Talanta 148 (2016) 463–471

Contents lists available at ScienceDirect

Talanta

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

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

ARTICLE INFO

Article history:
Received 13 August 2015
Received in revised form
29 October 2015
Accepted 4 November 2015
Available online 11 November 2015

Keywords:
Triacylglycerols
Signal supression
Regiospecific analysis
Liquid chromatography tandem mass spectrometric
High perfomance thin layer chromatography
Liquid–liquid extraction

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).

http://dx.doi.org/10.1016/j.talanta.2015.11.016
0039-9140/

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

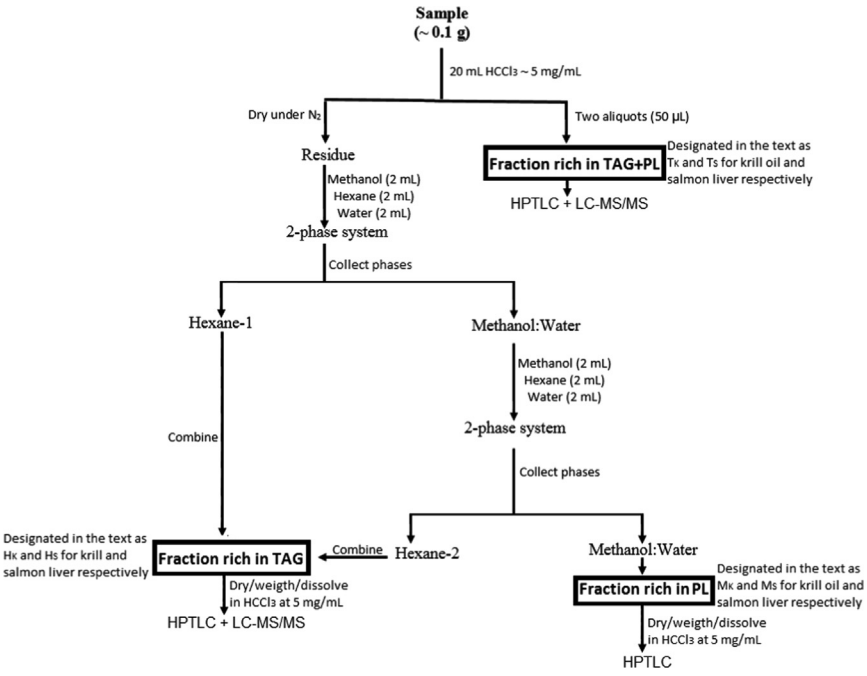

Fig. 1. Proposed liquid–liquid extraction procedure for separating phospholipids (PL) from triacylglycerols (TAG) in biological samples.

the organic phases in fractions H_K and H_S and the degree of TAG removal from the polar phase in fractions M_K and M_S.

The precision of the TAG determination, for krill oil (T_K, H_K) and salmon liver (T_S, H_S) in triplicate ($n=3$), was expressed as coefficient of variation (CV) by dividing the standard deviation (σ) by the averaged TAG concentrations (μ). The estimated levels of TAG in T_K, T_S, H_K and H_S along with the dilution volumes (DV) of the dried samples before and after implementing the LLE protocol will indicate the recovery of TAG by using the expression:

$$\text{Re covery} = \frac{DV_{H_i} \times TAG_{H_i}}{DV_{T_i} \times TAG_{T_i}} \times 100 \quad (1)$$

where the subscripted *i* indicates the amount of TAG derived from the HPTLC chromatograms either from krill oil (H_K, T_K) or salmon liver (H_S, T_S) samples. The term DV_{T_i} was 20 ml (0.1 g sample in 20 mL chloroform to give 5 mg/mL) for both krill and salmon liver, while the term DV_{H_i} was 4.9 ± 0.2 mL for krill oil (24.7 ± 0.8 mg) and 5.5 ± 0.7 ml for salmon liver (27.3 ± 3.3 mg).

2.3.2. Positional distribution of fatty acids on TAG

The krill (T_K, H_K) and salmon liver (T_S, H_S) fractions were submitted to LC–MS/MS analysis to determine qualitative changes in chromatographic behavior after implementing the LLE protocol portrayed in Fig. 1.

The signal ratio after (α) and before (β) submitting the sample to the proposed LLE protocol was computed to determine quantitatively whether the presence of PL overwhelm the signal intensity of TAG in fractions T_K and T_S and also to establish whether the proposed LLE protocol contributes effectively to eliminate any PL suppression effect in fractions H_K and H_S.

2.3.3. Effect of the volume of water on the extraction

Due to the partial miscibility of hexane in methanol, a significant reduction in the volume of the hexane layer (and increasing in the volume of the methanol phase) is visually observed after the addition of water to the samples. The subsequent addition of water increases the methanol polarity, prevents the formation of the azeotropic mixture between hexane:methanol and apparently restates the initial volume of hexane. Based on this observation, the effect of added water to the system hexane:methanol was investigated by submitting the krill oil sample to the LLE protocol at four different volumes of water (0–3 mL in triplicate) and the final volume of hexane phase measured. In addition, the various collected hexane volumes were dried, weighed, dissolved in chloroform, submitted to LC–MS/MS positional analysis and the TAG signal intensity at the different volumes compared statistically.

2.4. Instruments

2.4.1. HPTLC analysis

The various standards used for HPTLC were individually diluted to 0.1 mg/mL by adding chloroform (0.01% BHT). The HPTLC plates 20×10 cm^2, silica 60 were from Merck (Darmstadt, Germany). The plate was pre-cleaned by eluting the polar solution (KCl:methanol: chloroform:isopropanol:methyl acetate, 9:10:25:25:25, v/v) way up to the top of the plate in a 20×10 cm^2 glass tank. The plate was dried and activated in an oven at 110 °C for 30 min. Standards (1 µL) and samples (1 µL) were applied to the plate with a digital microdispenser (ATS4, Camag, Switzerland). Lipids were first eluted with a polar solution in an automatic development chamber (AMD2, Camag, Switzerland) until the elution goes up to 48 mm. After 30 min, the plate was wiped and neutral lipids were further eluted with a neutral solution (isohexane:diethyl ether: acetic acid, 80:20:1.5, v/v) up to 88 mm. The plate was dried for 20 min. After removing the plate from the development chamber, it was dipped into a glass tank containing a solution of 3% copper (I) acetate and 8% ortho-phosphoric acid and developed for about 10 s. The liquid was drained and dried in an oven at 160 °C for 15 min. The plate was cooled at room temperature and scanned by a D_2 lamp (Scanner3, Camag, Switzerland) at 350 nm. The lipid classes in the sample were identified by comparing with the standard band. Concentrations of the chromatographed compounds were determined automatically from the intensity of the absorption via peak areas using winCATS Planar Chromatography Manager version 1.3.3 (Camag, Switzerland). The weight ratio (WR) in mg lipid/g sample units was calculated by the expression:

