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Technical Report

Stop-flow Comprehensive Two-dimensional Liquid Chromatography Combined with Mass Spectrometric Detection for Phospholipid Analysis

LC×LC for phospholipids in milk and plasma samples

Paola Dugo[1, 2], Nermeen Fawzy[1], Francesco Cacciola[1], Paola Donato[1], Filomena Cichello[1], Luigi Mondello[1, 2]

Abstract:

A novel comprehensive two-dimensional liquid chromatographic (LC×LC) system for characterization of phospholipid (PL) molecular species belonging to six phospholipid classes was developed. To tackle such a task, a silica hydrophilic interaction liquid chromatography (HILIC) column was used as the first dimension (D1), and reversed-phase liquid chromatography (RP-LC) with a C18 column was used as the second dimension (D2) in combination with mass spectrometric detection. Fraction transfer from the D1 to the D2 was performed by means of a two-position ten-port switching valve, operated under stop-flow conditions. The capability of the investigated LC×LC approach was demonstrated in the separation of phospholipid molecular species contained in two Folch-extracted cow's milk and plasma samples.

Keywords: comprehensive LC, phospholipids, mass spectrometry, stop-flow

1. Introduction

Phospholipids (PLs) are an important class of biomolecules playing an important functional, structural and metabolic role in the human body as witnessed by recent studies which have given considerable evidence on the health-promoting effects such as antiinflammatory activity and risk reduction of cardiovascular diseases.

Several analytical methods have been developed for characterization of molecular species within different PL classes. From a chromatographic stand-point, it must be noted that the employment of a single technique can only provide useful information on either the different phospholipid classes or the molecular species within a particular PL class.

In this technical report, in order to simultaneously separate and identify the different PL classes together with the separation and identification of the different molecular species within each class, a fully comprehensive LC (LC×LC) method was developed for the first time. Such a system comprised of a silica hydrophilic interaction liquid chromatography (HILIC) column in the first dimension (D1) and an octadecylsilica column in the second dimension (D2) and was run under stop-flow conditions. The capability of such a system (Fig. 1) was evaluated for analysis of PLs contained in two Folch-extracted cow's milk and plasma samples (Fig. 2).

Fig. 1 LC×LC/MS instrumentation

2. Experimental

2-1. Samples and sample preparation

A crude cow's milk sample was provided by a Calabrian producer whereas the plasma sample was kindly donated by a sane volunteer.

Extraction of the lipid fraction was carried out from 10 mL and 1.5 mL, respectively, of the cow's milk and plasma sample, according to the Folch method in order to attain an exhaustive extraction of the whole lipid content. The total extract was evaporated under vacuum, and the final dry residue (400 and 150 mg for cow's and donkey's milk, respectively) was re-dissolved in chloroform/methanol 2:1 (v/v) and stored at –18 °C until use.

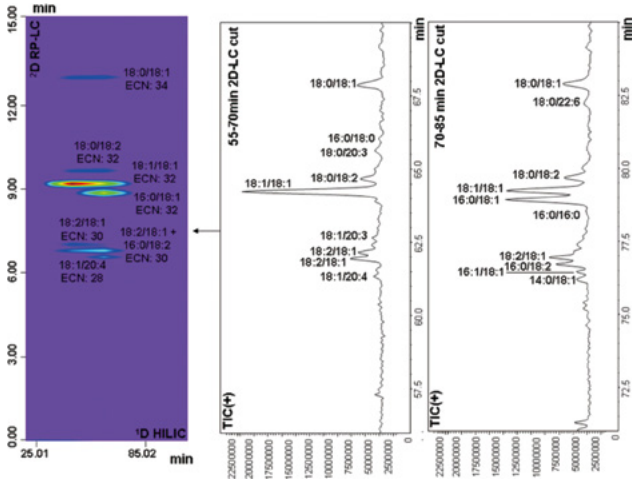

Fig. 2 Enlargement of the HILIC×RP-LC–ESI-MS contour plot for separation of the phosphatidylethanolamine (PE), along with the corresponding 2D raw data

1 University of Messina, Italy
2 Chromaleont S.r.l.

2-2. Reagents and Materials

For the extraction procedure, chloroform and methanol were obtained from VWR (Milan, Italy).

