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Hydrophilic interaction and reversed phase mixed-mode liquid chromatography coupled to high resolution tandem mass spectrometry for polar lipids analysis

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ABSTRACT

A hydrophilic interaction liquid chromatography (HILIC) fused-core column (150 × 2.1 mm ID, 2.7 μm particle size) and a short reversed-phase liquid chromatography (RPLC) column (20 mm × 2.1 mm ID, 1.9 μm) were serially coupled to perform mixed-mode chromatography (MMC) on complex mixtures of phospholipids (PL). Mobile phase composition and gradient elution program were, preliminarily, optimized using a mixture of phosphatidylcholines (PC), phosphatidylethanolamines (PE), their corresponding lyso-forms (LPC and LPE), and sphingomyelins (SM). Thus a mixture of PC extracted from soybean was characterized by MMC coupled to electrospray ionization (ESI) high-resolution Fourier-transform mass spectrometry (FTMS) using an orbital trap analyzer. Several previously undiscovered PC, including positional isomers (i.e. 16:0/19:1 and 19:1/16:0) of PC 35:1 and skeletal isomers (i.e. 18:1/18:2 and 18:0/18:3) of PC 36:3 were identified. Therefore, high-resolution MS/MS spectra unveiled the occurrence of isomers for several overall side chain compositions. The proposed MMC-ESI-FTMS/MS approach revealed an unprecedented capability in disclosing complexity of an actual lipid extract, thus representing a very promising approach to lipidomics.

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1. Introduction

Lipidomics investigation based on liquid chromatography-mass spectrometry (LC–MS) is a rapidly growing field, which has greatly benefited from the introduction of new MS analysers, featuring high mass resolution/accuracy [1–6], and novel LC technologies, enabling very selective and efficient separations with reduced analysis time and solvent consumption [7,8]. Lipidomics-related LC separations can be accomplished by (i) normal phase LC (NPLC), which typically leads to separation of lipids on the basis of their polar functionalities, (ii) hydrophobic interaction mode of reversed-phase LC (RPLC), that enables the separation of lipid species as a function of their lipophilic tails, and (iii) hydrophilic interaction liquid chromatography (HILIC), which can be a good alternative to RPLC for separating polar compounds [2,9–11]. Interestingly, HILIC combines the features of both NPLC and RPLC, as it employs polar stationary phases typical of NPLC, such as silica, and mobile phases typical of RPLC, such as solvent mixtures containing more than 50–70% acetonitrile, though with a reversed eluotropic strength [10,12].

The LC–MS coupling greatly helps to cope with several shortcomings occurring in the direct analysis by MS. Firstly, the chromatographic separation alleviates ionization suppression effects [13]. Secondly, the LC step could enables the separation of (intra- and/or inter-class) isobaric or isomeric PL, which, if co-eluted, would make MS identification ambiguous. However, some inherent limitations of each LC mode, potentially affecting MS detection, must be also accounted for. NPLC, for instance, is certainly suitable for phospholipids analysis, but the highly polar solvents typically employed possess low ionization efficiency thus negatively affecting the LC–MS coupling [8]. Conversely, RPLC uses MS compliant mobile phases, but partial or severe lipid co-elution is quite common especially for highly complex samples. HILIC, which is particularly effective in providing class separation, should avoid the co-elution of isobars belonging to different lipid classes, yet intra-class lipid competition for ionization could remain [14]. Moreover, the co-elution of lipids, belonging to the same class, hav-

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ing molecular masses differing by 2 Da leads to the unavoidable superposition of spectra referred to the M + 0 isotopologue of the target lipid and to the M + 2 isotopologue related to the 2 Da lighter one, garbling the interpretation of MS/MS spectra.

To overcome these issues, two-dimensional (2D) high-performance liquid chromatography coupled to mass spectrometry has been suggested. Specifically, a 2D LC approach can be performed on-line [15,16] or off-line [17] and offers the chance to analyze complex lipid mixtures [16,18] using orthogonality in the separation mechanism operating in the two dimensions [19,20]. Among 2D approaches, comprehensive LCxLC is certainly the most promising, in spite of the higher instrumental complexity required [21]; note, however, that a partial loss of orthogonality has been experienced when using RPLCxRPLC [22] or HILICxHILIC [23]. A viable and instrumentally simpler alternative approach for analysing highly complex samples could be represented by mixed-mode chromatography (MMC) [24,25], potentially offering high selectivity, high sample loadings and speed [2,25,26]. MMC is typically performed by: (i) serial connection of two columns packed with different stationary phases (ii) use of a single column packed with two different stationary phases or (iii) use of special stationary phases, possessing ad-hoc designed functional groups displaying several domains (e.g. hydrophilic, hydrophobic, ionic) and then capable of combining orthogonal separation principles [25]. Two *in series* (tandem) columns, with no interface between them, is the simplest way to implement MMC, provided that mobile phase and/or gradient elution are compatible with both columns [25]. Since PL are characterized by a polar head (class specific) and by hydrophobic acyl chains, a suitable column combination would be HILIC-RPLC, potentially capable of achieving between- and within-class separation, as demonstrated by HILIC-RPLC offline 2D-LC [17,27,28].