$$WR = \frac{(y-b) \times f}{a \times w}$$

where y is the corrected area of the absorption peaks, a and b are the slope and intercept of the calibration curve, f is the dilution factor and w is the weighed amount (g). The above-described HPTLC procedure is part of NIFES battery of methods for determining lipid classes in oils, tissue and biological fluids. The precision of the method was lower than 15% of the coefficient of variation (CV = $100 \times \sigma/\mu$), the recovery was between 80% and 105% and the limits of quantification for the various lipid classes were 0.029 μg/μL for phosphatidylinositol; 0.028 μg/μL for phosphatidylethanolamine; 0.026 μg/μL for lysophosphatidylcholine, sphingomyelin and phosphatidylcholine; 0.025 μg/μL for cholesterol and free fatty acids; 0.024 μg/μL for diacylglycerol and triacylglycerol; and 0.022 μg/μL for phosphatidylserine.

2.4.2. LC–MS/MS analysis

An Agilent 1100 series LC/MSD trap, SL model with an electrospray interface, a quaternary pump, degasser, autosampler, thermostatted column compartment, variable-wavelength UV detector and 10 μl injection volume was used. The Zorbax Eclipse-C8 RP 150 × 4.6 mm^2, 5 μm (Agilent Technologies, Palo Alto, CA) was kept in the column compartment at 40 °C. The mobile phase, delivered at 0.8 mL/min consisted of methanol:acetonitrile:water (45:30:25, v/v) (A) and methanol:acetonitrile (40:60, v/v) (B). Both solvents (A and B) contained 2.5 mM ammonium acetate and 10 μM L-serine. The gradient program was as follows: 40% B (0–9 min), ramped to 100% B in 15 min (9–24 min) kept at this concentration for 184 min (24–208 min) and returned to 40% B in 2 min (208–210 min) and kept at 40% B for 5 min (210–215 min). The ESI conditions were: capillary voltage −4000 V, nebulizer gas 45 psi, dry gas 8 L/min and dry temperature 280 °C. The MS conditions were: skimmer 40 V, capillary exit 166 V, octopole 1 and 2 (both in DC) 12 and 2.7 V respectively, octopole RF 200 Vpp, lens 1 and 2 at −5 and −60 V respectively and trap drive 80 V. The parameters of both ESI and ion trap were controlled by the software MSD trap control version 5.3 (Bruker Daltonik, GmbH Inc).

2.5. Data analysis

The Matlab based computational algorithm used for elucidating automatically the distribution of fatty acids on the backbone of TAG is described in detail elsewhere [20]. The total ion chromatograms (TIC) and corresponding tandem mass spectra (TIC+MS/MS) data were exported to netCDF and ASCII files using DataAnalysis for LC/MSD Trap Version 5.3 (Bruker Daltonik, GmbH Inc). The ASCII and netCDF files were then exported to Matlab and submitted to the developed automated TAG prediction algorithm for identification of TAG species along with their respective signal intensity in ion counts per second. The results were finally transferred to and saved in Excel format. The *t*-test and *F*-test at the 95% confidence levels were computed in Microsoft Excel 2013 (15.0.4711.1000).

