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Short communication

Determination of phosphatidylserine in milk-based nutritional products using online derivatization high-performance liquid chromatography

Qi Lin*, Jie Zhang, Weijie Pei, Chunyan Zhang, Jia Le Yew

Abbott Nutrition Research and Development, 20 Biopolis Way #09-01/02, 138668, Singapore

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ABSTRACT

Phosphatidylserine (PS) has received interest for its ability to improve cognitive abilities and behaviors. A new method for determining PS in milk-based nutritional products has been developed. The method includes a quick and simple sample preparation procedure, followed by high-performance liquid chromatography (HPLC) fluorescence detection (FLD) with an on-line 9-fluorenylmethyloxycarbonyl (FMOC) derivatization. The method allows PS to be determined in raw materials, milk powder and liquid milk products. The day-to-day (n = 3 days) average recovery of over spike-in (at 100% PS content level) was 100%, and the method quantification limit is 53 mg per kg milk powder.

1. Introduction

Phosphatidylserine (PS) belongs to the family of membrane phospholipids (PL) and it is commonly found in the membranes of plants and animals. The structure of PS consists of a glycerol backbone, a polar head group and a hydrophobic moiety. The *sn*-1 and *sn*-2 positions of the glycerol are esterified with a long chain fatty acid, while the *sn*-3 position is linked to a phosphate group, which is connected to a serine molecule. Natural-sourced PS is usually a class of compounds with different fatty acid chains.

Because of its high concentration in the brain, the effects of PS on brain cells have been studied intensively. PS activates protein kinase C [1] and stimulates (Na^+-K^+)-dependent ATPase [2]. In animal studies, PS is found to stimulate the increased release of the neurotransmitter acetylcholine in brain tissues [3]. PS also increases the availability of endogenous choline for *de novo* acetylcholine synthesis and release [4]. This ensures an adequate supply of acetylcholine. In aging rats, it has been shown that there is a reduction in acetylcholine release [5] and protein kinase C activity [6]. PS restores acetylcholine release [5] and protein kinase C activity [7]. Protein kinase C facilitates the release of acetylcholine as well [8]. The reduction of acetylcholine levels in the brain is thought be a possible cause of cognitive deficits in Alzheimer's disease and age associated memory impairment [9]. As such, treatment with PS is believed to play an important role in improving cognitive functions. Clinical studies have demonstrated that treatment with PS has positive effects on the cognitive abilities of the elderly and those with Alzheimer's disease [10,11]. In addition, PS treatment improves cognitive functions in the elderly with memory complaints [12]. Therefore, natural-sourced PS has been fortified in nutritional products (NP) such as milk.

Reported techniques to determine PS in dairy products following a lipid extraction include thin layer chromatography (TLC) [12], ^{31}P-Nuclear Magnetic Resonance (NMR) spectroscopy [13] and high-performance liquid chromatography coupled with an evaporative light scattering detector (HPLC-ELSD) [14]. While TLC is more suitable for qualitative and semi-quantitative purposes [15], ^{31}P-NMR is unsuitable for routine analysis due to its high cost. It has been reported that HPLC-ELSD response curves are typically complex and often sigmoidal as light scattering efficiency changes exponentially with particle size. The complex nature of ELSD response curves can adversely affect quantitation including reproducibility and accuracy (i.e., underestimation at lower analyte concentrations and overestimation at higher concentrations) [16]. The ELSD determination of phospholipids in milk powder has an intermediate precision of more than 10% [14].

* Corresponding author. Tel.: +65 68016342; fax: +65 64789010.
E-mail address: qi.lin@abbott.com (Q. Lin).

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LCMS was also reported for the determination of inherent PS in bovine milk [17]. However, our collaborative study with Avanti Polar Lipids, Inc. regarding LCMS determination of PS suggested that it is not appropriate to use a single PS internal standard (21:0–22:6 PS, for example) and a single PS reference standard (one of the PS analytes) to quantitate the class of various natural PS in milk. Individual PS with different fatty acid chain length showed significant difference in instrumental response. While a $[M-H]^-$/serine fragment (m/z 87) MRM pair was used for mass detection, it was discovered that the 17:0–20:4 PS response is twice that of 21:0–22:6 PS (data not shown).

In this study, we developed a method using HPLC-FLD with an online FMOC derivatization. Other fluorescent reagents such as 1-dimethylaminonapthalene-5-sulfonyl chloride (DNS chloride) [18] and succinimidyl 2-naphtoxyacetate [19] have been used to derivatize PL with amino group such as PS, phosphatidylethanolamine (PE), lyso-phosphatidylserine (Lyso-PS) and lyso-phosphatidylethanolamine (Lyso-PE) and subsequently quantify them in rat brain extract. However, the derivatization process using the above mentioned reagents required a reaction time as long as 3 h, rendering them unsuitable for routine analysis. FMOC is known to be a good quantitative derivatization reagent for primary amine and secondary amine for fluorescence detection and is widely used for amino acid analysis [20]. Therefore, for the first time, the application of FMOC derivatization to the quantification of PS was explored. Because of the high selectivity of fluorescence detection, a simple and quick sample preparation protocol was developed where no time-consuming liquid–liquid phase lipid extraction is needed.

