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A simple liquid extraction protocol for overcoming the ion suppression of triacylglycerols by phospholipids in liquid chromatography mass spectrometry studies

Pedro Araujo [a,*], Ephrem Tilahun [a], Joar Fjørtoft Breivik [a], Bashir M. Abdulkader [a], Livar Frøyland [a], Yingxu Zeng [b]

[a] National Institute of Nutrition and Seafood Research (NIFES), PO Box 2029 Nordnes, N-5817 Bergen, Norway
[b] Department of Chemistry, University of Bergen, N-5020 Bergen, Norway

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ABSTRACT

It is well-known that triacylglycerol (TAG) ions are suppressed by phospholipid (PL) ions in regiospecific analysis of TAG by mass spectrometry (MS). Hence, it is essential to remove the PL during sample preparation prior to MS analysis. The present article proposes a cost-effective liquid–liquid extraction (LLE) method to remove PL from TAG in different kinds of biological samples by using methanol, hexane and water. High performance thin layer chromatography confirmed the lack of PL in krill oil and salmon liver samples, submitted to the proposed LLE protocol, and liquid chromatography tandem MS confirmed that the identified TAG ions were highly enhanced after implementing the LLE procedure.

1. Introduction

Matrix effects in liquid chromatography mass spectrometry (LC–MS) analysis are generally defined as changes in the ionization efficiency of an analyte by the presence of co-eluting components present in the sample [1]. For instance, the MS signals of triacylglycerols (TAG) are highly suppressed by the presence of co-eluting phospholipids (PL) in the sample. The PL have been labeled as one of the major contributors to matrix effects in LC–MS/MS [2]. However, the exact mechanisms by which matrix components cause ionization suppression are not clear [3]. It has been suggested that the physicochemical properties of the analyte [3,4] and the competition between nonvolatile matrix components and analyte ions for access to the droplet surface for transfer to the gas phase [5] can have an influence on the degree of suppression of ionization. The postulated competition process may decrease or increase the ionization efficiency of targeted analyte ions present at the same concentrations in the electrospray interface [3,5].

The positional distribution of fatty acids on triacylglycerols (TAG) is characteristic for different nutritional products and a valuable indicator for food authenticity [6,7]. In addition, it could be used as a potential biomarker in nutritional interventions [8]. In recent times, several mass spectrometry (MS) and liquid chromatography tandem MS (LC–MS/MS) base techniques for regiospecific analysis of TAG molecules, such as matrix assisted laser desorption ionization time of flight MS (MALDI-TOF-MS) [9–12], electrospray ionization MS (ESI-MS) [13–15], LC atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS) [16,17], LC-ESI-MS/MS [18–28], have been proposed as alternatives to cumbersome and time consuming enzymatic treatments. The main disadvantage of these techniques is that TAG signals are suppressed by phospholipids (PL) [10,28,29]. Therefore, the successful implementation of these techniques for regiospecific analysis will be highly dependent on the effective and a priori removal of the PL from the sample.

Sample treatment methods for separating and isolating neutral and polar lipids have been developed and most of them are based on preparative thin-layer chromatography (TLC) [30–32], solid-phase extraction (SPE) [33,34] and column chromatography [35]. Direct protein precipitation (or in conjunction with SPE), liquid–liquid extraction (LLE) and colloidal silica in combination with anions and cations have been proposed as valuable alternatives for

* Corresponding author.
E-mail address: pedro.araujo@nifes.no (P. Araujo).

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removing PL from different matrices [28,36,37]. Direct analysis of lipid chloroform extract for TAG profiling and quantification through direct infusion has been also reported in the literature [38].

Regardless of its simplicity, preparative TLC is sensitive to sample load and is prone to oxidation of polyunsaturated fatty acids (PUFAs) during the separation process. In addition, manual collection of the lipids from the plates for quantitative analysis generates silica dusts, adds trace contaminants (e.g. silica and fluorescent dyes) and is in general a time and labour consuming process with low yields of scraped lipids which in turn demand repeating the TLC process several times [39]. Over the years, SPE has become a popular technique for isolation and fractionation of lipids due to its simplicity, speed, decreased solvent requirements and low cost [40]. However, its main disadvantages are the lack of reproducibility between commercial cartdriges in addition to the potential co-extraction of contaminants from the cartridges [40–42]. The isolation of lipids by column chromatography requires expensive equipment, copious amounts of solvents and is in general a time-consuming method [35,43,44].

Protein precipitation methods have been reported to lack selectivity due to coelution of endogenous compounds such as PL [36]. TAG and other neutral lipids can be extracted quite selectively into water immiscible organic solvents [2]. However, LLE methods are prone to ion-suppression due to the co-extraction of amphipathic PL along with TAG [45]. For instance, in a recent study five different LLE systems were tested and the presence of both TAG and PL was confirmed in different tested organic phases (e.g chloroform, tert-butyl methyl ether and hexane) [45]. It seems that none of the mainstream methods (SPE, LLE and protein precipitation) can separate PL from the analytes of interest due to the complexity of lipid extracts and the presence of polar and non-polar groups in the PL structures [36].