3. Results and discussion

3.1. Protocol evaluation

3.1.1. Lipid classes before and after LLE

The HPTLC results before submitting the krill oil to LLE (fraction

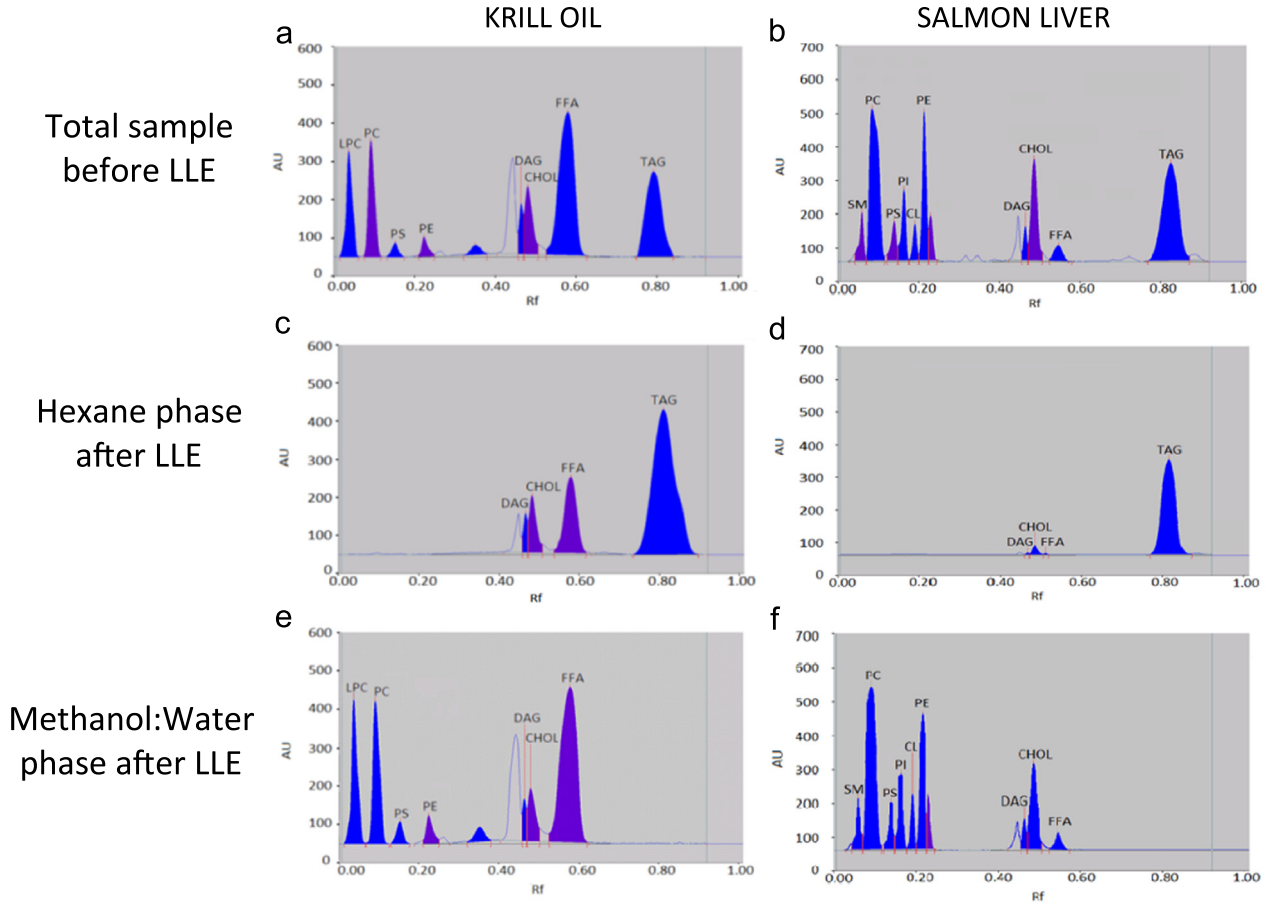

Fig. 2. HPTLC chromatograms before and after submitting the samples to the proposed liquid–liquid extraction (LLE) procedure. The embedded symbols indicate LPC=lysophosphatidylcholine; PC=phosphatidylcholine, PS=phosphatidylserine; PI=phosphatidylinositol, PE=phosphatidylethanolamine; SM=sphingomyelin; CL=cardiolipin, DAG=diacylglycerol; CHOL=cholesterol; FFA=free fatty acid; TAG=triacylglycerol.

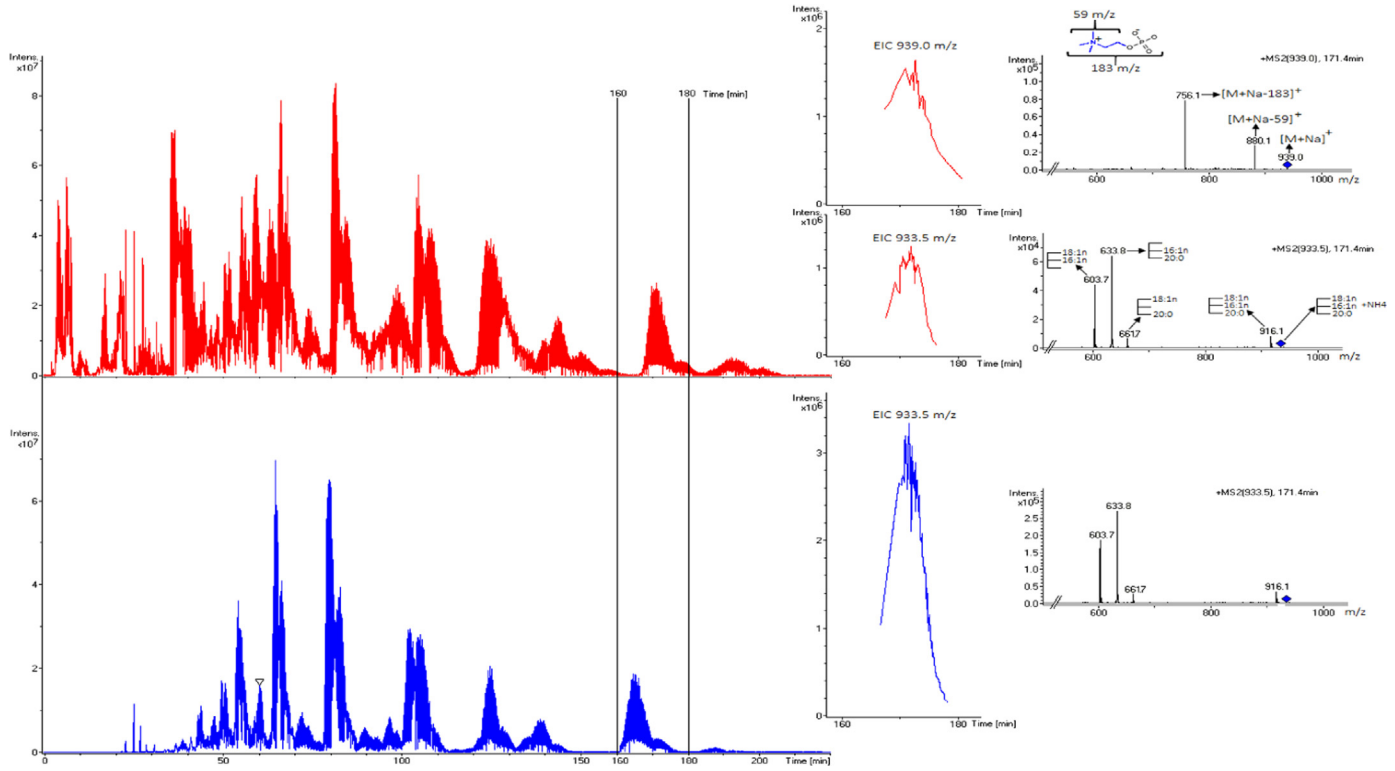

Fig. 3. Total ion chromatograms for (a) T_K (TAG+PL rich fraction) and (b) H_K (TAG rich fraction) exhibiting clear changes in behavior after implementing the proposed LLE protocol. The ammoniated adduct $[TAG+NH_4]^+$ at 933.5 *m/z* was identified as 18:1/16:1*n*/20:0 and was suppressed in T_K due to the coeluting PL sodiated adduct $[PL+Na]^+$ at 939.0 *m/z*. The mass spectra of $[TAG+NH_4]^+$ in T_K and H_K show the direct loss of ammonia at 916.1 *m/z* from 933.5 *m/z*, the preferential formation of diacylglycerol fragments from the loss of fatty acids (18:1 and 20:0) from the *sn*−1 and sn−3 positions at 633.8 and 603.7 *m/z* respectively and the less favorable loss of the central fatty acid (16:1*n*) from the *sn*−2 position (661.7 *m/z*). The $[PL+Na]^+$ adduct shows the characteristic loss of trimethylamine (59 *m/z*) and phosphatydilcholine (183 *m/z*) groups.

T_K) (Fig. 2a) revealed that the highest class corresponds to FFA (1.91 ± 0.15 μg/μL) followed by PL (1.57 ± 0.13 μg/μL), TAG (1.06 ± 0.09 μg/μL), cholesterol (0.29 ± 0.05 μg/μL) and diacylglycerol (0.09 ± 0.00 μg/μL). The PL were distributed as PtdCho (0.70 ± 0.03 μg/μL), lyso-PtdCho (0.68 ± 0.09 μg/μL), PtdEtn (0.11 ± 0.01 μg/μL) and PtdSer (0.08 ± 0.00 μg/μL). The chromatogram for H_K was characterized by the absence of PL and the the main lipid classes in this fraction were TAG (3.62 ± 0.10 μg/μL), FFA (0.91 ± 0.01 μg/μL), cholesterol (0.35 ± 0.04 μg/μL) and diacylglycerol (0.12 ± 0.00 μg/μL) for krill oil (Fig. 2c).

