Journal of Chromatography A, 1432 (2016) 39–48

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Determination of phospholipids in milk using a new phosphodiester stationary phase by liquid chromatography-matrix assisted desorption ionization mass spectrometry

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ARTICLE INFO

Article history:
Received 9 October 2015
Received in revised form 3 December 2015
Accepted 29 December 2015
Available online 4 January 2016

Keywords:
Phosphodiester stationary phase
Phospholipids
Fatty acids
HPLC
MALDI-TOF MS

ABSTRACT

A methodology employing high performance liquid chromatography coupled with matrix-assisted laser desorption and ionization time-of-flight mass spectrometry has been utilized to determine the quality of phospholipid classes. Home-made phosphoester chemically bonded stationary phase containing diol, phosphate and octadecyl groups (Diol-P-C18) has been employed in the separation of polar lipids from milk. Each phospholipid fraction was collected manually and identified by MALDI-TOF MS.

1. Introduction

Milk is a natural source of various mineral compounds which are present there in amounts and proportions that allow their optimal absorption from the gastrointestinal tract into the blood. The main energetic component of milk is a fat, which is distinguished among natural fats for its bioavailability resulting from the ease of emulsification [1]. The importance of milk fat is associated with its very complex composition, characterized by the presence of 400–500 fatty acids [2]. The unique feature of the fat of cow's milk is the presence of short-chain fatty acids, which constitute a source of easily available energy necessary for the functioning of heart, liver, nervous system and muscles. Diverse composition of fatty acids (FA) shows a beneficial effect on human health, for example: anticancer and antimicrobial [3]. In the composition of bovine milk fat omega acids are particularly noteworthy. Bovine milk lipids in 98% consist of triacylglycerols (TAG) and small amounts of phospholipids (PL) (1%), free fatty acids (FFA) (0.1%), cholesterol esters and cholesterol diacylglycerols (DAG) (less than 0.5%) [4]. Phospholipids that represent approximately 1% of milk lipids consist of five fractions of different structure and biological activity [5]. Milk and dairy product contain: phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI), and phosphatidylserine (PS) [6].

Milk phospholipids play an important role in human body. Phospholipids have demonstrated a potentially favorable role in reducing the risk of cardiovascular and cancer disease [1], inflammation and gastrointestinal infection [5], Alzheimer's and Parkinson's diseases as well as diabetes [7,8]. Additionally, PLs decrease the triglycerides and cholesterol concentration in the blood. Sphingolipids, especially sphingomyelin, are fundamental components in the central nervous system [5,9]. Phospholipids are also used in pharmaceutical and food industries to obtain emulsion stability, gelation and heat stability [1,9]. Together with proteins, the phospholipids are mainly situated in the milk fat globule membrane (MFGM). The MFGM plays a significant role in emulsification process of the membrane by amphiphilic properties [5]. Research has shown that the MFGM is a potential therapeutic agent in a number of diseases [10]. It has been shown that one of the proteins contained in the MFGM is a potential anticancer agent in the case of breast cancer cells [11]. The composition and quality of milk depends on different factors. The most important factors are: genetic and environmental conditions, physiological state of animals, stage of lactation, season and feeding [6].

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http://dx.doi.org/10.1016/j.chroma.2015.12.083
0021-9673/

Table 1
Parameters of calibration curves for individual lipid classes.

Lipid class	t_R (min)	Slope	Intercept	R^2	RSD (%)	Accuracy (%)
PI	2.212	7794	104215	0.999	1.93	100.3
PE	2.789	25729	38512	0.9981	2.84	100.4
SM	3.695	20482	27861	0.9981	2.77	98.2
LPC	4.127	23547	17981	0.9994	2.18	100.5
PS	5.007	31699	-30919	0.9995	2.45	98.4
PC	5.824	40534	23012	0.9984	2.03	99.5

Among the most popular methods of phospholipids separation are chromatographic techniques such as liquid chromatography (LC), thin layer chromatography (TLC), and gas chromatography (GC) for separation of fatty acids [5]. High performance liquid chromatography coupled with mass spectrometry (MS) has been used for the characterization and quantification of PL. Soft ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) have been often used in lipidomics [15]. The application of electrospray ionization, is a good solution for the LC–MS analysis, but it has some disadvantages. Ion source produces ions with multiple charge, which allows a reliable results but makes the interpretation of data difficult [16]. Another problem in the application of the ESI is high sensitivity to the salinity of the sample. This problem may be partially solved using MALDI. MALDI is a fast method and only a few nanograms of lipids are enough for MS analysis. Ions created in MALDI are endowed with a single charge, which facilitates interpretation of the data and increases the sensitivity of the system [15]. MALDI is more sensitive than ESI [17].

The aim of this study was to develop a new HPLC-UV method to separate the most abundant classes of phospholipids in food products. The separation mechanism is based on the differences in the polarity of phospholipids "headgroup". The mobile phase used in HILIC-HPLC separation was methanol and water with 0.1% formic acid. It allows to obtain good separation of the PLs classes in short time. The unique nature of this adsorbent is a result of the selected functional groups. Quantitative analysis was performed using MALDI-TOF MS. All the identified fatty acids contain from 10 to 22 carbon atoms and from 1 to 6 double bonds.

