Journal of Chromatography A, 1487 (2017) 179–186

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Full length article

Non-target analysis of phospholipid and sphingolipid species in egg yolk using liquid chromatography/triple quadrupole tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 31 August 2016
Received in revised form 18 January 2017
Accepted 22 January 2017
Available online 24 January 2017

Keywords:
Phospholipids
Fatty acyls
Egg yolk
MRM
HPLC–MS/MS

ABSTRACT

In this work, phospholipids extracted from egg yolk (control group, experimental group) were identified using high performance liquid chromatography coupled with electrospray ionization-tandem mass spectrometry (HPLC-ESI–MS/MS). Combinations of fatty acyls occurring in 11 classes of phospholipids from egg yolk were investigated. Differences between the profile of fatty acyls from hens fed traditionally and the ones that received special diet supplementation were observed. Experimental findings were complemented with multivariate chemometric analysis. Multiple reaction monitoring mass spectrometry mode was utilized and 123 distinct combinations of fatty acyls occurring in phospholipids were identified. From these, large portions are polyunsaturated fatty acyls from the omega-3 and omega-6 family. HPLC MS/MS analysis allows for quick, accurate and precise determination of biologically active compounds, found in low concentrations within the tested material.

1. Introduction

Phospholipids (PLs) are basic building blocks of cell membranes omnipresent in all living organisms. At the same time, they have numerous other functions related to signal transduction, transformation of energy, membrane transport, the formation of intracellular cytoplasmic compartments, or cellular metabolism [1,2]. Phospholipids play crucial roles in signaling mechanisms for cell proliferation and cell death and decrease triacylglycerols and cholesterol concentration in the blood [3,4]. Moreover, together with cholesterol, they form a part of the so-called “lipid rafts”, which play an important role in regulation of membrane fluidity, the processes of signal transduction, apoptosis and cell activation in immune reactions [5].

Hen’s egg yolk is a rich source of phospholipids. It contains approximately 31% lipids, out of which about 28.3% are phospholipids. Most widely occurring fractions of phospholipids are phosphatidylcholine (PC, 77%), phosphatidylethanolamine (PE, 18%), sphingomyelin (SM, 3%), and others (2%) [6]. Phosphatidylcholine is a major natural phospholipid. It performs a huge role in constructing and maintaining of cell membranes. Structural and dynamic properties of PC-based membranes can be considerably influenced by its chain length, number and position of double bonds in the acyl chains. PLs are considered a source of arachidonic acid released by phospholipase A2, which is then converted to eicosanoids, prostaglandins, prostacyclin and leukotrienes which are mediators of inflammation [7]. Fatty acyls included in the phospholipids are usually linked by means of an ester bond with glycerol in position sn-1 and sn-2. Carbon chain length influences the characteristics of the fatty acyls, as well as the degree of unsaturation. PL includes both saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) [8]. Phospholipid compounds that are of particular importance are omega-3 (ω-3) and omega-6 (ω-6) fatty acyls. Mammalian organisms are not capable of de novo synthesis of PUFA because they do not possess desaturase, an enzyme responsible for the formation of double bonds in positions of the ω-3 and ω-6. PUFAs must be delivered to a mammalian organism as a food supplement [9,10]. The lower the degree of unsaturation in the chain of PUFAs, the membrane is more packed and stable. Consequently, this has a positive influence on the effectiveness of intracellular metabolism.

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http://dx.doi.org/10.1016/j.chroma.2017.01.055
0021-9673/© 2017 Published by Elsevier B.V.

The commonly used techniques for separation of PLs are chromatography techniques, such as: thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) for separation of methyl fatty acids [11]. HPLC is the most frequent and widely used technique of separation. Phospholipids can be determined by normal phase liquid chromatography (NP-LC) [12,13], reversed-phase liquid chromatography (RP-LC) [14], hydrophilic interaction liquid chromatography (HILIC) [15], and two dimensional liquid chromatography (LC × LC) [16]. Separation in NP-LC and HILIC systems is based on the differences in the polarity of the phospholipid "headgroups". In RP-LC mode, separation mechanism is based on differences in chain lengths, number of double bonds and geometry of acyl chains. In lipidomics research soft ionization techniques, such as electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) have often been used [11,17]. Qualitative analysis can be achieved with two approaches. The first is direct infusion (shotgun analysis) of phospholipids crude extract into ESI–MS, and the second is LC–MS/MS (liquid chromatography-tandem mass spectrometry) in which LC is used for initial separation of analytes, followed by MS/MS [11,18]. The LC–MS/MS approach is more often used, since the combination of HPLC and MS decreases the effect ion suppression of some PLs, generated by the presence of polar contamination [7,19]. The shotgun approach is used in nontargeted analysis using a precursor ion and neutral loss scans for the identification of many lipid classes [20]. Tandem mass spectrometry allows for the study of structure, qualitative, as well as quantitative determination of compounds.

Within the scope of this work, a rapid and effective non-target HPLC–MS/MS method for identification of combination of fatty acyls occurring in phospholipids extracted from hens egg yolk was developed. Analysis was carried out for 2 different extracts (control group, experimental group). Multiple reaction monitoring (MRM) was used for characterization of the fatty acyls composition, especially polyunsaturated fatty acyls, comprising phospholipids. Finally, a qualitative profile of fatty acyls in phosphatidylcholine species present in egg yolk before and after a supplementation diet was determined with using chemometric analysis. For all the analyses, a column working in HILIC mode was used.