For salmon liver the main lipid classes in the T_S fraction (Fig. 2b) were PL (3.25 ± 0.20 μg/μL), TAG (1.44 ± 0.11 μg/μL), cholesterol (0.47 ± 0.03 μg/μL), FFA (0.10 ± 0.01 μg/μL) and diacylglycerol (0.08 ± 0.01 μg/μL). The PL were distributed as PtdCho (1.85 ± 0.07 μg/μL), PtdEtn (0.73 ± 0.08 μg/μL), PtdIns (0.22 ± 0.02 μg/μL), CerPCho (0.18 ± 0.01 μg/μL), PtdSer (0.16 ± 0.01 μg/μL) and Ptd_2Gro (0.12 ± 0.01 μg/μL).The results for the H_S fraction (Fig. 2d) were distributed as TAG (4.65 ± 0.08 μg/μL), cholesterol (0.23 ± 0.03 μg/μL), FFA (0.05 ± 0.01 μg/μL) and diacylglycerol (0.08 ± 0.01 μg/μL).

The HPTLC chromatograms for krill oil and the salmon liver without and with LLE treatment (Fig. 2) demonstrated that it is possible to remove the observed PL from the T_K and T_S fractions (Fig. 2a and b) by collecting the initial hexane phases from the LLE protocol and washing them two times with methanol and water (H_K and H_S) (Fig. 2c and d). The observed low levels of PL and TAG compared to FFA in krill oil (Fig. 2a) are the result of degradation through the time. It has been reported that inappropriate storage of krill oil can lead to degradation of PL and TAG [19]. In addition, a krill oil manufacturer has mentioned that the presence of FFA and water makes the krill oil prone to hydrolysis [46].

The coefficient of variation and the recovery of the TAG determinations were 2.9–8.8% and 82.3–87.2% for krill oil and 1.7–7.8% and 82.7–98.6% for salmon liver respectively. These values were within the range of precision and accuracy of the HPTLC method.

The HPTLC results for the fractions M_K and M_S from krill oil (Fig. 2e) and salmon liver (Fig. 2f) revealed very low levels of TAG (< 0.02 μg/μL) respectively. These low levels strengthen the argument that most of the TAG were extracted into the hexane phase (Fig. 2c and d). By washing the initial polar phases from krill oil and salmon liver with hexane, the TAG fractions were eliminated and rich PL fractions were obtained (results not shown).

3.1.2. *Positional distribution of fatty acids on TAG before and after LLE*

The positional distribution of fatty acids (FA) on the backbone of TAG of the above analyzed krill oil (T_K and H_K) and salmon liver (T_S and H_S) fractions was determined by submitting the recorded TIC and corresponding tandem mass spectra chromatograms (TIC+MS/MS) to a developed algorithm for prediction of TAG structures [20]. The algorithm in question is based on the well-known fact that during LC–MS/MS analysis the relative abundance of diacylglycerol (DAG) fragments depends on the stereospecific position (*sn*−1, *sn*−2, and *sn*−3) of the FA on the backbone of TAG molecules. For example, it has been reported consistently that fragmentation of $[TAG+NH_4]^+$ and also $[TAG+H]^+$ adducts leads to the preferential formation of DAG fragments from the loss of FA from the *sn*−1 and *sn*−3 positions [13,14,20–22]. The adduct formation from FA at position *sn*−2 (center) is less favorable, probably as consequence of steric hindrance. Hence, the mass intensity signals from the loss of FA at *sn*−1 and *sn*−3 positions are always higher than fatty acids at *sn*−2 position. Some authors have indicated that the less favorable loss of the fatty acid from the center position appears to be universal for all TAG [22].

For krill oil, the comparison between the total ion chromatograms (TIC) for T_K and H_K (Fig. 3a and b) shows that early eluting components in Fig. 3a (fraction T_K) are no longer observed in Fig. 3b (fraction H_K), indicating that the LLE procedure is effectively removing some polar components from the krill oil sample. Comparison of the extracted ion chromatograms (EIC) before and

Table 1
Identified TAG species in krill oil. The equivalent carbon number (ECN) is defined as ECN = CN − 2DB. where CN and DB are the total number of carbon atoms and double bonds respectively. The ratio between the signals after (α) and before (β) implementing the LLE procedure is expressed as average (α/β) ± standard deviation (s) of triplicate samples ($n=3$). The calculated α/β values were compared against a nominal value of 1 to estimate whether removing the phospholipids from the sample has an impact on the intensity of the TAG signals. The Student t was calculated by the expression $t_{\text{calculated}} = \frac{(\alpha/\beta)-1}{s} \times \sqrt{n-1}$ and compared against a ttheoretical value of 4.303 ($p=0.05$. $n=3$).

