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A fast liquid chromatography-mass Spectrometry methodology for membrane lipid profiling through hydrophilic interaction liquid chromatography

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ABSTRACT

In this paper, we report the development of a new method based on HILIC-ESI-MS for the separation of several different membrane lipid classes and their detection on a triple quadrupole mass spectrometer using Precursor Ion (PIS) and Neutral Loss (NL) scanning in positive ion mode. Four different columns were tested for their ability to separate, under different conditions, a mixture of 14 lipid standards containing 7 glycerophospholipids (GPL), 2 glycosphyngolipids (GSL), 3 glycolipids (GL) and 2 betaine lipids (BL).

The best separation was obtained using a Lichrosphere DIOL column as stationary phase and water (10 mm ammonium acetate)/acetonitrile gradient elution as mobile phase which allows the separation of the 14 lipid classes within 35 min runtime. Our method was successfully tested for the separation and analysis of crude lipid extracts obtained from a green alga (*Jaoa bullata*), a dinoflagellate (*Peridinium cinctum*) and a plant (*Vitis vinifera* cv. Corvina)

1. Introduction

Lipidomics, or "the system-level analysis and characterization of lipids and factors that interacts with them" [1,2], is an emerging area within the field of "omics" sciences [3]. The term "lipids" defines a vast array of chemically diverse substances, which are mostly water-insoluble and contain fatty acids or their derivatives [4]. Lipids have been categorized into eight major classes: fatty acids, glycerolipids, glycerophospholipids, polyketides, prenyl lipids, saccharolipids, sphingolipids and sterols [5].

Lipids are involved in many cellular processes and aberrant lipid metabolism is a determinant of the onset of some important diseases, such as diabetes, atherosclerosis, obesity, Alzheimer's disease and some cancer types [1]. It is thus clear why the comprehensive analysis of the lipidome, the complete set of lipids within a cell, tissue or organism, is of great interest.

Various analytical methodologies have been applied for lipid analysis, such as thin-layer-chromatography (TLC), high-performance liquid chromatography (HPLC) coupled with mass spectrometry (HPLC-MS), diode array (HPLC-DAD) or evaporative light scattering detector (HPLC-ELSD), nuclear magnetic resonance (NMR) and gas-chromatography (GC) coupled to mass spectrometry and/or flame ionization detector (GC-MS/FID).

The use of HPLC has been widely exploited as it is possible to achieve a fast and consistent separation of many components of a mixture, and given the large sample loading compared to TLC, the ease of automation and the hyphenation with a wide array of detectors are advantageous [1,5,6]. Separation of lipids can be achieved either by normal phase (NP) or reverse-phase-liquid chromatography (RP-LC), as depicted in Fig. 1.

NP methods exploit silica, diol or amine-bonded stationary phases in combination with mixtures of hexane/isopropanol/water as mobile phases; under NP-LC lipids are separated according to different polarity of the heads [5,7–9]. On the other hand, RP-based separation on octasilyl (C8)- and octadecylsilyl (C18)- derivatized stationary phases is achieved in combination with solvent systems usually composed of mixtures of methanol, acetonitrile and water. Under RP-based conditions, the separation of membrane lipids mainly relies on the general chemical features of their acyl (or alkyl in plasmalogens) chains such as length, number and position of unsaturation [5,9–13]. For this reason, RP-and NP-based separations are considered complementary because the former is particularly suitable to establish the diversity of molecular lipid species within the same lipid class (intra-class differentiation) whilst the latter can be exploited to establish the total lipid species belonging to different membrane lipids classes in a given sample

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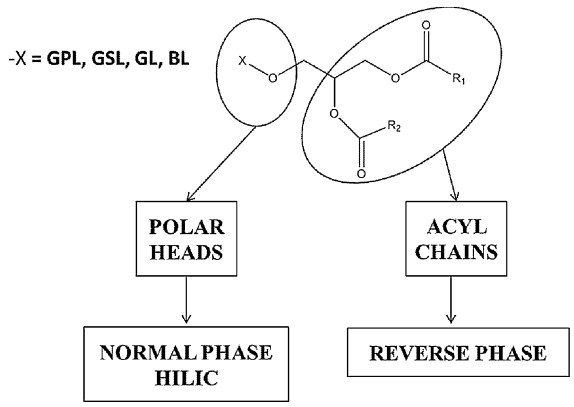

Fig. 1. Chromatographic methods exploited in lipid analysis: lipids can be separated in NP and HILIC according to the different polarity of their heads or in RP according to the hydrophobicity of their acyl chains.

(inter-classes differentiation) [9]. Of course, since the partition processes determining the chromatographic separations are multiple and complex, using an RP-column is often possible to roughly separate (especially when the lipid classes have quite different polarity) not only the lipid species belonging to a given class but also lipid species belonging to different lipid classes [13–15].

In recent years, lipid analysis has benefitted from the development of hydrophilic interaction liquid chromatography (HILIC), which allows one to overcome (i) the scarce reproducibility observed in NP-LC due to the low miscibility of hexane/isopropanol/water solvent systems and (ii) the low ESI compatibility of NP-based mobile phases. The principle of HILIC lipid separation is the same as for NP-LC but with the difference that mobile phases are similar to those used for RP-LC. An HILIC stationary phase is hydrophilic and/or charged, with solvent systems consisting of acetonitrile/methanol/water (by eventual addition of an ion-pairing reagent as volatile buffer). Much of the lipid separation using HILIC chromatography has been done for the separation of glycerophospholipids (GPLs) and glycerosphingolipids (GSLs) classes from various matrices, as tissues, protozoans or milk [16–19].