For LC×LC–MS analyses, water, acetonitrile, methanol, tetrahydrofuran, isopropanol, all LC–MS grade, and formic acid were purchased from Riedel-de Haën (Seelze, Germany). Ammonium formate was obtained from Alfa Aesar GmbH & Co., KG (Karlsruhe, Germany). The pH of buffered mobile phases was adjusted to 5.5 by adding a few drops of formic acid. The standards of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyielin (SM) and lysophosphatidylcholine (LPC) were purchased from Sigma–Aldrich/Supelco (Bellefonte, PA, USA).

Chromatographic separations were carried out using different columns provided by Supelco (Bellefonte, PA, USA): Ascentis Express HILIC (150 mmL. × 2.1 mmI.D., 2.7 µm d.p.), and Ascentis Express C18 (150 mmL. × 4.6 mmI.D., 2.7 µm d.p.).

2-3. LC×LC instrumentation and software

- Shimadzu CBM-20A controller
- two Shimadzu LC-20AD dual-plunger parallel-flow pumps
- Shimadzu LC-20AB dual-plunger parallel-flow pumps
- Shimadzu DGU-20A5 degassing unit
- Shimadzu CTO-20A column oven
- Shimadzu SIL-20AC autosampler
- Shimadzu SPD-M20A photo diode array detector (2.5 µL detector flow cell)
- Shimadzu LCMS-2020 mass spectrometer

For connecting the two dimensions: 2-position 10-port switching valve (Supelco, Bellefonte, PA, USA) placed inside the column oven and equipped with two identical 20 µL sample loops.

2-4. Software

- Shimadzu LabSolutions (Version 5.41 SP1)

2-5. 2D Software

- ChromSquare (Version 2.0) from Chromaleont, Messina, Italy

2-6. LC×LC-MS conditions

D1 separations: Ascentis Express HILIC	
Flow rate	: 0.1 mL.mL^{-1}
Mobile phases	: (A) acetonitrile/ammonium formate (10 mM) buffer pH 5.5 (90:10) and (B) acetonitrile/methanol/ammonium formate (10 mM) buffer pH 5.5 (55:35:10)
Gradient elution	: 0 min, 0% B; 20 min, 0% B; 25 min, 100% B; 210 min, 100% B; 211 min, 0% B
Injection volume	: 10 µL.

D2 separations: Ascentis Express C18	
Mobile phase	: (A) ammonium formate buffer (10 mM; pH 5.5)/ isopropanol/tetrahydrofuran (30:55:15) and (B) acetonitrile. Isocratic elution (40% B)
Flow rate	: 3.0 mL.min^{-1}. Prior to MS detection, the mobile phase flow rate was reduced to 0.3 mL.mL^{-1} through a T-piece union.
Modulation time of the switching valve : 15 min	

MS conditions

MS acquisition performed using the ESI interface operating in both positive and negative ionization modes:

mass spectral range: 200–1100 *m/z*; event time: 1 sec; scan speed: 938 amu/s; nebulizing gas (N_2) flow: 1.5 L.min^{-1}; drying gas (N_2) flow: 15 L.min^{-1}; interface temperature: 350°C; heat block temperature: 200°C; desolvation line (DL) temperature: 250°C; DL voltage: –34 V; probe voltage: +4.5 kV; Qarray DC voltage: 1 V; Qarray RF voltage: 100 V; detection gain: 0.8 kV.

3. Results and discussion

The objective of this work was to develop an HILIC×RP-LC system in combination with mass spectrometric detection for analysis of PL molecular species contained in Folch-extracted cow's milk and plasma samples. Prior to HILIC×RP-LC separations the two dimensions were optimized independently.