Instrument base alternatives have been also proposed to eliminate the detrimental effect of PL on TAG signals. For instance, the classical resolution of the sample by HPLC [10,11], the coupling of TLC to MALDI-MS [10] and the separation of components using a silica gel cation exchanger [10,12]. More recently, the use of gold nanoparticle-assisted laser desorption/ionization MS has been recommended as superior strategy for the analysis of TAG directly from crude lipid mixtures with no pretreatment [29].

The present article proposes a LLE system consisting of methanol, hexane and water to remove the PL fraction from biological samples (krill oil and salmon liver) prior to the regiospecific analysis of TAG by LC-ESI-MS/MS. The success of the proposed LLE strategy in eliminating the PL fraction is demonstrated by means of high performance thin layer chromatography (HPTLC) and LC-ESI-MS/MS.

2. Experimental

2.1. Reagents

Chloroform, diethyl ether, methyl acetate, potassium chloride, copper(I) acetate, ortho-phosphoric acid, isohexane, butylated hydroxytoluene (BHT), acetic acid, ammonium acetate ($\geq$98%), hexane and methanol (HPLC grade $>$99.9%) used for LLE and HPTLC were from Merck (Darmstadt, Germany). Isopropanol used for HPTLC and HPLC was from Kemetyl (Norway). Acetonitrile (LC grade, $\geq$99.8%), ammonium acetate (mass spectrometry grade, 99%), acetone and the various standards used for HPTLC analysis including lysophosphatidylcholine (lyso-Ptd-Cho), sphingomyelin (CerPCho), phosphatidylcholine (PtdCho), phosphatidylinositol (PtdIns), phosphatidylethanolamine (PtdEtn), linolenic acid as free fatty acid (FFA), trilinolenin, cholesterol, linolenate cholesteryl, methyl linolenate, monolinoleninglycerol and 1,3-dilinoleinglycerol were from Sigma-Aldrich (St. Louis, MO, USA). Phosphatidylserine (PtdSer), phosphatidic acid (PtdOH), cardiolipin (Ptd_2Gro) standards for HPTLC were from Avanti Polar Lipids (Alabaster, Alabama, US). Linoleyl behenate for HPTLC was from Larodan Fine Chemicals (Malmö, Sweden). L-serine (TLC 99%) was from Sigmma (Steinheim, Germany). De-ionized and purified water in a Milli-Q system was used throughout the experiments (Millipore, Milford, USA). The krill oil (stored at room temperature) was from Neptune Krill Oil (Québec, Canada). The salmon liver (from a wild salmon salar) was kindly donated by Professor Rune Waagbø (NIFES).

2.2. Sample preparation

2.2.1. Krill oil

Pure krill oil (0.1 g) from a commercial capsule was dissolved in chloroform at 5 mg/mL. Reactive charcoal ($\sim$15 mg) was added to remove the astaxanthin, vortex-mixed for 1 min, centrifuged at 4500*g* for 5 min and the clean and bright chloroform phase is collected and designated as total krill oil solution T_K (rich in PL and TAG). Two aliquots of 50 μL of the T_K solution are saved and the remaining solution is dried under a stream of nitrogen before submitting it to the proposed LLE protocol as follows: the dried residue is dissolved in successive 2 ml aliquots of methanol, hexane and water, vortex-mixed for 30 s, centrifuged at 1620*g* for 10 min and the upper hexane layer collected. Aliquots of 2 mL of methanol and 2 mL of water were added into the collected hexane layer, vortex-mixed and centrifuged at 1620*g* for 10 min. After phase separation, the polar phase was saved and the hexane phase washed one more time with successive 2 mL aliquots of methanol and water. The final collected hexane layer, designated as H_K (TAG rich fraction) and the initially saved methanol:water layer designated as M_K (PL rich fraction) were dried under a stream of nitrogen, weighed and redissolved in chloroform at 5 mg/mL. The described procedure was implemented on three capsules of commercial krill oil.

2.2.2. Salmon liver

Salmon liver (0.1 g) weighed in a pyrex test tube was added an equal volume of glass pellets, suspended in chloroform at 5 mg/mL and vortex-mixed 5 times at interval of 1 min, sealed under nitrogen and left at -20 °C overnight. The sample was filtered using a sample processing manifolds (VacMaster, Biotage, Uppsala. Sweden). The filtrate was centrifuged at 4500*g* for 5 min, the bright chloroform phase collected, designated as total salmon liver solution T_S (rich in PL and TAG) and two aliquots (50 μL) of this phase saved for further analysis. The remaining T_S solution was dried under a stream of nitrogen and the residue submitted to the above described LLE protocol for krill oil. The final hexane and methanol:water fractions for salmon liver were designated as H_S (TAG rich fraction) and M_S (PL rich fraction) respectively. The described procedure was performed in triplicate samples from the same liver.

A general diagram of the proposed LLE procedure is presented in Fig. 1.

2.3. LLE protocol evaluation

2.3.1. Lipid classes

The collected fractions from krill oil (T_K, H_K, M_K) and salmon liver (T_S, H_S, M_S) were submitted to HPTLC analyses to determine the lipids classes before (T_K, T_K) and after (H_K, H_S, M_K, M_S) implementing the proposed LLE protocol (Fig. 1). The HPTLC chromatograms should provide information about the amount of PL and TAG in the fractions T_K and T_S, the degree of PL removal from