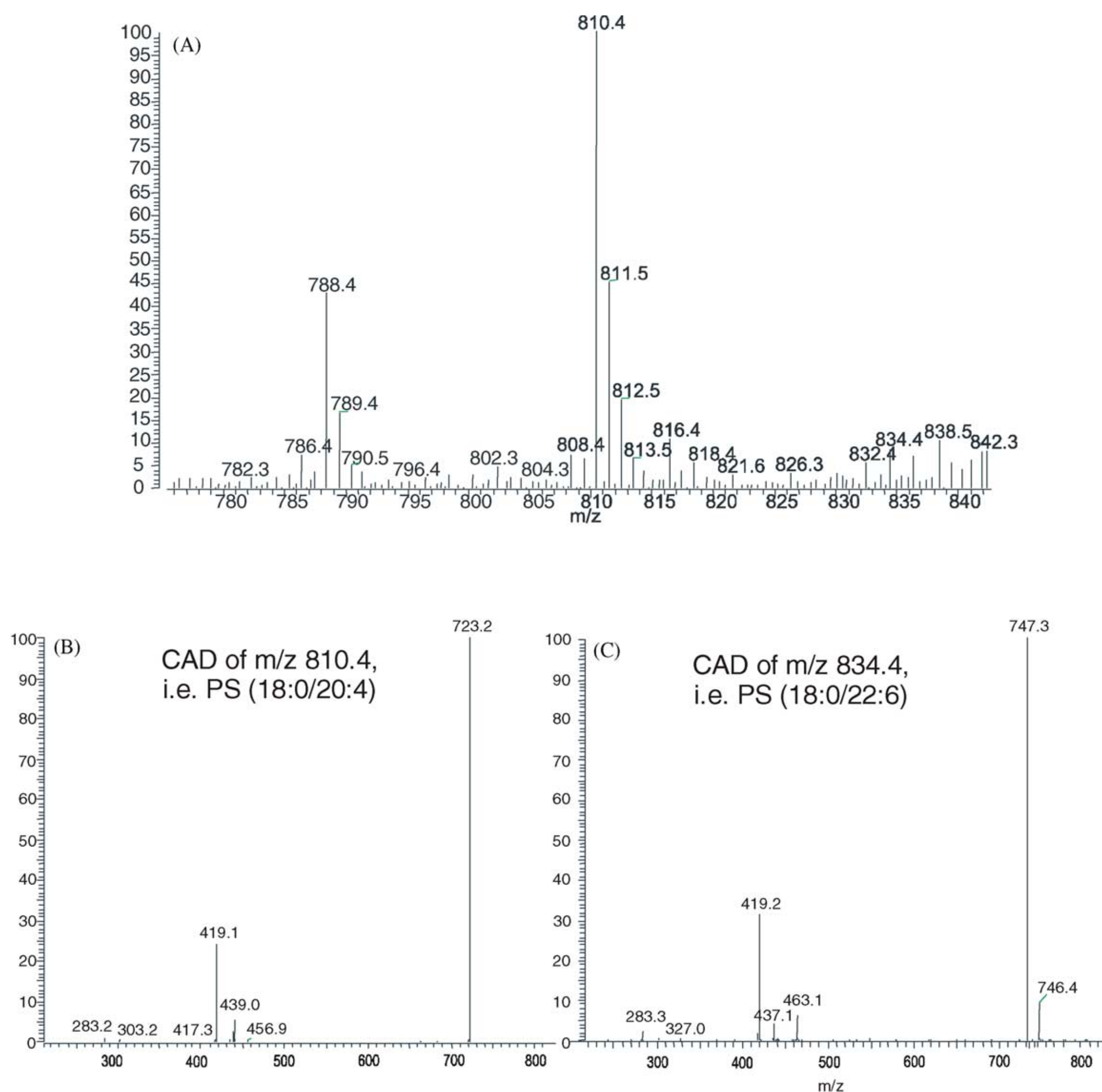

Fig. 5. Negative-ion HPLC–ESI-MS analysis of PS molecular species from blood mononuclear cells. (A) Mass spectrum of PS (from 12.0 to 12.5 min of the classes shown in Fig. 1D); (B) CAD of *m/z* 810.43, i.e. PS (18:0/20:4); (C) CAD of *m/z* 834.42, i.e. PS (18:0/22:6).

major [LPE–H]$^-$ fragment at 452.2 (due to loss of C 20:4). These features identify the parent as PE (16:0/20:4). Moreover, other minor peaks (not highlighted in Fig. 3C) revealed the presence of small amounts of PE (18:2/18:2) as isobaric specie. In Fig. 3E, the tandem spectrum of *m/z* 766.4 ion contains C 18:0 (*m/z* 283.3) and C 20:4 (*m/z* 303.1) anions and an ion at *m/z* 480.1, reflecting loss of C 20:4, thus identifying the parent as PE (18:0/20:4). Other minor peaks revealed the presence of small amounts of PE (16:0/22:4) as isobaric species.