4. Optimization of D1 and D2 separation systems

HILIC separation can be described either as liquid–liquid partition chromatography, or a version of NP-LC, run with partially aqueous mobile phases. Compounds are separated by passing normally an organic mobile phaseacross a neutral hydrophilic stationary phase, thus solutes are eluted in order of increasing hydrophilicity; the separation selectivity is, therefore, complementary to that in reversed phase mode. The use of higher-organic content mobile phases is advantageous in providing larger diffusion constants of analytes during their migration through the column, allowing a partial separation of molecular species and also better ionization efficiency in electrospray ionization.

Fig. 3 shows the total ion current (TIC) chromatogram of a HILIC-ESI-MS analysis of three different samples, namely, PL standard mixture (A), Folch-extracted cow's milk (B), Folch-extracted plasma sample (C). Baseline separation of the six PL classes was achieved, under gradient conditions, within a run time of 30 min, according to decreasing polarity viz. PI eluted first followed by PE and PS; the PL classes, containing the phosphocholine head group (PC, SM and LPC), were the most retained and thus the latest to elute. Identification was carried out by the inspection of both $[M+H]^+$ and $[M–H]^-$ ions, the latter employed for better ionization of PI.

For the 2D separations, a C18 column packed with 2.7 mm particles was employed. RP-LC separation is mainly achieved on the basis of the difference in chain length and the number of fatty acid double bonds (i.e. essentially on the lipophilicity), viz. increasing equivalent carbon number (ECN), defined as the total carbon number (CN) of fatty acids minus two times the double bond (DB) number (ECN = CN – 2DB).

Fig. 4 shows the TIC chromatogram from the positive-ion LC–ESI-MS analysis of a PC standard. Baseline separation of six of them was achieved, at a flow rate of 0.9 mL/min, under isocratic conditions, within a run time of 15 min. The retention of molecular species increased proportionally to the ECN, from 24 to 34).

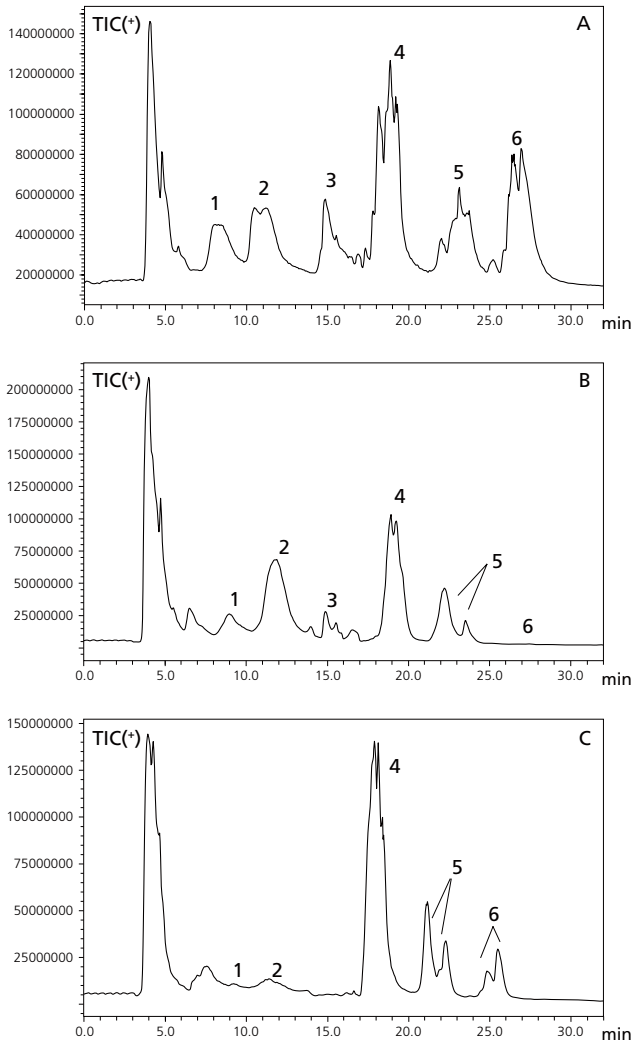

Fig. 3 Positive-ion HILIC-ESI-MS TIC (total ion current) chromatogram of a phospholipid standard mixture (A), Folch-extracted cow's milk (B), Folch-extracted plasma sample (C)
(1) Phosphatidylinositol (PI); (2) Phosphatidylethanolamine (PE); (3) Phosphatidylserine (PS); (4) Phosphatidylcholine (PC); (5) Sphingomyelin (SM); (6) Lysophosphatidylcholine (PLC).