ECN	sn-1	sn-2	sn-3	α/β	$t_{calculated}$	ECN	sn-1	sn-2	sn-3	α/β	$t_{calculated}$	ECN	sn-1	sn-2	sn-3	α/β	$t_{calculated}$
34	18:4n-3	14:00	18:4n-3	d/nd	×	**40**	16:00	14:00	18:4n-3	3.53 ± 0.04	87.37	**42**	18:1n	16:00	EPA	d/nd	×
34	18:4n-3	14:00	EPA	d/nd	×	**40**	18:4n-3	14:00	18:1n	1.94 ± 0.06	23.17	**42**	16:00	16:1n	18:3n	d/nd	×
34	14:00	18:4n-3	18:4n-3	d/nd	×	**40**	16:00	14:00	EPA	2.10 ± 0.04	37.59	**42**	16:00	18:1n	EPA	d/nd	×
34	14:00	EPA	18:4n-3	d/nd	×	**40**	14:00	16:00	18:4n-3	1.49 ± 0.03	27.78	**42**	16:00	18:4n-3	18:1n	d/nd	×
36	16:4n-3	16:1n	16:1n	d/nd	×	**40**	18:4n-3	16:1n	18:1n	3.36 ± 0.13	25.16	**42**	16:00	EPA	18:1n	d/nd	×
36	16:00	18:4n-3	EPA	d/nd	×	**40**	16:1n	16:2n-4	16:1n	1.98 ± 0.06	24.06	**42**	16:00	18:4n-3	16:00	2.24 ± 0.00	432.71
36	16:00	EPA	18:4n-3	d/nd	×	**40**	18:1n	16:4n-3	18:1n	4.04 ± 0.10	43.26	**42**	18:3n	14:00	18:1n	1.28 ± 0.05	7.12
36	18:4n-3	16:00	18:4n-3	1.73 ± 0.04	29.16	**40**	14:00	18:1n	18:4n-3	2.52 ± 0.10	22.05	**42**	16:00	14:00	18:3n	1.42 ± 0.02	28.57
36	18:4n-3	18:1n	18:4n-3	2.00 ± 0.08	18.47	**40**	16:1n	18:1n	18:4n-3	3.05 ± 0.06	50.48	**42**	14:00	16:00	18:3n	3.24 ± 0.13	24.78
36	16:00	18:4n-3	18:4n-3	1.44 ± 0.08	8.27	**40**	16:4n-3	18:1n	18:1n	1.89 ± 0.08	15.16	**42**	16:2n-4	16:00	16:1n	1.84 ± 0.02	66.51
38	16:1n	16:1n	18:4n-3	3.07 ± 0.03	92.41	**40**	14:00	18:3n	16:1n	1.20 ± 0.01	23.11	**42**	16:1n	16:00	18:3n	2.04 ± 0.04	35.83
38	16:1n	18:4n-3	16:1n	2.24 ± 0.03	51.49	**40**	14:00	18:4n-3	18:1n	3.10 ± 0.10	29.65	**42**	16:00	16:00	18:4n-3	2.24 ± 0.09	19.62
40	18:1n	14:00	EPA	d/nd	×	**40**	14:00	EPA	16:00	1.86 ± 0.08	15.12	**42**	16:00	16:00	EPA	2.99 ± 0.08	35.30
40	16:1n	18:4n-3	18:1n	d/nd	×	**40**	14:00	18:4n-3	16:00	1.25 ± 0.03	10.80	**42**	16:1n	16:1n	16:1n	2.41 ± 0.07	28.91
40	14:00	EPA	18:1n	d/nd	×	**42**	18:4n-3	16:00	18:1n	d/nd	×	**42**	16:2n-4	16:1n	16:00	1.91 ± 0.09	14.47
42	16:1n	16:2n-4	16:00	4.06 ± 0.09	47.25	**44**	14:00	18:1n	16:1n	3.54 ± 0.04	95.94	**46**	16:1n	18:1n	18:1n	2.49 ± 0.08	27.34
42	16:1n	16:2n-4	18:1n	3.23 ± 0.02	158.27	**44**	16:2n-4	18:1n	18:1n	2.04 ± 0.01	107.21	**48**	16:1n	14:00	20:00	1.85 ± 0.03	41.72
42	14:00	18:1n	18:3n	2.52 ± 0.09	23.54	**44**	16:4n-3	18:1n	20:00	2.30 ± 0.06	30.86	**48**	18:1n	14:00	18:00	3.22 ± 0.12	25.30
42	16:00	18:1n	18:4n-3	1.97 ± 0.03	40.79	**44**	16:00	18:1n	18:3n	1.17 ± 0.04	6.35	**48**	18:3n	16:00	20:00	1.89 ± 0.10	13.26
42	18:4n-3	18:1n	18:1n	1.17 ± 0.00	102.00	**44**	16:00	18:3n	18:1n	1.13 ± 0.07	2.75	**48**	16:00	16:00	18:1n	1.28 ± 0.01	31.83
42	14:00	18:3n	16:00	2.05 ± 0.09	16.64	**44**	16:4n-3	20:00	16:00	2.87 ± 0.04	63.51	**48**	18:1n	16:00	18:1n	2.86 ± 0.05	51.83
42	14:00	18:3n	18:1n	1.84 ± 0.05	22.57	**44**	16:4n-3	20:00	18:1n	2.39 ± 0.06	32.42	**48**	14:00	16:1n	20:00	1.28 ± 0.05	7.21
42	16:1n	18:3n	16:00	3.03 ± 0.02	142.13	**46**	18:4n-3	16:00	20:00	d/nd	×	**48**	16:2n-4	16:00	20:00	1.77 ± 0.04	24.46
42	18:1n	18:4n-3	18:1n	2.28 ± 0.08	22.06	**46**	14:00	16:2n-4	20:00	d/nd	×	**48**	16:1n	16:1n	20:00	3.43 ± 0.01	440.75
42	16:00	EPA	16:00	1.84 ± 0.02	67.83	**46**	16:00	18:4n-3	20:00	d/nd	×	**48**	16:00	16:2n-4	20:00	2.25 ± 0.08	22.43
44	16:1n	14:00	18:1n	2.54 ± 0.05	43.54	**46**	14:00	20:00	16:2n-4	d/nd	×	**48**	14:00	18:00	18:1n	1.86 ± 0.02	55.71
44	16:1n	16:00	16:1n	2.90 ± 0.07	38.10	**46**	16:00	20:00	18:4n-3	d/nd	×	**48**	16:00	18:1n	16:00	1.42 ± 0.05	11.65
44	16:4n-3	16:00	20:00	3.94 ± 0.12	34.35	**46**	16:00	14:00	18:1n	1.27 ± 0.03	13.70	**48**	16:00	18:1n	18:1n	3.23 ± 0.06	49.22
44	18:3n	16:00	18:1n	2.94 ± 0.05	55.14	**46**	18:1n	14:00	18:1n	1.88 ± 0.07	17.12	**48**	14:00	18:1n	18:00	2.90 ± 0.11	23.89
44	16:1n	16:1n	18:1n	3.25 ± 0.15	20.95	**46**	16:2n-4	14:00	20:00	1.78 ± 0.02	47.25	**48**	18:1n	18:1n	18:1n	3.04 ± 0.06	49.93
44	14:00	16:1n	18:1n	4.81 ± 0.08	64.86	**46**	14:00	16:00	18:1n	1.96 ± 0.07	18.93	**48**	16:00	18:3n	20:00	1.94 ± 0.05	27.93
44	16:1n	16:1n	16:00	2.50 ± 0.05	43.94	**46**	16:1n	16:00	18:1n	3.39 ± 0.05	72.75	**48**	14:00	20:00	16:1n	1.35 ± 0.04	12.56
44	16:00	16:2n-4	18:1n	6.05 ± 0.03	224.00	**46**	16:00	16:1n	18:1n	1.85 ± 0.04	31.51	**48**	16:1n	20:00	16:1n	1.96 ± 0.05	27.02
44	18:1n	16:2n-4	18:1n	1.40 ± 0.04	13.09	**46**	18:1n	16:1n	18:1n	1.58 ± 0.00	6173.88	**48**	16:00	20:00	18:3n	2.04 ± 0.05	31.81
44	16:00	16:4n-3	20:00	3.42 ± 0.03	103.72	**46**	14:00	18:1n	16:00	1.05 ± 0.04	1.94	**50**	16:00	14:00	20:00	2.43 ± 0.04	49.04
44	16:1n	18:1n	16:1n	5.53 ± 0.21	29.90	**46**	14:00	18:1n	18:1n	1.79 ± 0.03	42.78	**50**	20:1n	14:00	20:1n	1.28 ± 0.03	12.08
44	16:2n-4	18:1n	16:00	5.12 ± 0.16	36.59	**46**	16:1n	18:1n	16:00	4.08 ± 0.13	32.45	**50**	18:1n	14:00	22:1n	1.90 ± 0.06	20.20
50	14:00	16:00	20:00	1.36 ± 0.01	70.40	**50**	14:00	22:1n	18:1n	1.82 ± 0.04	28.11	**52**	18:1n	18:1n	22:1n	3.19 ± 0.02	131.16
50	16:1n	16:00	20:00	1.17 ± 0.02	13.82	**50**	18:1n	20:1n	18:1n	1.64 ± 0.02	50.78	**52**	20:1n	18:1n	20:1n	2.11 ± 0.05	30.84
50	18:1n	16:00	18:00	3.20 ± 0.08	39.60	**50**	18:1n	14:00	20:00	2.11 ± 0.05	32.66	**52**	16:00	20:00	16:00	5.12 ± 0.10	58.17
50	18:1n	16:00	20:1n	1.72 ± 0.06	17.29	**50**	18:1n	18:00	18:1n	3.03 ± 0.01	282.39	**52**	18:1n	20:1n	20:1n	2.38 ± 0.06	34.15
50	16:00	16:1n	20:00	1.31 ± 0.05	8.54	**50**	18:1n	18:1n	18:00	2.65 ± 0.07	34.50	**52**	16:00	22:1n	18:1n	3.19 ± 0.02	189.56
50	18:1n	16:1n	20:00	3.00 ± 0.06	46.28	**50**	14:00	18:1n	22:1n	3.34 ± 0.01	222.76	**52**	18:1n	22:1n	18:1n	2.61 ± 0.06	39.94
50	16:00	18:00	18:1n	1.78 ± 0.03	43.38	**50**	14:00	20:00	18:1n	1.86 ± 0.02	57.87	**54**	18:00	18:00	18:00	2.24 ± 0.05	38.66
50	14:00	18:1n	20:00	2.90 ± 0.11	23.83	**52**	18:1n	16:00	20:00	3.87 ± 0.00	1635.71	**54**	18:1n	18:00	20:00	1.99 ± 0.07	19.63
50	16:1n	18:1n	20:00	2.35 ± 0.01	232.48	**52**	16:00	18:1n	20:00	1.89 ± 0.04	29.76	**54**	20:1n	18:00	20:1n	4.05 ± 0.12	35.05
50	16:00	18:1n	18:00	1.92 ± 0.04	35.04	**52**	18:1n	18:1n	20:00	2.43 ± 0.08	25.08	**54**	18:00	18:1n	20:00	2.84 ± 0.06	45.33
50	16:00	18:1n	20:1n	1.39 ± 0.03	16.36	**52**	16:00	20:00	18:1n	6.39 ± 0.25	30.16	**54**	20:1n	18:1n	20:00	3.26 ± 0.12	25.59
50	18:1n	18:1n	20:1n	2.12 ± 0.05	34.54	**52**	18:1n	20:00	18:1n	1.60 ± 0.06	14.74	**54**	18:1n	20:00	18:00	2.86 ± 0.03	79.29
50	14:00	20:00	16:00	1.74 ± 0.06	17.80	**52**	16:00	16:00	20:00	3.01 ± 0.05	57.29	**54**	18:1n	20:00	20:1n	2.65 ± 0.04	60.54
50	16:1n	20:00	16:00	1.31 ± 0.04	9.88	**52**	18:1n	16:00	22:1n	4.04 ± 0.15	29.44	**54**	18:00	20:1n	20:1n	2.07 ± 0.08	18.74
50	16:1n	20:00	18:1n	3.23 ± 0.05	66.60	**52**	18:1n	18:00	18:00	2.29 ± 0.02	118.56	**54**	18:1n	20:1n	20:00	7.04 ± 0.27	31.82
50	16:00	20:1n	18:1n	2.85 ± 0.12	21.40	**52**	18:00	18:1n	18:00	2.35 ± 0.00	392.45						
50	14:00	20:1n	20:1n	1.55 ± 0.04	17.44	**52**	16:00	18:1n	22:1n	2.35 ± 0.01	318.87						