CAD spectra of pPE molecular species (Fig. 3B and D) were similar to those obtained with PE species but fragmentation of the vinyl–ether bond did not occur. However, there was an ion resulting from the fragmentation of the ester bond with loss of H_2O (*m/z* 446.3 in Fig. 3D). In Fig. 3B, the tandem spectrum of *m/z* 722.4 ion showed a carboxylate anion fragment at *m/z* 303.2 (C 20:4) and a fragment at *m/z* 436.2 (due to loss of C 20:4), thus identifying the parent as pPE (p16:0/20:4). In Fig. 3D, the tandem spectrum of *m/z* 750.4 ion showed a carboxylate anion fragment at *m/z* 303.2 (C 20:4) and two ion fragments (*m/z* 464.1 and *m/z* 436.2) resulting from the fragmentation of the ester bond, thus identifying the parent as pPE (p18:0/20:4).

3.4. PI species

CAD of $[M - H]^-$ ions from PI species produced abundant ions resulting from the fragmentation at the ester bond ([LPI–H]$^-$) followed by neutral loss of H_2O ([LPI–H_2O–H]$^-$) and by loss of the headgroup ([LPA–H_2O–H]$^-$), whereas carboxylate anion fragments were less abundant. In Fig. 4B is reported the tandem spectrum of *m/z* 885.5, the most abundant $[M - H]^-$ ion from PI species of extracted PBMC. The two prominent fragment ions at *m/z* 581.3 and at *m/z* 419.2 correspond to the [LPI–H_2O–H]$^-$ and [LPA–H_2O–H]$^-$ ions resulting

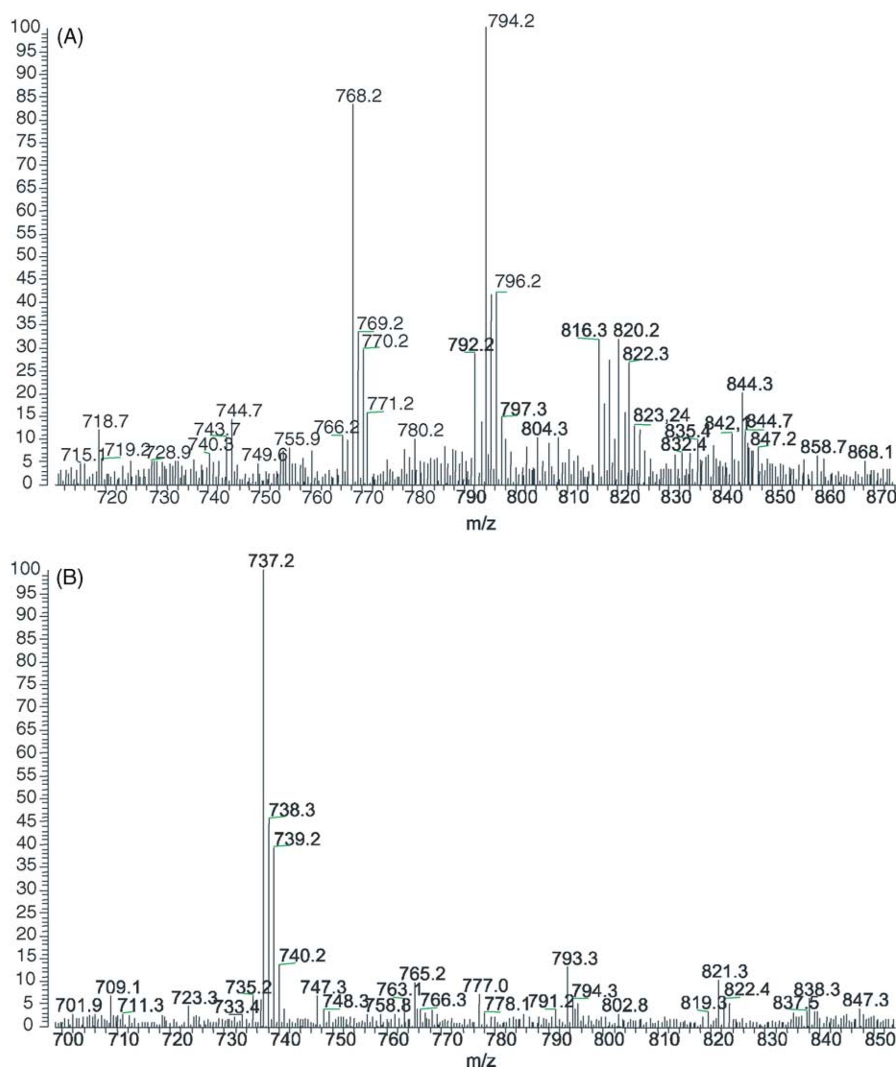

Fig. 6. Negative-ion HPLC–ESI-MS analysis of PC and Sph molecular species from blood mononuclear cells. (A) Mass spectrum of PC (from 12.5 to 13.5 min of the classes shown in Fig. 1E); (B) mass spectrum of Sph (from 14.5 to 15.5 min of the classes shown in Fig. 1F).

from the loss of C 20:4. The fragment ions at *m/z* 601.2 and *m/z* 439.2 correspond to the $[LPI{-}H_2O{-}H]^-$ and the $[LPA{-}H_2O{-}H]^-$ ions resulting from the loss of C 18:0, and the fragment ions at *m/z* 283.1 and *m/z* 303.1 correspond to the carboxylate anions. This fragmentation pattern identifies the parent as PI (C 18:0/C 20:4).