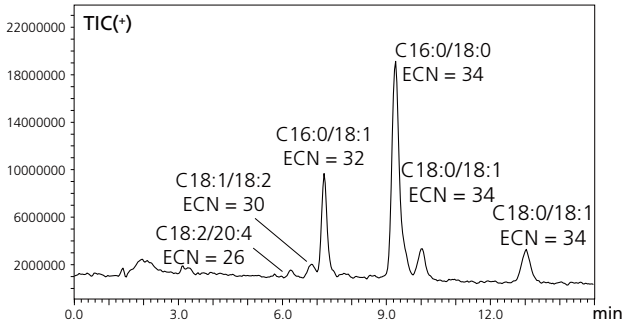

Fig. 4 Positive-ion RP-LC–ESI-MS TIC chromatogram of the different molecular species identified in a PC standard

5. HILIC×RP-LC for phospholipid (PL) separation

After a proper optimization of the two different separation systems, an HILIC×RP-LC system was tuned.

For both samples, the PC turned out the richest in terms of molecular species. As an example, an enlargement of the HILIC×RP-LC–ESI-MS contour plot, with the corresponding 2D raw data for the plasma sample, is reported in Fig. 5. Up to sixteen and fourteen molecular species, belonging PC classes, over two 15 min modulation cycles were positively identified in cow's milk and plasma samples, respectively. The observed chromatographic pattern fits to the expected PL separation based on increasing hydrophobicity, viz. increasing ECN values, ranging from 26 to 34. It is worth mentioning that the employed 2D mobile phase allowed to successfully separate also isobaric species. A list of the major species contained in a phospholipid standard mixture, cow's milk sample and a plasma sample is reported in Table 1.

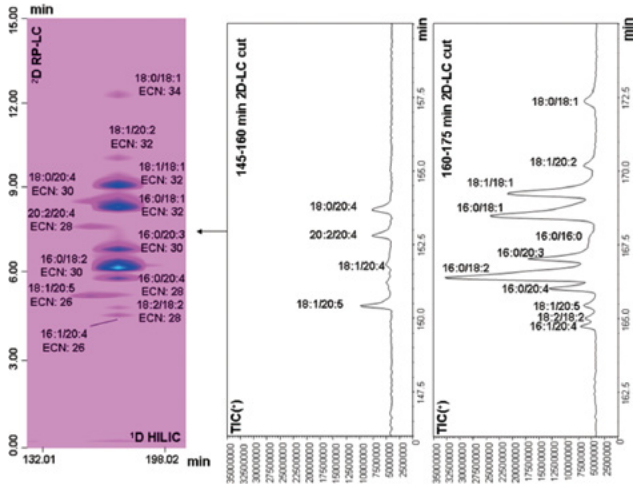

Fig. 5 Enlargement of the HILIC×RP-LC–ESI-MS contour plot along with the corresponding 2D raw for separation of the PC molecular species contained in the plasma sample

6. Conclusions

The aim of the present research was to separate simultaneously the PL fraction belonging to different classes along with the molecular species corresponding to those classes, by using a comprehensive HILIC × RP-LC–ESI-MS system in stop-flow mode.

The combination of HILIC and RP-LC techniques with ESI-MS as detection system, allowed to achieve separation of individual molecular species contained in two Folch-extracted cow's milk and plasma samples. In particular, PC turned out to be the most complex one, and up to 16 and 14 different species were identified, respectively.

The only drawback was the long analysis time due to the stop-flow mode employed even though this is well compensated by the enhanced resolving power and the greater amount of analyte information obtained.