d/nd= detected in α/non-detected in β

after implementing the proposed LLE (Fig. 3a and b) revealed a significant decrease in the mass signal of some TAG species as a result of coeluting PL. For example, fraction T_K in Fig. 3a showed two types of adducts, namely $[M+NH_4]^+$ and $[M+Na]^+$ eluting at 171.4 min with masses of 933.5 and 939.0 m/z respectively. The ammoniated adduct, identified as TAG (18:1/16:1n/20:0) by the Matlab based computational algorithm, exhibited some $[M+H]^+$ ions at 916.5 m/z which were attributed to the direct loss of ammonia from the precursor ion (Fig. 3a and b). The coeluting sodiated adduct at 939.0 m/z (Fig. 2a) was identified as as PL due to the loss of trimethylamine (59 m/z) and phosphatidylcholine (183 m/z) groups which yield the mass signals of 880.1 and 756.1 m/z respectively (Fig. 3a). After implementing the LLE procedure (H_K fraction), the PL at 939.0 m/z was not longer detected and a considerable increase in the signal of the identified TAG (8:1/16:1n/20:0) was observed (Fig. 3b).

For salmon liver, the comparison between the TIC for fractions T_S and H_S (Supplementary Fig. 1) showed both, the disappearance and the decrease of some chromatographic peaks in the former fraction after implementing the LLE procedure. Some suppressed peaks in T_S (e.g., between 80 and 110 min in Supplementary Fig. 1a) were highly increased in H_S (Supplementary Fig. 1b).

The positional distribution results for krill oil (T_K and H_K) and salmon liver (T_S and H_S) are presented in Tables 1 and 2 respectively. The stereospecific position of the various FA is arranged in increasing number of equivalent carbon number (ECN) and the signal ratio after (α) and before (β) submitting the sample to the proposed LLE protocol is designated as α/β in these tables.

Table 2
Identified TAG species in salmon liver ($n=3$). The symbols, terms and statistical analysis as in Table 1.