3.5. PS species

CAD of $[M-H]^-$ ions from PS species produced an abundant ion resulting from the loss of the head group ($[PA{-}H]^-$), one or two ions resulting from the fragmentation at the ester bond followed by the loss of the headgroup ($[LPA{-}H]^-$) and small carboxylate anion fragments. This is illustrated in Fig. 5B and C, where the fragmentations of *m/z* 810.3 and of *m/z* 834.3 ions are reported. In Fig. 5B, the two carboxylate anion fragments at *m/z* 283.2 and at 303.2 and the two $[LPA{-}H]^-$ ions at 419.1 and at 439.0 identify PS (18:0/20:4) as the parent ion, whereas in Fig. 5C, the two carboxylate anion fragments at *m/z* 283.3 and at *m/z* 327.0 and the two $[LPA{-}H]^-$ ions at 419.1 and at 463.1 identify PS (18:0/22:6) as the parent ion.

3.6. PC and Sph species

CAD of $[M+Cl]^-$ ions from PC and Sph produced only the $[M-15]^-$ ion fragment. To identify PC species, a positive ion scan analysis and CAD of $[M+H]^+$ molecular ions were performed, as reported in Fig. 7A. The CAD spectra were compared to those obtained with standard solutions of POPC, OPPC, PDPC, DPPC. The tandem mass spectra obtained from CAD of $[M+H]^+$ ions displayed abundant fragment ions resulting from the fragmentation at the ester bond ($[LPC+H)]^+$) followed by neutral loss of H_2O ($[LPC - H_2O + H]^+$). In Fig. 7B is represented the CAD spectrum of *m/z* 760.74, where a $[LPC+H)]^+$ ion fragment