2. Materials and methods

2.1. Reagents and materials

The PS reference standard materials, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (18:1–18:1 PS, purity of >99%), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1–18:1 PE, purity of >99%), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-L-serine (sodium salt) (18:1 Lyso PS, purity of >99%), and L-α-lysophosphatidylethanolamine (Lyso PE mixture, purity of >99%) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, United States). HPLC grade chloroform, methanol, ammonium hydroxide and isopropanol were purchased from Sigma–Aldrich (Singapore). Borate buffer (0.4 M in water, pH 10.2) and 9-fluorenylmethyloxycarbonyl chloride were purchased from Agilent (Singapore) as a kit. The ultrapure water used was purified through a Barnstead Nanopure system (Thermo Scientific, Singapore). PS raw material with a purity of 63% (determined by ^{31}P-NMR) was purchased from Enzymotec, Israel. Samples of milk-based nutritional products (NP) NP A-G were obtained from Abbott Nutrition, a division of Abbott Laboratories, and samples of NP H-L were purchased from local supermarkets. NP A and NP B were natural-PS fortified milk powder and liquid, respectively. NP C-L were milk products without PS fortification.

2.2. Instrumentation

Direct HPLC determination of PS was performed using two Agilent systems: an Agilent model 1200 HPLC system (Agilent Technologies, Singapore) with a model G1321A fluorescence detector (FLD, Agilent Technologies, Singapore) and an Agilent model 1260 HPLC system (Agilent Technologies, Singapore) with a model G1321B fluorescence detector (FLD, Agilent Technologies, Singapore). The FLD excitation wavelength was 266 nm, and the emission wavelength was 305 nm. FMOC-PS derivatization was performed using an Agilent 1329A autosampler (Agilent Technologies, Singapore). The autosampler temperature was maintained at 4 °C with a model G1330B autosampler thermostat. The method used a Zorbax RX-SIL column (5 μm, 4.6 mm × 250 mm; Agilent Technologies, Singapore) and a Zorbax RX-SIL guard column (5 μm, 4.6 mm × 12.5 mm; Agilent Technologies, Singapore). The columns were maintained at 40 °C with a model G1316A thermostatted column compartment (Agilent Technologies, Singapore). The HPLC mobile phase A was a mixture of chloroform/methanol/25% ammonium hydroxide (80/19.5/0.5; v/v/v), and the mobile phase B was a mixture of chloroform/methanol/water/25% ammonium hydroxide (60/34/5.5/0.5; v/v/v/v). The flow rate was 1.0 mL/min. The elution program started at 100% A for 2 min before ramping to 100% B for 14 min, holding at 100% B for 8 min, back to 100% A within 1 min, and finally holding at 100% A for 15 min.

The HPLC autosampler was programmed to perform the online FMOC derivatization of PS (see Table 1).

Table 1
HPLC autosampler program for FMOC derivatization of PS.

Step	Action	Vial [a]	Volume (μL)
1	Draw	1	5
2	Draw	4	3
3	Mix [b]		8
4	Draw	Sample	2
5	Draw	3	0
6	Mix [b]		10
7	Draw	2	4
8	Mix [c]		14
9	Wait 3 min		
10	Inject		

[a] Vial 1: borate buffer solution; vial 2: FMOC reagent; vial 3: rinse solvent (chloroform/methanol, 1:1, v/v); vial 4: isopropanol.
[b] In air; default speed; five times.
[c] In air; default speed; ten times.

2.3. Sample preparation

2.3.1. Powder milk formulation

A total of 0.75 g of milk powder sample was weighed directly into a 25 mL volumetric flask, while 1.5 mL of water was added rapidly to prevent gumming. A total of 9 mL of methanol was also added to the flask. The flask was capped and vortexed immediately for 60 s. A total of 10 mL of chloroform was then added and vortexed intensively for 60 s before diluting to 25 mL with chloroform. The sample was filtered with a 0.45 μm PTFE syringe filter into an HPLC autosampler vial.

2.3.2. Liquid milk formula

A total of 1.5 mL of liquid milk was weighed directly into a 25 mL volumetric flask, and 9 mL of methanol was added to the flask. The flask was capped and vortexed immediately for 60 s. A total of 10 mL of chloroform was then added and vortexed intensively for 60 s before diluting to 25 mL with chloroform. The sample was filtered with a 0.45 μm PTFE syringe filter into an HPLC autosampler vial.

2.3.3. PS raw material

A total of 30 mg of powder sample was weighed directly into a 25 mL volumetric flask, and 1.5 mL of water was added to disperse the powder well. A total of 9 mL of methanol was added to the flask, followed by diluting to volume with chloroform. A total of 1 mL of this solution was diluted to 25 mL with methanol/chloroform (1:1, v/v) and filtered with a 0.45 μm PTFE syringe filter into an HPLC autosampler vial.