2. Materials and methods

2.1. Chemicals

Acetonitrile (HPLC grade) was purchased from Sigma-Aldrich (Steinheim, Germany). Water was obtained running it through the Milli-Q RG apparatus by Millipore (Millipore Intertech, Bedford, MA, USA) in our laboratory. Standards of: L-α-phosphatidylcholine (≥99%) (PC), L-α-phosphatidylethanolamine (≥98%) (PE), sphingomyelin (≥95%) (SM), L-α-lysophosphatidylcholine (≥98%) (LPC), L-α-lysophosphatidylethanolamine (≥97%) (LPE), L-α-phosphatidylglycerol (≥98%) (PG), L-α-lysophosphatidylglycerol (≥98%) (LPG), L-α-phosphatidylinositol (≥98%) (PI), L-α-lysophosphatidylinositol (≥98%) (LPI), phosphatidic acid (≥98%) (PA), and lysophosphatidic acid (≥98%) (LPA) from egg yolk were purchased from Larodan Lipids (Malmö, Sweden). Pure phospholipids fraction from egg yolk [21] was obtained from Wroclaw University of Environmental and Life Sciences.

2.2. HPLC–MS/MS conditions

Separation of phospholipids was performed on a Shimadzu HPLC-ESI–MS/MS 8050 liquid chromatographic system (Tokyo, Japan). The instrument includes a binary solvent delivery system (LC-30 AD), controller (CBM 20A), an autosampler (SIL-30A), and a column thermostat (CTO-20AC). For instrument control, data acquisition and processing LabSolution 5.8 software was used. Individual phospholipids classes were determined by analyzing total ion current chromatograms in both positive and negative ion mode in the *m*/*z* of 400–1000. The optimal parameters of MS were as follows: nebulizing gas flow was 3.0 L min^{-1}, the heating gas flow was 10 L min^{-1}, while the temperature of the drying gas was 400 °C. Characteristic head-group products are summarized in Supplementary info. For quantification of phospholipids, species were monitored in the scheduled multiple reaction monitoring (MRM) mode. The total dwell time was one second. Phospholipids were separated using a Luna HILIC column dimensions of 150 × 4.6 mm and 5 μm with the pore size of 20 nm (Phenomenex, Torrance, CA, USA), flow rate of 0.4 mL min^{-1}, injection volume of 1 μL, separation temperature of 25 °C, and the isocratic mobile phase consisting of 90% of acetonitrile and 10% of water.

2.3. Chemometric analysis

In order to get a deeper insight into the differences in the largest phospholipid fraction: phosphatidylcholine (PC) between the two egg yolk samples principal component analysis (PCA) [22] was employed. Principal components were computed using Singular Value Decomposition (SVD) [23]. For the construction of models, areas under chromatographic peaks belonging to different combinations of fatty acyls within the PC fractions before (F1) and after supplementation diet (F2) were used as variables. Three repetitions of samples F1 and F2 were used as cases. Score, loading and contribution plots were employed for analysis and interpretation of the PCA results. Contributions of distinct combinations of fatty acyls to the type of supplementation were computed using the following equation [24]:

$$SC_k = \left(\bar{\mathbf{x}}_i - \bar{\mathbf{x}}_j\right) \sqrt{\sum_a \left(\frac{\bar{\mathbf{t}}_i - \bar{\mathbf{t}}_j}{s_{\mathbf{t},a}}\right)^2 \mathbf{p}_a{}^2} \qquad (1)$$

where **x** corresponds to an average of cases for a latent variable (a) and a variable of **X**-space (k), whereas **t** corresponds to **X**-space scores, i corresponds to left ("from") observation or group of observations, j corresponds to right ("to") observation or group of observations, s corresponds to standard deviation of scores, and **p** corresponds to **X**-space loadings.

Computations were performed using ProSensus Multivariate 16.08 (ProSensus, Ancaster, ON, Canada) software on a personal computer equipped with an AMD A8–7650 K Radeon R7 processor with a frequency of 3.30 GHz, 14 computing cores (8 CPU, 6 GPU), and 8 GB (6.94 GB usable) of RAM.

3. Results and discussion

3.1. Qualitative aspects of phospholipids

3.1.1. Qualitative characteristics of phospholipids

Phospholipids are amphiphilic molecules that ionize in both positive and negative modes [11]. In the negative ion mode, molecules of PE, LPE, PI, and PS appear on the mass spectrum as deprotonated molecules: $[M-H]^-$. The molecules of PC, LPC, and SM are efficiently analyzed in positive ion mode, wherein the protonated molecules $[M+H]^+$ are generated. In this mode, sodiated adduct $[M+Na]^+$ can also be formed [25,26]. Distinct scan modes used in mass spectrometry allow for detection of particular phospholipid classes and molecules within each class. For detection of PI, PE, LPE, and PS, as well as, PC, LPC, and SM neutral loss, precursor ion scan mode is most frequently used. Ion mode, type of MS/MS scan,