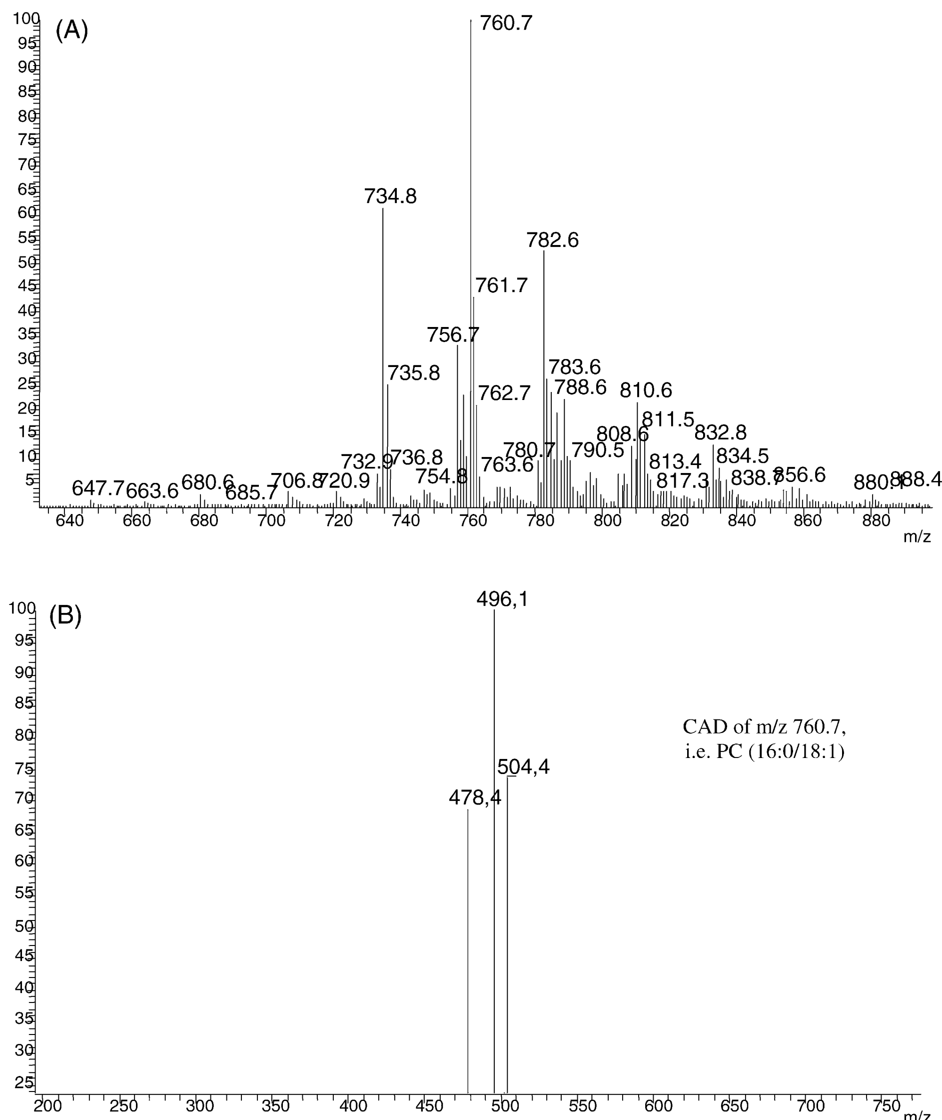

Fig. 7. Positive-ion HPLC–ESI-MS analysis of PC molecular species from blood mononuclear cells. (A) Mass spectrum of PC (from 12.5 to 13.5 min of the chromatogram shown in Fig. 1E); (B) CAD of *m/z* 760.7, i.e. PC (16:0/18:1).

(*m/z* 496.1) and two $[LPC - H_2O + H]^+$ ion fragments (*m/z* 478.4 and *m/z* 504.4) identify the parent ion as PC (16:0/18:1). Sph molecular species carry a single fatty acid chain, thus they were identified by simple MS analysis in Fig. 6B.

3.7. Semi-quantitative analysis of phospholipid molecular species in blood mononuclear cells

The molecular species composition of each phospholipid class from extracted blood mononuclear cell of CF and normal subjects were compared. The relative abundance of individual molecular species within a phospholipid class was calculated from the single ion current responses, with the Interactive Chemical Information System (ICIS) peak detection algorithm software provided by Finnigan, after correction for the contribution from the ^{13}C isotope effect. The ICIS allows a very good peak detection efficiency especially at low MS signal levels. Moreover, the identification of the peaks with this method allows to discriminate among PLs species and isobaric interfering substances (which are identified by the tandem MS spectra), even when the difference in the chromatographic retention times is small. The relative abundance data were calculated by the ratio between the single phospholipid species area and the sum of all the phospholipid species areas within each class. The complete quantification of the phospholipid in the experimental samples has not been performed because certain variability was observed in the response of the instrument for each class. Therefore, an internal standard for each class should be used in order to perform a reproducible quantitative analysis. However, because the intensity response among phospholipid species within each

Table 3
Molecular species of phosphatidylethanolamine and plasmalogen PE from blood mononuclear cells of cystic fibrosis and normal subjects by negative-ion HPLC–ESI-MS analysis

PE and pPE molecular species ($[M-H]^-$)				Relative abundance in percentage (mean ± S.D.)	
m/z	Combined chain length	Double bonds	Major species*	CF ($n = 7$)	N ($n = 7$)
698.2	34	2	p16:0/18:2	0.6 ± 0.2	0.6 ± 0.2
700.4	34	1	p16:0/18:1	1.4 ± 0.4	1.4 ± 0.5
714.4	34	2	16:0/18:2	1.1 ± 0.5	1.3 ± 0.6
716.6	34	1	16:0/18:1	2.5 ± 1.1	2.9 ± 0.5
722.4	36	4	p16:0/20:4	13.4 ± 1.3	12.1 ± 1.6
726.6	36	2	p18:0/18:2 + p18:1/18:1	0.9 ± 0.3	0.7 ± 0.2
728.3	36	1	p18:0/18:1	0.7 ± 0.4	0.7 ± 0.2
738.4	36	4	16:0/20:4 + 18:2/18:2	2.8[a] ± 0.6	3.7 ± 1.0
740.5	36	3	16:0/20:3 + 18:1/18:2 + 18:0/18:3	1.0 ± 0.6	1.4 ± 0.4
742.4	36	2	18:0/18:2 + 18:1/18:1 + 16:0/20:2	3.2 ± 0.7	4.1 ± 1.4
744.8	36	1	18:0/18:1	2.8 ± 1.1	2.3 ± 0.9
746.6	38	6	p16:0/22:6	1.3[a] ± 0.2	1.8 ± 0.3
748.4	38	5	p18:1/20:4 + p18:0/20:5 + p16:0/22:5	6.9 ± 0.9	6.5 ± 0.5
750.5	38	4	p18:0/20:4 + p16:0/22:4	19.4 ± 2.8	18.9 ± 2.1
762.5	38	6	16:0/22:6	1.1 ± 0.4	1.0 ± 0.3
764.4	38	5	18:1/20:4 + 18:0/20:5 + 16:0/22:5	3.7 ± 1.1	4.1 ± 0.3
766.5	38	4	18:0/20:4 + 16:0/22:4	18.7 ± 1.9	17.5 ± 2.0
772.3	38	1; 7	18:0/20:1; p18:1/22:6	1.3 ± 0.4	1.5 ± 0.3
774.4	40	6	p18:0/22:6 + p18:1/22:5	3.0[a] ± 0.6	3.8 ± 0.5
776.5	40	5	p18:0/22:5 + p18:1/22:4	3.6 ± 0.7	3.5 ± 0.6
778.5	40	4	p18:0/22:4	5.2 ± 0.7	4.6 ± 1.0
790.4	40	6	18:0/22:6 + 18:1/22:5 + 18:2/22:4	1.5 ± 0.4	1.7 ± 0.4
792.5	40	5	18:0/22:5 + 18:1/22:4	1.6 ± 0.7	1.7 ± 0.5
794.6	40	4	18:0/22:4 + 20:0/20:4	2.4 ± 0.8	2.0 ± 1.1

* p, plasmalogen.
[a] Significantly different from normal subjects; $P < 0.05$.