ECN	sn-1	sn-2	sn-3	α/β	$t_{calculated}$	ECN	sn-1	sn-2	sn-3	α/β	$t_{calculated}$	ECN	sn-1	sn-2	sn-3	α/β	$t_{calculated}$
38	18:2n	18:2n	EPA	2.03 ± 0.15	9.58	**40**	18:1n	20:4n	20:4n	7.44 ± 0.42	21.81	**40**	20:4n	18:1n	20:4n	1.32 ± 0.12	3.69
38	18:3n	18:1n	EPA	1.62 ± 0.09	9.42	**40**	18:1n	EPA	20:3n	2.01 ± 0.14	10.32	**42**	14:00	18:1n	18:3n	1.50 ± 0.10	6.76
38	18:3n	18:1n	DHA	2.44 ± 0.20	10.31	**40**	18:2n	18:4n-3	18:1n	3.11 ± 0.25	11.80	**42**	14:00	18:2n	18:2n	1.27 ± 0.12	3.34
40	14:00	18:2n	18:3n	2.49 ± 0.19	11.32	**40**	18:2n	18:3n	18:2n	6.98 ± 0.61	13.76	**42**	16.00	18:2n	18:3n	4.78 ± 0.40	13.48
40	16.00	18:4n-3	18:2n	1.85 ± 0.15	8.23	**40**	18:2n	EPA	18:1n	6.44 ± 0.42	18.39	**42**	16.00	18:3n	18:2n	1.65 ± 0.13	6.91
40	16.00	18:3n	18:3n	6.96 ± 0.59	14.27	**40**	18:2n	20:4n	18:2n	2.52 ± 0.20	10.67	**42**	16.00	18:1n	EPA	5.00 ± 0.54	10.39
40	16.00	18:2n	18:4n-3	1.40 ± 0.14	4.02	**40**	18:2n	18:1n	EPA	1.32 ± 0.12	3.64	**42**	16.00	EPA	18:1n	0.98 ± 0.07	-0.39
40	16.00	EPA	18:2n	2.37 ± 0.19	10.03	**40**	18:2n	16.00	DHA	2.49 ± 0.16	13.01	**42**	16.00	18:1n	DHA	2.79 ± 0.17	14.86
40	16.00	DHA	18:2n	3.61 ± 0.37	10.00	**40**	18:3n	16.00	18:3n	2.33 ± 0.16	11.42	**42**	16.00	DHA	18:1n	2.19 ± 0.18	9.53
40	16.00	18:2n	DHA	2.90 ± 0.25	10.69	**40**	18:3n	18:1n	18:3n	1.20 ± 0.11	2.75	**42**	16:1n	16:1n	16:1n	1.60 ± 0.15	5.81
40	16:1n	18:2n	18:3n	3.11 ± 0.27	11.06	**40**	18:3n	18:2n	18:2n	1.56 ± 0.10	7.85	**42**	16:1n	20:4n	18:1n	1.55 ± 0.15	5.36
40	16:1n	18:3n	18:2n	2.34 ± 0.21	8.95	**40**	18:3n	16:1n	18:2n	4.17 ± 0.31	14.26	**42**	18:1n	18:4n-3	18:1n	19.33 ± 1.87	13.85
40	16:1n	18:1n	EPA	2.62 ± 0.24	9.60	**40**	18:3n	18:3n	18:1n	6.87 ± 0.41	20.34	**42**	18:1n	18:2n	20:4n	5.98 ± 0.63	11.27
40	18:1n	18:2n	EPA	9.58 ± 0.76	15.95	**40**	18:3n	18:1n	20:4n	3.52 ± 0.32	10.98	**42**	18:1n	EPA	18:1n	1.82 ± 0.12	9.84
40	18:1n	18:3n	20:4n	1.51 ± 0.11	6.78	**40**	18:3n	20:4n	18:1n	8.26 ± 0.90	11.43	**42**	18:1n	18:1n	EPA	3.28 ± 0.25	12.97
40	18:1n	16:1n	EPA	2.58 ± 0.18	12.20	**40**	18:4n-3	18:1n	18:2n	3.21 ± 0.26	12.03	**42**	18:1n	18:3n	20:3n	2.36 ± 0.15	13.07
40	18:1n	20:3n	EPA	4.10 ± 0.27	16.48	**40**	18:4n-3	20:3n	18:1n	4.32 ± 0.35	13.45	**42**	18:1n	20:3n	20:4n	1.82 ± 0.13	8.82
42	18:1n	20:4n	20:3n	6.32 ± 0.49	15.33	**42**	20:3n	18:2n	20:3n	2.40 ± 0.19	10.17	**44**	18:1n	20:3n	20:3n	3.77 ± 0.25	15.45
42	18:1n	16.00	EPA	4.00 ± 0.34	12.33	**42**	20:4n	18:1n	20:3n	4.24 ± 0.39	11.70	**44**	18:1n	18:00	EPA	2.90 ± 0.28	9.68
42	18:1n	16.00	DHA	2.07 ± 0.19	8.06	**44**	14:00	18:1n	18:2n	3.88 ± 0.32	12.82	**44**	18:1n	18:4n-3	20:1n	1.85 ± 0.15	7.89
42	18:2n	18:3n	18:1n	2.22 ± 0.16	10.62	**44**	14:00	18:2n	18:1n	4.77 ± 0.46	11.53	**44**	18:1n	22:5n	18:1n	3.99 ± 0.32	13.24
42	18:2n	18:1n	20:4n	6.65 ± 0.53	15.00	**44**	14:00	16:1n	18:1n	1.88 ± 0.18	6.86	**44**	18:1n	18:1n	22:5n	1.47 ± 0.12	5.78
42	18:2n	20:3n	18:2n	3.99 ± 0.36	11.68	**44**	16.00	18:2n	18:2n	11.98 ± 0.83	18.70	**44**	18:1n	EPA	18:00	3.97 ± 0.28	14.91
42	18:2n	14:00	18:2n	1.64 ± 0.14	6.69	**44**	16.00	18:3n	16.00	6.79 ± 0.55	14.80	**44**	18:1n	EPA	20:1n	1.95 ± 0.16	8.26
42	18:2n	20:4n	18:1n	6.31 ± 0.42	17.95	**44**	16.00	18:1n	18:3n	1.75 ± 0.17	6.40	**44**	18:1n	20:1n	EPA	1.31 ± 0.11	4.22
42	18:2n	18:2n	20:3n	2.43 ± 0.19	10.60	**44**	16.00	16:1n	18:2n	1.76 ± 0.11	9.36	**44**	18:2n	18:1n	18:2n	4.65 ± 0.31	16.76
42	18:2n	20:3n	20:3n	4.65 ± 0.34	14.97	**44**	16:1n	16:1n	18:1n	4.36 ± 0.33	14.45	**44**	18:2n	18:2n	18:1n	1.41 ± 0.11	5.29
42	18:2n	18:2n	18:2n	9.40 ± 0.49	24.37	**44**	16:1n	14:00	18:1n	1.07 ± 0.09	1.10	**44**	18:2n	18:1n	20:3n	4.73 ± 0.39	13.41
42	18:3n	16.00	18:2n	5.00 ± 0.48	11.70	**44**	16:1n	16.00	16:1n	1.79 ± 0.13	8.71	**44**	18:2n	20:3n	18:1n	4.57 ± 0.40	12.72
42	18:3n	18:1n	18:2n	4.71 ± 0.32	16.20	**44**	18:00	18:1n	EPA	4.05 ± 0.25	17.56	**44**	18:3n	18:1n	18:1n	0.99 ± 0.06	-0.26
42	18:3n	18:2n	18:1n	1.21 ± 0.09	3.32	**44**	18:1n	18:3n	18:1n	8.37 ± 0.58	17.86	**44**	18:4n-3	18:1n	20:1n	1.77 ± 0.13	8.69