Few attempts to use MMC in lipid (mainly triacylglycerols – TAGs) analysis have been reported so far. Profiling of TAGs in plant oils on a mixed-mode phenyl-hexyl chromatographic column providing hydrophobic as well as π–π interactions has been described by Hu et al. [29]. TAGs separation could also be achieved by MMC on a single column packed with silver ion-modified octyl and sulfonic co-bonded silica, providing hydrophobic as well as complexation interactions [30]. Using a commercially available octadecylsilane column (end-capped with trimethylsilane moieties) and a mobile phase composed of methanol and isopropanol containing *para*-toluenesulfonic acid (as ion pairing agent) Lima and Synovec [31] demonstrated the separation of PL based on both non-polar fatty acid chain length and polar head group functionality. However, this approach cannot be classified as one of the three MMC modes above described so, to the best of our knowledge, no application of genuine MMC to phospholipidomics has been reported so far.

Starting from these considerations, an MMC approach based on the serial coupling of HILIC and RPLC columns, not requiring additional equipments (e.g. pumps, switching valves or T-pieces) has been developed for the separation of complex PL mixtures prior to MS detection. Its optimization and subsequent application to the characterization of a complex mixture of phosphatidylcholines is described here.

2. Materials and methods

2.1. Chemicals and standards

Water, methanol and acetonitrile (LC–MS grade), chloroform (HPLC grade) and ammonium acetate were obtained from Sigma–Aldrich (Milan, Italy). The standard L-α-phosphatidylcholine from soybean (Sigma Aldrich) was dissolved in methanol and a solution of 100 μg/mL was used for analysis. A standard mixture containing 10 μg/mL each of PE 18:0/22:6, PE 18:1/18:1, LPE 18:0, LPE 22:6, PC 16:0/20:4, PC 12:0/12:0, LPC 18:1 (Sigma Aldrich) and 10 μg/mL of a SM standard mixture from chicken egg yolk was prepared. Note that the lipid nomenclature described by Liebisch et al. [32] was adopted throughout this paper.

2.2. LC–MS instrumentations and operating conditions

Ultra high performance (UHP) LC – high resolution MS was performed using an Ultimate 3000 UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled to a Q-Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA), including a quadrupole connected to an Orbitrap analyzer. Sample injection (5 μL) was performed by a RS Autosampler (Thermo Scientific, Waltham, MA, USA). The column effluent was transferred into the Q-Exactive spectrometer through a heated electrospray ionization (HESI) interface. The main electrospray and ion optics parameters were the following: sheath gas flow rate, 35 arbitrary units (a.u.); auxiliary gas flow rate, 15 a.u.; spray voltage, $\pm$3.5 kV (positive/negative polarity); capillary temperature, 320 °C; S-Lens RF Level, 60 a.u. MS spectra were acquired in the m/z range 120–1200, at a mass resolving power of 140000 (measured at m/z 200). The Orbitrap fill-time was set to 200 ms and the automatic gain control (AGC) level was set to 2.5×10^6. The Q-Exactive spectrometer was calibrated using a solution containing caffeine, the MRFA peptide and Ultramark, provided by Thermo Scientific. Mass accuracy ranged between 0.15 and 0.16 ppm in positive polarity and between 0.43 and 0.74 ppm in negative polarity.

In order to retrieve molecular information on the separated PL, additional targeted-MS^2 acquisitions were performed during each chromatographic run using a resolving power of 70,000 (at m/z 200), an Orbitrap fill-time of 100 ms and an AGC value of 5×10^5. The isolation window for precursor ions was 1.0m/z unit wide, whereas the normalized collision energy (NCE) used for the higher energy collision dissociation (HCD) cell, was stepped at 15, 25 and 35%. A stepwise fragmentation of the precursor ions (selected from an inclusion list of exact m/z values) was performed, then all generated fragments were collected and sent to the Orbitrap analyzer for single scan detection. All ions fragmentation (AIF) with multiple dissociation techniques, i.e. in source collision induced dissociation (sid) and HCD, providing MS and MS/MS data was also employed to increase the amount of retrievable information. AIF spectra were acquired using a NCE value of 35% and the same resolving power, trap-fill time and AGC value adopted for MS acquisitions. The control of LC–MS instrumentation and the first processing of data were performed by the Xcalibur software 3.0.63 (Thermo Scientific).

RPLC, HILIC and MMC were tested separately. RPLC was performed at 30 °C on an Accucore Polar Premium C18 column (150 × 2.1 mm ID, 2.6 μm particle size) equipped with a Accucore Polar Premium C18 (10 × 2.1 mm ID) security guard cartridge (Thermo Scientific, Waltham, MA, USA), using the following elution program, based on water (solvent A) and methanol (solvent B), both containing 2.5 mmol/L of ammonium acetate: 0–3 min at 60% (v/v) solvent B, 3–5 min linear to 100% solvent B; 5–30 min isocratic, 30–35 min back to the initial composition, followed by 10 min equilibration time. The flow rate was 0.2 mL/min.

HILIC separations were performed at ambient temperature on a narrow-bore fused-core [33] Ascentis Express HILIC column (150 × 2.1 mm ID, 2.7 μm particle size) equipped with an Ascentis Express HILIC (5 × 2.1 mm ID) security guard cartridge (Supelco, Bellefonte, PA, USA), using the following elution program: 0–6 min, isocratic at 90% solvent B; 6–15 min, linear from 90% to 60% solvent B; 15–25 min, isocratic at 60% solvent B; 25–30 min, linear to the initial composition, followed by 10 min equilibration time. The flow rate was 0.3 mL/min.

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