Table 4
Molecular species of phosphatidylinositole from blood mononuclear cells of cystic fibrosis and normal subjects by negative-ion HPLC–ESI-MS analysis

PI Molecular species ($[M-H]^-$)				Relative abundance in percentage (mean ± S.D.)	
m/z	Combined chain length	Double bonds	Major species	CF ($n = 7$)	*N* ($n = 7$)
833.5	34	2	16:0/18:2	0.5 ± 0.3	0.8 ± 0.3
835.5	34	1	16:0/18:1; 16:1/18:0	1.1 ± 0.6	1.6 ± 0.5
857.5	36	4	16:0/20:4; 18:2/18:2	2.4 ± 0.6	2.7 ± 0.7
859.5	36	3	16:0/20:3; 18:1/18:2; 18:0/18:3	0.8 ± 0.4	0.9 ± 0.4
861.5	36	2	18:0/18:2; 18:1/18:1	2.3 ± 0.9	2.2 ± 0.2
863.5	36	1	18:0/18:1	1.3 ± 0.7	1.1 ± 0.6
881.5	38	6	16:0/22:6	0.8 ± 0.5	0.9 ± 0.3
883.5	38	5	18:1/20:4; 16:0/22:5; 18:0/20:5	5.5 ± 1.2	6.1 ± 0.6
885.5	38	4	18:0/20:4; 16:0/22:4; 18:1/20:3	76.9 ± 2.9	76.5 ± 1.9
887.5	38	3	18:0/20:3	3.8 ± 1.4	3.1 ± 1.3
907.5	40	7	18:1/22:6	1.5 ± 0.6	0.9 ± 0.5
909.5	40	6	18:0/22:6; 18:1/22:5	0.7 ± 0.2	0.9 ± 0.1
911.5	40	5	18:0/22:5; 18:1/22:4; 20:1/20:4	1.4 ± 0.5	1.2 ± 0.3
913.5	40	4	18:0/22:4; 20:0/20:4	1.3 ± 0.4	1.1 ± 0.3

class was stable, the calculations were based upon relative abundances.

3.8. PE and pPE molecular species

PE and pPE molecular species relative abundance is reported in Table 3. The major molecular species for PE and pPE were 18:0/20:4 (*m/z* 766.4) and p18:0/20:4 (*m/z* 750.4). Including the contribution of minor isobaric species, PE 18:0/20:4 and p18:0/20:4 accounted for approximately 36–38% of total PE and pPE molecular species in both CF and normal subjects. A substantial amount was also represented by p16:0/20:4 (*m/z* 722.4) which, from CAD spectrum, seems to have no isobaric species. There was a significant lower amount of *m/z* 774.4 ion (corresponding to the sum of p18:0/22:6 and p18:1/22:5 molecular species), of *m/z* 738.4 (corresponding to the sum of 16:0/20:4 and 18:2/18:2) and of p16:0/22:6 in CF compared to normal subjects. As reported

Table 5
Molecular species of phosphatidylserine from blood mononuclear cells of cystic fibrosis and normal subjects by negative-ion HPLC–ESI-MS analysis

PS molecular species ($[M-H]^-$)				Relative abundance in percentage (mean ± S.D.)	
m/z	Combined chain length	Double bonds	Major species	CF (*n* = 7)	*N* (*n* = 7)
760.4	34	1	16:0/18:1	0.9 ± 0.4	1.1 ± 0.5
762.4	34	0	16:0/18:0	0.3 ± 0.2	0.4 ± 0.3
782.3	36	4	16:0/20:4; 18:1/18:3	0.5 ± 0.4	0.5 ± 0.2
784.6	36	3	16:0/20:3; 18:1/18:2; 18:0/18:3	0.4 ± 0.2	0.6 ± 0.2
786.4	36	2	18:0/18:2; 18:1/18:1	4.2 ± 0.6	4.7 ± 0.7
788.4	36	1	18:0/18:1; 16:0/20:1	28.6 ± 4.8	25.7 ± 3.7
808.4	38	5	18:0/20:5; 18:1/20:4; 16:0/22:5	2.8 ± 0.6	2.9 ± 0.5
810.4	38	4	18:0/20:4; 16:0/22:4; 18:1/20:3	42.4 ± 1.7	42.7 ± 3.3
812.4	38	3	18:0/20:3	3.9 ± 0.5	4.0 ± 1.7
814.3	38	2	18:0/20:2	0.9 ± 0.6	1.0 ± 0.4
832.4	40	7	18:1/22:6	1.2 ± 0.5	1.0 ± 0.5
834.4	40	6	18:0/22:6; 18:1/22:5	5.9 ± 1.6	6.2 ± 1.0
836.4	40	5	18:0/22:5; 18:1/22:4	4.2 ± 1.2	5.3 ± 1.6
838.4	40	4	18:0/22:4	3.9 ± 1.0	4.0 ± 0.8