Since each second-dimension peak corresponds to a single PL species, which is eluted according to increasing hydrophobicity, the developed 2D-LC system can be used also in absence of tandem mass spectrometry detection in favour of less expensive techniques such as single quadrupole MS or ELS, for analysis of other lipid classes of different origin.

Table 1 List of the major species contained in a phospholipid standard mixture, cow's milk sample and a plasma sample

	Standard mixture		Cow's milk sample		Plasma sample	
PL class	*m/z*	FAs	*m/z*	FAs	*m/z*	FAs
Phosphatidylinositol (PI)	$[M-H]^-$		$[M-H]^-$		$[M-H]^-$	
	831.5	16:1/18:2	835.5	16:0/18:1	885.5	18:0/20:4, 18:1/20:3
	833.5	16:0/18:2	859.5	18:3/18:0		
	835.5	16:0/18:1	861.5	18:0/18:2, 18:1/18:1		
	857.6	16:0/20:4, 18:2/18:2	863.5	18:0/18:1		
	859.5	18:2/18:1	887.5	20:3/18:0		
	861.5	18:0/18:2, 18:1/18:1				
Phosphatidylethanolamine (PE)	$[M+H]^+$		$[M+H]^+$		$[M+H]^+$	
	744.5	18:0/18:2	690.6	14:0/18:1	744.5	18:1/18:1, 18:0/18:2
			692.5	16:0/16:0	764.6	16:0/22:6
			716.6	16:0/18:2, 16:1/18:1	768.5	18:0/20:4
			718.6	16:0/18:1	792.5	18:0/22:6
			742.6	18:2/18:1		
			744.6	18:1/18:1, 18:0/18:2		
			746.6	18:0/18:1		
			766.7	18:1/20:4		
			768.5	18:1/20:3		
			770.6	18:0/20:3		
			792.5	18:0/22:6		
Phosphatidylserine (PS)	$[M+H]^+$		$[M+H]^+$		n.d.	n.d.
	788.6	18:0/18:2	786.5	18:2/18:1		
	790.6	18:0/18:1	788.5	18:0/18:2		
	836.6	16:0/20:3	790.6	18:0/18:1		
	838.6	16:0/20:2				
	840.5	16:1/20:0				
Phosphatidylcholine (PC)	$[M+H]^+$		$[M+H]^+$		$[M+H]^+$	
	732.5	16:0/16:1	706.6	16:0/14:0	885.5	18:0/20:4,18:1/20:3
	758.6	16:0/18:1	720.5	15:0/16:0		
	760.5	16:0/20:4	732.5	16:0/16:1		
	782.5	18:2/18:2	734.5	16:0/16:0		
	784.5	18:1/18:2	746.5	15:0/18:1		
	786.5	18:1/18:1	748.6	15:0/18:0		
	788.5	18:0/18:1	756.5	16:0/18:3		
	790.5	18:1/22:6	758.6	16:0/18:2		
	792.6	18:0/22:6	760.5	16:0/18:1		
	806.5	18:2/20:4	762.5	16:0/18:0		
	810.5	18:2/20:2	774.5	17:0/18:1		
	818.6	20:1/22:6	782.5	18:2/18:2		
	834.6	18:0/20:4	784.6	18:1/18:2		
	836.6	18:0/22:5	786.6	18:0/18:2, 18:1/18:1		
			788.6	18:0/18:1		
Sphingomyielin (SM)	$[M+H]^+$		$[M+H]^+$		$[M+H]^+$	
	703.6	16:0	675.6	14:0	675.5	14:0
	705.6	18:0	689.5	15:0	701.5	18:1
	731.5	18:0	703.6	16:0	703.5	16:0
			705.6	16:0	729.5	18:1
			759.5	20:0	731.5	18:0
			773.5	21:0	759.6	20:0
			785.6	22:1	785.6	22:1
			787.6	22:0	787.5	22:0
			799.6	23:1	811.5	24:2
			801.5	23:0	813.5	24:1
			813.5	24:1	815.5	24:0
			815.5	24:0		

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