2. Experimental

2.1. Materials

Methanol, chloroform (both HPLC grade) and sodium chloride used for the extraction procedure were obtained from Sigma–Aldrich (Steinheim, Germany). SPE cartridges were from Sigma–Aldrich (Steinheim, Germany). Methanol and formic acid (both HPLC gradient grade) for HPLC analyses were purchased from Sigma–Aldrich (Steinheim, Germany). Ultra-pure water was obtained from a Milli-Q water system (Millipore, Bedford, MS, USA) and it was used throughout the study. The standard phosphatidylinositol (≥98%) (PI), phosphatidylserine (≥98%) (PS), phosphatidylcholine (≥99%) (PC), phosphatidylethanolamine (≥97%) (PE), lysophosphatidylcholine (≥98%) (LPC) and sphingomyelin (≥95%) (SM) were obtained from Larodan Lipids (Malmö, Sweden). MALDI matrix 2,5-dihydroxybenzoic acid (DHB) (addition and without addition of 0.1% trifluoroacetic acid in acetonitrile (both high purity for LC–MS) for positive and negative mode, respectively) was obtained at the highest commercially available purity from Fluka Feinchemikalien (Neu-Ulm, Germany; a subsidiary of Sigma–Aldrich) and used as supplied. Ground steel targets (Bruker Daltonik, Bremen, Germany) were used for sample deposition. Two milk samples were analyzed: raw cow's milk purchased at local dairy and powdered milk by a local producer.

2.2. Extraction of lipids

The extraction of the lipid fraction of liquid milk and powdered milk was carried out according to the Folch et al. procedure [18]. Milk samples (2 mL of liquid milk and 2 g of powdered milk) were dissolved in 50 mL of chloroform/methanol (2:1, v/v) mixture and 5 mL of sodium chloride (0.05 M NaCl), respectively. The solutions were shaken for 20 min on a rotary shaker (200 rpm) and centrifuged at 5000 rpm for 20 min. After centrifugation, the lower chloroform layer was removed and the process was repeated adding 35 mL of chloroform to the upper (methanol) phase. The chloroform phases were combined together, evaporated on a Labconco CentriVap DNA concentrator (Kansas City, USA) and stored at −20 °C until analysis.

2.3. Solid phase extraction SPE

Milk lipids extracts (50 mg) were dissolved in 1 mL of chloroform/methanol (95:5, v/v) mixture and cleaned using SPE cartridge containing 500 mg of silica gel (Roth, Germany). The SPE cartridges were first conditioned with 5 mL of chloroform/methanol (95:5, v:v) mixture [19]. Neutral lipids were eluted with 10 mL of chloroform/methanol (95:5, v:v) mixture. Phospholipids were eluted with 5 mL of methanol and 5 mL of chloroform/methanol/water (3:5:2, v/v/v) mixture. The solutions were evaporated at 37 °C and re-dissolved in 1 mL of chloroform/methanol (2:1, v/v) mixture for LC analysis.

2.4. Chromatographic system

Separation of phospholipid classes was accomplished in a Shimadzu Prominence HPLC system (Tokyo, Japan). The absorbance was measured at 206 nm using a diode array detector (SPD-M20A). HPLC instrument consists of a binary solvent delivery system (LC-10AD), an autosampler (SIL-20A), a column thermostat (CTO-10AS VP). Instrument control, data acquisition and processing were performed with LabSolution software for HPLC.

A home-made phosphodiester stationary bonded phase (Diol-P-C18) with pore size 300 Å basis on Kromasil 300 silica gel (Akzo Nobel, Bohus, Sweden) was used [20]. The coverage density of alkyl ligands was 0.47 μmol/m^2, and hydrogen and carbon percentage were 0.729% and 2.114%, respectively. The specific surface of bare silica area (S_{BET}) was 300 m^2 g^{-1}, and the silanol activity of the silica gel was 7.81 μmol/m^2 [21]. Fig. 1 shown structure of stationary bonded phase. The synthesis method and surface characteristic of the stationary phase were presented elsewhere [20]. Stationary phase were packed into 125 × 4.6 mm stainless steel column.

The injection volume was 1 μL. Analyses were performed at 30 °C. Analyses were done using isocratic mobile phase: 90% of methanol and 10% of water with addition 0.1% formic acid with

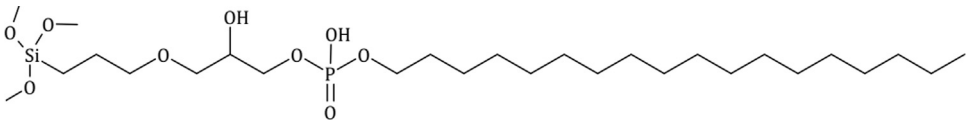

Fig. 1. The structure of stationary bonded phase.