Table 6
Molecular species of phosphatidylcholine from blood mononuclear cells of cystic fibrosis and normal subjects by negative-ion HPLC–ESI-MS analysis

PC molecular species ($[M+35]^-$)				Relative abundance in percentage (mean ± S.D.)		
m/z	Combined chain length	Double bonds	Major species	CF (*n* = 7)	*N* (*n* = 7)	CF (Pins)* (*n* = 5)
712.4	28	0	14:0/14:0	0.4 ± 0.3	0.4 ± 0.2	0.4 ± 0.3
740.4	30	0	14:0/16:0	1.1 ± 0.3	1.0 ± 0.3	1.2 ± 0.7
766.4	32	1	16:0/16:1	1.4 ± 0.3[a]	0.9 ± 0.3	1.6 ± 0.3[a]
768.4	32	0	16:0/16:0	11.7 ± 3.0	12.5 ± 1.7	12.6 ± 3.4
782.4	33	0	16:0/17:0	0.6 ± 0.2	0.8 ± 0.3	0.8 ± 0.2
792.4	34	2	16:0/18:2	5.5 ± 1.7	6.4 ± 0.8	3.8 ± 0.7[a]
794.4	34	1	16:0/18:1	23.7 ± 1.8	21.8 ± 1.8	24.1 ± 2.8
796.4	34	0	16:0/18:0	5.4 ± 0.3	5.8 ± 0.6	5.3 ± 0.2
814.4	36	5	16:0/20:5; 16:1/20:4	0.8 ± 0.5	0.4 ± 0.2	0.9 ± 0.5
816.4	36	4	16:0/20:4	6.4 ± 1.3	6.2 ± 0.8	6.5 ± 1.2
818.4	36	3	16:0/20:3	4.9 ± 0.6	4.4 ± 0.8	4.7 ± 0.4
820.4	36	2	18:0/18:2; 18:1/18:1	5.8 ± 0.7	6.4 ± 0.6	5.2 ± 0.4[a]
822.4	36	1	18:0/18:1	5.3 ± 0.9	5.7 ± 1.1	5.3 ± 1.2
824.4	36	0	18:0/18:0	1.5 ± 0.4	1.2 ± 0.4	1.6 ± 0.6
840.4	38	6	16:0/22:6	1.9 ± 0.8	1.7 ± 0.3	1.7 ± 0.4
842.4	38	5	16:0/22:5; 18:0/20:5; 18:1/20:4	3.4 ± 0.5[a]	4.2 ± 0.6	3.4 ± 0.6[a]
844.4	38	4	18:0/20:4; 18:1/20:3; 16:0/22:4	9.6 ± 1.6	9.3 ± 0.9	10.7 ± 0.7
846.4	38	3	18:0/20:3; 18:1/20:2	3.2 ± 0.5	3.1 ± 0.8	3.8 ± 1.1
848.4	38	2	18:0/20:2	1.0 ± 0.6	0.9 ± 0.4	1.0 ± 0.6
850.4	38	1	18:0/20:1	0.5 ± 0.2	0.4 ± 0.2	0.4 ± 0.3
866.4	40	7	18:1/22:6	1.0 ± 0.4	1.0 ± 0.4	1.2 ± 0.5
868.4	40	6	18:0/22:6; 18:1/22:5	2.3 ± 1.0	2.4 ± 0.5	1.8 ± 0.5
870.4	40	5	18:0/22:5; 18:1/22:4	1.8 ± 0.5	2.1 ± 0.4	1.5 ± 0.5
872.4	40	4	18:0/22:4; 20:0/20:4	0.7 ± 0.2	0.9 ± 0.3	0.8 ± 0.7

* CF (P_{ins}) = CF subjects with pancreatic insufficiency.
[a] Significantly different from normal subjects; $P < 0.05$.

in the CAD spectrum of *m/z* 738.4 (Fig. 3C), the contribution of other isobaric species is negligible. Therefore, changes in *m/z* 738.4 can be regarded as changes in PE (16:0/20:4).

3.9. PI molecular species

As shown in Table 4, the most abundant PI molecular species was 18:0/20:4 (*m/z* 885.5) which, including the contribution of other isobaric species, accounted for approximately 76% of total PI molecular species. The contribution of minor isobaric species (16:0/22:4; 18:1/20:3) was found to be poor, as shown in CAD spectra of *m/z* 885.5. The PI molecular species relative abundance was found to be similar in CF compared to normal subjects.

