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Lipidomic profiling of dried seahorses by hydrophilic interaction chromatography coupled to mass spectrometry

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ABSTRACT

Dried seahorse is a precious raw food material for cooking soups. In this study, a lipidomics strategy using the techniques of solid-phase extraction (SPE) and hydrophilic interaction chromatography–tandem mass spectrometry (HILIC–QTOF/MS) was developed for extraction, visualization, and quantification of phospholipids in dried seahorses. The parameters of SPE were optimized, and 1 mL of sample and chloroform/methanol (1:2, v/v) were found to be the best loading volume and eluting solvent, respectively. Afterwards, each phospholipid class was successfully separated on a HILIC column and analyzed by mass spectrometry. A total of 50 phospholipid molecular species were identified and determined, including 15 phosphatidylcholines (PCs), 14 phosphatidylethanolamines (PEs), 12 phosphatidylinositols (PIs) and 9 phosphatidylserines (PSs). In comparison to previously methods, this strategy was robust and efficient in extraction, characterization, and determination of phospholipids. The dried seahorse was found to contain large amounts of polyunsaturated fatty acyl phospholipids which are beneficial to human health.

1. Introduction

Seahorse, belonging to the Syngnathidae of Syngnathiformes in the Actinopterygii Class of vertebrate phylum, is a useful component of medicinal foods (Li, Qian, & Kim, 2008). Despite a lack of scientific studies or clinical trial, the collection and consumption of seahorses are intensive, where they are destined for functional food and aquarium market. In China, seahorse is wildly used as an important ingredient of seahorse soup recipe, primarily for the treatment of erectile dysfunction, wheezing, fatigue and caducity, as well as nocturnal enuresis (Lin, Lin, Lu, & Li, 2008). Previous studies on the bioactive components in seahorse had mainly focused on analysis of trace elements and amino acids (Zhang et al., 2003). Nevertheless, the characterization of the phospholipids, which are important health beneficial compounds was neglected. Therefore, this prompts us to analyse the series of bioactive phospholipids (Chen & Liu, 2013; Leng, Kinnun, Shaikh, Wassall, & Feller, 2015) in seahorse.

Phospholipids are the primary structural constituents of biological membranes, possessing important physiological and biological functions, and positive nutritional properties (Chen & Liu, 2013; Cullis & de Kruijff, 1979; Pacetti, Lucci, Boselli, & Frega, 2009). Moreover, some species of phospholipids were serve as potential markers for detecting adulteration (Calvano, Ceglie, Monopoli, & Zambonin, 2012; Calvano, de Ceglie, Aresta, Facchini, & Zambonin, 2013), monitoring food rancidity (Shen, Yang, & Cheung, 2015), and differentiating species (Shen et al., 2012, 2013). However, the molecular structures of phospholipids are quite complicated mainly due to the length of the fatty acid chains and the presence of double bonds in the fatty acyl on the glycerol backbone (Pazos, Iglesias, Maestre, & Medina, 2010). In addition, it should be noted that the UV absorption of phospholipids is quite low due to the lack of chromophores. Therefore, it is a challenge to characterize and determine all the phospholipid species.

The rapid expanding research field, lipidomics, is built on the advances in multiple technologies, such as liquid chromatography (LC), gas chromatography (GC), mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and computational

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol; B&D, Bligh & Dyer; HILIC–QTOF/MS, hydrophilic interaction chromatography–quadruple-time of flight/mass spectrometry.

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methods (Calvano, van der Werf, Sabbatini, & Palmisano, 2015; Chen, Belikova, & Subbaiah, 2012; Chen & Li, 2007; Liu et al., 2014; Schiller et al., 2004). Among them, LC–MS is the most commonly used method for the separation and detection of phospholipids, due to its resolution, sensitivity and reproducibility. For the separation of phospholipids, it is of norms to use normal phase column with a chloroform mobile phase (Boselli, Pacetti, Lucci, & Frega, 2012; Karlsson, Michelsen, Larsen, & Odham, 1996). To avoid toxic organic solvent system, hydrophilic interaction chromatography (HILIC) was developed (Shen & Cheung, 2014). For example, Donato et al. (2011) developed a HILIC–MS/MS method using an Ascentis Express fused-core column to achieve the separation of major phospholipid classes in cow's and donkey's milk. Zhu et al. (2012) used a diol column to separate phospholipids in HILIC mode.

The aim of the present work was to establish a method for the qualitative and quantitative analysis of phospholipids in dried seahorses. With this purpose, the performance of HILIC–MS/MS was evaluated, and the parameters were optimized. The developed method was applied to five different species of dried seahorses.