Bacteria, eukaryotic microorganisms and higher plants are sources of other lipid classes such as the glycolipids (GL) mono- and digalactosyldiacylglycerols (MGDG and DGDG) and sulfoquinovosyldiacylglycerol (SQDG), and the betaine (BL) ether-linked glycerolipids diacylglyceryl-hydroxymethoxy-trimethyl-β-alanine (DGTA), diacylglyceryl-trimethyl-homoserine (DGTS) and diacylglyceryl-carboxymethylcholine (DGCC) [20–26]. These lipids are co-extracted with GPL using common extraction procedures [22,27,28].

In few studies, betaine lipids and glycolipids were separated from GPL using HILIC chromatography in combination with hexane, isopropanol and water solvent systems [21,24] while in plant lipidomics, glycolipids were separated from phospholipids using mixtures of methanol, acetonitrile and water [23,29].

In a recent work, Kind and co-workers developed an HILIC method based on acetonitrile, water, ammonium acetate and acetic acid for the qualitative determination by high resolution-MS of lipids secreted by algae [22]. Lipid classes, mainly GPLs phosphatidylcholine and phosphatidylinositol, the glycolipid SQDG, the betaine DGTS, and triacylglycerols, were separated within 20 min-run time but many species were still coeluted and, therefore, a longer run time would be needed for a clearer separation.

Here we present a simple but detailed diol-based HILIC-ESI-MS method for the separation and detection of fourteen different lipid classes that constitute cellular membranes of various micro- and macrorganisms: 7 GPLs (phosphatidic acid (PA), phosphatidylcholine (PC) and lyso-PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS)), 2 GSL (sphingomyelin (SM) and ceramide CER), 3 GLs (MGDG, DGDG and SQDG) and 2 BLs (DGTS and DGCC). The separation of lipids was tested by using four different columns with acetonitrile, water and ammonium acetate as mobile phases and then further optimized on the column giving the best chromatographic results. Lipid classes were separated within a 35 min-run time, showing a good repeatability for most of lipid classes. The method was further tested on complex lipid extracts, such as the dinoflagellate *Peridinium cinctum*, the green alga *Jaoa bullata* and the berries of *Vitis vinifera*.

2. Materials and methods

2.1. Chemicals

Analytical grade acetonitrile, HPLC grade chloroform and reagent grade methanol were purchased from VWR (VWR International PBI, Milan, Italy); deionized water, filtered at 0.2 μm, was obtained from Elix Water Purification System (Merck Millipore, Billerica, MA, USA). Reagent grade ammonium acetate was purchased from Rudi Pont (Chimica Rudi Pont, Torino, Italy); reagent grade ammonium formate was purchased from Carlo Erba (Carlo Erba Reagents, Milan, Italy); reagent grade acetic acid was purchased from Merck (Merck KGaA, Darmstadt, Germany) and LC-MS grade formic acid was purchased from Fisher Scientific (Fisher Scientific, Illkirch, France).

2.2. Commercial standards

1,2-Dioleoyl-*sn*-glycero-3-phospho-L-serine (PS sodium salt), 1,2-Dioleoyl-*sn*-glycero-3-phosphate (PA sodium salt), L-α-phosphatidylinositol mixture extracted from soy (PI sodium salt), L-α-phosphatidylethanolamine mixture extracted from soy (PE sodium salt), 1,2-Distearoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (PG sodium salt), 1,2-Dilinolenoyl-*sn*-glycero-3-phosphocholine (PC), 1-Myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lyso-PC), N-palmytoyl-D-*erythro*-sphingosylphosphorylcholine (SM), N-lignoceroyl-D-*erythro*-sphingosine (CER) and 1,2-Dipalmitoyl-*sn*-glycero-3-O-4′-(*N,N,N*-trimethyl)-homoserine (DGTS) were purchased from Avanti Polar Lipids (Avanti Polar Lipids Inc., Alabaster, AL, USA). Saturated monogalactosyldiacylglycerols- (MGDG) and digalactosyldiacylglycerols (DGDG) were purchased from Matreya (Matreya LLC, Pleasant Gap, PA, USA).

2.3. Extraction of non-commercially available standard compounds

Sulfoquinovosyldiacylglycerols (SQDG) were isolated from *Codium bursa* raw extract by Solid Phase Extraction (SPE) on a 2 g RP 18 cartridge (Phenomenex, Torrance, CA, USA)using methanol-water 85:15.

The DGCC were isolated from *Peridinium aciculiferum* raw extract by SPE on a 2 g silica cartridge (Phenomenex, Torrance, CA, USA). A gradient elution of hexane/ethyl acetate was used to remove non-polar lipids, as sterols and triacylglycerols, and pigments; then polar lipids were eluted using 45 ml of methanol acidified with 0.1% of formic acid. The fraction was dried under rotary evaporation (Büchi Labortechnik AG, Flawil, Swiss), re-dissolved into chloroform and subsequently injected in HPLC-HILIC-UV system to collect single molecular species. The 1-myristoyl-2-docosahexaenoyl-*sn*-glycero-3-O-diacylglyceryl-carboxymethylcholine was selected as a standard.