3.10. PS molecular species

PS molecular species relative abundance is reported in Table 5; the most part of PS species contained C18:0. Indeed, the major molecular species for PS were *m/z* 810.4

Table 7
Molecular species of sphyngomyeline from blood mononuclear cells of cystic fibrosis and normal subjects by negative-ion HPLC–ESI-MS analysis

Sph molecular species ($[M+35]^-$)				Relative abundance in percentage (mean ± S.D.)	
m/z	Combined chain length	Double bonds	Major species	CF ($n = 7$)	N ($n = 7$)
709.4	14	0	14:0	3.0 ± 1.2	2.9 ± 0.5
735.3	16	1	16:1	2.1 ± 0.4	2.2 ± 0.2
737.3	16	0	16:0	48.0 ± 2.5	47.2 ± 2.6
761.3	18	2	18:2	1.2 ± 0.8	1.1 ± 0.5
763.3	18	1	18:1	3.3 ± 1.3	3.0 ± 1.4
765.3	18	0	18:0	4.7 ± 0.6	5.2 ± 0.7
791.3	20	1	20:1	1.2 ± 0.2	1.3 ± 0.3
793.3	20	0	20:0	5.9 ± 1.0	6.1 ± 0.8
819.3	22	1	22:1	2.8 ± 0.5	2.6 ± 0.3
821.3	22	0	22:0	8.7 ± 1.6	11.0 ± 2.4
847.3	24	1	24:1	11.9 ± 1.5	10.7 ± 1.7
849.3	24	0	24:0	7.1 ± 1.8	6.6 ± 1.9

(i.e. 18:0/20:4) and *m/z* 788.4 (i.e. 18:0/18:1), which accounted, respectively, for 42–43 and 26–29% of total PS molecular species of both CF and normal subjects, including the contribution of minor isobaric species. However, as reported in Fig. 5B, the contribution of isobaric species other than 18:0/20:4 to the total ion intensity is negligible. There were no significant differences between the two experimental groups.

3.11. PC molecular species

In contrast with PI and PS classes, the relative abundance of the PC molecular species were comparable except for *m/z* 794.4 (i.e. 16:0/18:1), which is the dominant species (about 23%). As reported in Table 6, there was a significant greater amount of the ion *m/z* 766.4 (i.e. 16:0/16:1) and a significant lower amount of the ion *m/z* 842.4 (corresponding to the sum of 16:0/22:5; 18:0/20:5; 18:1/20:4) in the PC molecular species from CF subjects with respect to normal subjects. PC molecular species were also compared between normal and CF subjects with pancreatic insufficiency (P_{ins}). This subgroup ($n = 5$) was obtained by the exclusion of subjects with pancreatic sufficiency (P_{suff}) from the whole CF group. There was significantly less *m/z* 794.4 (i.e. 16:0/18:2) (3.8 versus 5.5%) and *m/z* 820.4 (corresponding to the sum of 18:1/18:1 and 18:0/18:2) (5.2 versus 6.4%) in the blood mononuclear cell of CF with PI as compared with normal subjects.

3.12. Sph molecular species

Sph molecular species are reported in Table 7. These were almost exclusively saturated and monounsaturated molecular species with a relevant amount of long chain fatty acid containing species (i.e. C 22:0, C 24:0, C 24:1). The most abundant $[M + 35]^-$ ion was *m/z* 737.3 (corresponding to 16:0 species) which accounted for approximately 46–48% of total Sph molecular species. There were no significant differences between the two experimental groups.

3.13. Sensitivity and reproducibility

The sensitivity of the present method is strictly dependent upon the phospholipid class analyzed. By making dilutions of the standard solutions, the following detection limits (signal-to-noise ratio: higher than 3) were estimated (values given as total amount of injected substance): 0.5 ng DPPC, 0.5 ng DPPE, 0.5 ng Sph, 1.0 ng PI (estimated from the peak of 18:0/20:4) and 5.0 ng DPPS. The reproducibility was calculated by the relative standard deviation (R.S.D.) obtained for the main PLs with triplicate analysis of the same species. R.S.D. at concentrations of 0.06 and 0.01 mg/mL was 12 and 13% for PE (16:0/16:0), 10 and 11% for PE (18:2/16:0), 3 and 2% for PI (20:4/18:0), 4 and 5% for PS (16:0/16:0), 7 and 15% for PC (22:6/16:0), 2 and 12% for PC (18:1/16:0), 2 and 14% for PC (16:0/16:0), respectively.