2. Materials and methods

2.1. Chemicals and solvents

The chemical standards of PC (14:0/14:0), PE (15:0/15:0), PI (16:0/16:0), and PS (14:0/14:0), PA (14:0/14:0) and PG (15:0/15:0) were bought from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and prepared in MeOH/$CHCl_3$ (1:1, v/v) to a final concentration of 1 μg mL^{-1}. Acetonitrile, methanol and formic acid (FA) were chromatographic grade and obtained from Merck (Darmstadt, Germany). High purity water with a resistivity of 18.2 MΩ cm^{-1} was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). Other chemicals and solvents used were of analytical grade (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Sample preparation

Five species of male seahorse, including *Hippocampus histrix* (*H. histrix*), *Hippocampus trimaculatus* (*H. trimaculatus*), *Hippocampus japonicus* (*H. japonicus*), *Hippocampus kelloggi* (*H. kelloggi*), and *Hippocampus spinosissimus* (*H. spinosissimus*) were bought from Tongren Co., Ltd. (HongKong, China), and authenticated by Prof. Zhifeng Zhang (Southwest University for Nationalities). The confirmed samples have been deposited in the Laboratory of Natural Products, City University of Hong Kong (Nos. 357761, 357762, 357763, 357764 and 357765, respectively). The seahorse samples were grounded into powder, and 0.01 g sample powder (particle sizes < 250 μm) was weighted and suspended in 1 mL chloroform/methanol (1:2, v/v) and homogenized vigorously on a vortex. Then, 1.25 mL of chloroform was added, followed by ultrasound assisted extraction for 15 min. Finally, an aliquot of 1.25 mL water was added for the separation of hydrophilic and hydrophobic phases. After centrifuging at 13,400*g* for 5 min, the lower organic phase was collected. The aqueous phase was re-extracted with 2.0 mL of chloroform for another two times as described before. The collected organic phases were combined and evaporated under a flow of nitrogen.

2.3. Solid-phase extraction

The aminopropyl silica (100 mg) was packed into a 1 mL cartridge. Polypropylene were set as frits at the both sides to hold the sorbent. Before use, the cartridge was conditioned with 3 mL hexane and washed with 3 mL chloroform to remove interference. Then, 1 mL of phospholipid standards or crude extract was loaded and passed through the cartridge at a flow rate of 0.5 mL min^{-1}. Subsequently, the cartridge was eluted with 1 mL mixture of chloroform and methanol (1:2, v/v), and the eluent was then analyzed by MALDI–TOF/MS.

2.4. HILIC conditions

Chromatographic analysis was performed on an Agilent series 1100 HPLC instrument (Agilent Technologies, Palo Alto, CA, USA), equipped with a quaternary pump, an autosampler, a vacuum degasser and column compartment. The separation was achieved on an YMC-Triart Diol-HILIC column (250 mm × 4.6 mm; i.d., 3 μm, YMC, Kyoto, Japan). A gradient method was employed with mobile phase A: 10 mM Ammonium formate (AmFm) and 0.2% FA aqueous solution and mobile phase B: acetonitrile with 0.2% FA. The gradient profile was carried out as following: 98% B (initial), 98% B (0–15 min), 98–60% B (15–60 min), 60% B (60–70), 60–98% B (70–80 min). The flow rate, column temperature and injection volume were set at 0.2 mL min^{-1}, 50 °C, and 5 μL, respectively. Before each sample injection, the column was cleaned, readjusted to the initial conditions and equilibrated.

2.5. MS/MS conditions

All mass spectrometric analysis were done on a QqTOF-tandem mass spectrometers (API QSTAR, Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization (ESI) ion source. Instrument control, data acquisition and the processing were performed using the Analyst 1.5.1 software. To obtain maximum sensitivity for identification and detection of phospholipids, the ion source temperature (TEM) was set at 500 °C, and ion spray voltages (IS) were set at −4.5 kV in negative ion mode. Ion source gas1 (GS1) and ion source gas2 (GS2) were used as the drying and nebulizer gases at a back pressure of 35 psi and 30 psi, respectively. Curtain gas (CUR) was 35 psi.

2.6. Statistical analysis

Statistical analysis and the calculation of mean, standard deviation, and level of significance were performed by using Kingsoft WPS software. Principal component analysis (PCA) was applied to determine the main sources of variability present in the data sets and to establish the relation between samples (objects) and phospholipids (variables). The MS data were imported into the statistical software package MarkerView, version 1.2.1 (AB Sciex, Concord, ON, Canada), and the software would look for the difference in the mass fragments (m/z) and the abundance.

3. Results and discussion

3.1. Optimization of SPE conditions

Preconcentration and purification of the target analytes are critical and significant for the performance of the analytical method. A home-made SPE column was packed with aminopropyl silica material and further used for the extracting of the phospholipids. Before extraction, the cartridge was conditioned with 3 mL hexane and washed with 3 mL chloroform to remove interference. The sample loading capacity and the type of elution solvent were evaluated to assess the performance of aminopropyl silica based SPE process, and search for the optimal extraction conditions with a minimum effort and cost.

The loading capacity of SPE was tested using phospholipid standards and real sample by varying the loading volume from 1 to