4. Conclusions

A rapid normal phase HPLC method coupled on-line to ESI-MS has been developed for the analysis of blood mononuclear cell (lymphocyte and monocyte) phospholipid molecular species. The method was used for the separation, identification and characterization of individual molecular species of PE, pPE, PI, PS, PC and Sph extracted from human PBMC. HPLC–ESI-MS was used previously to analyze phospholipid species in the lipid extract from the whole human blood [26] and ESI-MS was used to characterize the major phospholipid molecular species in human red blood cells (RBC) [34]. The former reported also a detailed characterization of LPC species, which in our samples were found only in traces. Therefore, we have deduced that LPC species originate mainly from serum, where they are known to compose the serum lipoproteins [35]. On the contrary, PE and pPE species and their relative abundances were very similar to those identified in the RBC and in the whole human blood, where pPE (p18:0/20:4) and PE (18:0/20:4) were found to be the most representative in these phospholipid classes. However, the relative amounts of pPE (p18:1/20:4) (plus its

isobaric species), and that of PE and pPE species containing docosahexaenoic acid (C 22:6) appear to be lower with respect to the study performed in the human blood, probably because these species are more abundant in RBC. In analogy with the cited studies, the prominent species for PI was found to be PI (18:0/20:4). This species accounted about 75–80% of all PI species, which is a considerably higher amount of that found in the whole blood (approximately 45% converting the data normalized to the base peak into relative percentages). This is in agreement with the pro-inflammatory role of PBMC, which have been shown to metabolize a considerable amount of arachidonic acid bound to PI into 12-hydroxy-eicosatetraenoic acid (12-HETE) [36]. A comparison of PS species identified in our work to that identified in the whole blood and in RBC confirms that: (a) PS (18:0/20:4) is the prominent species in human blood cells; (b) PS species containing docosahexaenoic acid are well represented in this class; (c) PS species containing palmitic acid are scarcely represented. However, a higher amount of PS (18:0/18:1) and a lower amount of PS (18:0/22:6) was found in the present work. The major difference between this study and that performed in the whole blood consists in the abundance of the most representative PC species, PC (16:0/18:1) and PC (16:0/18:2), which were found to be 18 and 30%, respectively (converting the base peak normalized data into relative percentages) in the blood, and 23 and 6%, respectively, in this study. This could be due both to a great presence of PC (16:0/18:2) in serum (and/or in the RBC) and to a high metabolic conversion rate of LA, in the mononuclear blood cell membrane, into more unsaturated and longer fatty acids. Moreover, 12 different species of Sph were identified and analyzed in the present work.

The study of phospholipid molecular species in PBMC of CF patients could be of interest because PBMC normally express cystic fibrosis transmembrane protein (CFTR), whereas in lymphocytes from CF subjects, normal chloride conductance is restored only after transfection of wild-type CFTR [34]. Moreover, a defective regulation of arachidonic acid, resulting in an increased turnover, but a normal functional glucocorticoid receptor was found to occur in lymphocyte from CF subjects [37].

The results show that despite the disease status (pancreas condition or mutation), there are not unusual phospholipid species and no qualitative differences were seen among the subjects. However, this preliminary study suggests that PC and pPE relative molecular species distribution can be different in CF PBMC compared to normal subjects. A significant greater amount of PC (16:0/16:1) was found in PBMC from CF subjects with respect to normal subjects and the relative amount of PC molecular species carrying C 18:2 in the glycerol backbone was found to be lower in CF subjects with pancreas insufficiency compared to normal subjects.

These abnormalities were in agreement with previous studies, where high levels of palmitoleic acid (PO) and low levels of linoleic acid (LA) were found in serum [38], platelet [39] PLs, and in tissues [3] from CF subjects.

The differences found in pPE consisted of lower amount of some species carrying docosahexaenoic acid in CF PBMC compared to normal subjects. Docosahexaenoic acid deficiency in CF subjects has been previously reported by studying the fatty acid composition of serum [4] and red blood cell PLs [40], but, as far as we know, the localization of this deficiency in particular molecular species is reported in this paper for the first time. This result might be important because pPE species are known to have an antioxidative action involved in scavenging radicals at the vinyl–ether linkage, and to protect membrane against peroxidation [41–43].

The differences reported between normal persons and CF patients in the PE/pPE and PC classes are small considering that the targeted species are approximately 1–4% of the total of these classes. This difference, generally lower than that reported in literature could be attributed to a concomitant increase of some isobaric species and decrease of others, which mask or reduce the changes observed for a particular ion. On the contrary, statistical differences found between normal and CF patients with P_{ins}, in the PC species corresponding to m/z 820.4 and m/z 792,4, account for 3,8%, and the sum of targeted species constitute 12% of this class in normal subjects. Therefore, these data might be considered reliable and fit with the ones from literature obtained in other blood cells by means of GC analysis of fatty acids [40].

Therefore, the analytical method presented in this work is proposed as an effective approach in order to study CF lipidic deficiencies and to evaluate the effects of lipidic supplements aimed at their correction. Further research using HPLC–MS to study the phospholipid molecular species distribution in cells and tissues of CF subjects should be encouraged in order to understand whether lipidic deficiencies in CF are related to intestinal malabsorption or malnutrition or also to the existence of a relationship with the basic defect of CF, as recently hypothesized [5].

Acknowledgements

The authors wish to thank Dr. Rolando Gagliardini (C.R.R. Centro Fibrosi Cistica, Divisione di Pediatria e Neonatologia Ospedale dei Bambini ULSS no. 12, Ancona, Italy) for kindly providing the blood samples.

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