42	18:3n	20:3n	18:1n	4.28 ± 0.27	17.43	**44**	18:1n	18:2n	20:3n	5.24 ± 0.38	15.95	**44**	18:4n-3	20:1n	18:1n	2.07 ± 0.15	10.05
42	18:3n	18:3n	20:1n	3.36 ± 0.29	11.54	**44**	18:1n	20:4n	18:1n	4.71 ± 0.33	15.95	**44**	20:3n	18:1n	20:3n	2.89 ± 0.19	14.23
42	18:4n-3	18:1n	18:1n	4.06 ± 0.30	14.51	**44**	18:1n	18:1n	20:4n	3.93 ± 0.29	14.05	**44**	20:4n	18:2n	20:1n	1.26 ± 0.09	3.88
44	EPA	18:1n	20:1n	1.35 ± 0.08	6.30	**48**	16.00	16.00	16.00	1.85 ± 0.14	8.90	**50**	18:1n	18:1n	20:1n	0.90 ± 0.07	-2.08
46	14:00	18:1n	16.00	2.46 ± 0.15	13.76	**48**	16.00	18:1n	16.00	1.15 ± 0.10	2.15	**50**	18:1n	16.00	18:00	3.97 ± 0.30	13.94
46	16.00	18:2n	16.00	9.34 ± 0.92	12.83	**48**	16.00	16.00	18:1n	2.00 ± 0.17	8.27	**50**	18:1n	18:1n	18:00	1.13 ± 0.10	1.73
46	16.00	16:1n	18:1n	4.38 ± 0.26	18.18	**48**	16.00	18:1n	18:1n	0.83 ± 0.07	-3.43	**52**	16.00	18:00	18:00	5.12 ± 0.29	20.19
46	16:1n	18:1n	16.00	6.14 ± 0.60	12.10	**48**	18:1n	16.00	18:1n	3.68 ± 0.21	18.42	**52**	18:00	16.00	18:00	1.82 ± 0.10	11.54
46	18:1n	18:2n	18:1n	0.85 ± 0.06	-3.79	**48**	18:1n	18:2n	20:1n	0.94 ± 0.07	-1.26	**52**	18:00	18:1n	18:00	1.67 ± 0.12	8.16
46	18:1n	20:3n	18:1n	1.74 ± 0.09	12.02	**48**	18:1n	18:00	20:3n	3.29 ± 0.26	12.27	**52**	18:00	18:1n	20:1n	0.84 ± 0.07	-3.42
46	18:1n	18:3n	20:1n	1.16 ± 0.10	2.30	**48**	18:1n	20:3n	20:1n	5.90 ± 0.47	14.63	**52**	18:1n	18:00	18:00	1.21 ± 0.10	2.90
46	18:1n	18:00	20:4n	4.92 ± 0.44	12.57	**48**	18:1n	20:1n	20:3n	3.55 ± 0.28	12.85	**52**	18:1n	18:00	20:1n	5.12 ± 0.34	17.25
46	18:1n	20:4n	18:00	1.29 ± 0.08	4.89	**48**	18:2n	20:1n	18:1n	2.93 ± 0.27	10.14	**52**	18:1n	20:00	18:1n	0.90 ± 0.04	-3.54
46	18:1n	18:1n	20:3n	0.82 ± 0.07	-3.88	**48**	18:2n	18:1n	20:1n	1.63 ± 0.13	6.81	**52**	18:1n	18:1n	22:1n	4.56 ± 0.35	14.26
46	18:1n	20:4n	20:1n	3.83 ± 0.25	16.33	**48**	20:3n	18:1n	20:1n	1.89 ± 0.11	11.53	**52**	18:1n	20:1n	20:1n	3.14 ± 0.27	11.38
46	18:2n	18:1n	18:1n	0.92 ± 0.07	-1.61	**50**	16.00	18:00	16.00	10.93 ± 0.99	14.21	**52**	18:1n	22:1n	18:1n	4.36 ± 0.29	16.61
46	18:2n	18:2n	20:1n	4.96 ± 0.52	10.79	**50**	16.00	18:00	18:1n	5.15 ± 0.41	14.23	**52**	18:1n	18:1n	20:00	2.39 ± 0.15	13.22
46	18:3n	18:1n	20:1n	2.41 ± 0.22	9.13	**50**	16.00	18:1n	18:00	1.38 ± 0.12	4.33	**52**	18:1n	20:1n	18:00	4.40 ± 0.38	12.59
46	18:3n	20:1n	18:1n	7.92 ± 0.59	16.65	**50**	18:1n	18:00	18:1n	3.90 ± 0.31	13.29	**52**	20:1n	18:1n	20:1n	3.57 ± 0.24	15.35
46	20:4n	18:1n	20:1n	3.46 ± 0.28	12.60	**50**	18:1n	20:1n	18:1n	1.44 ± 0.11	5.66	**54**	18:00	18:00	18:00	0.97 ± 0.06	-0.62

Although the stereospecific positions $sn-1$ and $sn-3$ are indistinguishable, the algorithm assigned the FA at these specific positions automatically and they are kept in Tables 1 and 2 for the sake of presentation.

A total of 139 and 160 TAG structures were identified in fractions T_K and H_K from krill oil respectively (Table 1). The α/β ratios, expressed as average and standard deviation for triplicate T_K and H_K samples, were consistently higher than 1 in all the computed cases and ranged from 1.05 to 7.04. The estimated α/β ratios were statistically higher than 1 as reflected from the $t_{calculated}$ values which were larger than 4.303 ($p=0.05$. $n=3$) in 98% of the cases.

The observed discrepancy in Table 1 between the total number of identified TAG structures in T_K and H_K (139 and 160 structures respectively) along with the computed α/β range of 1.05–7.04, demonstrated that 13% of TAG molecules were adversely affected by the presence of PL; that washing the hexane phase with methanol and water brings about a consistent increase in the TAG signals ($\alpha/\beta > 1$); and that the proposed LLE procedure is an effective strategy to remove and prevent the detrimental effect of PL on TAG signals.

For salmon liver, the analysis of the recorded TIC+MS/MS chromatograms revealed the same number of TAG (153 structures) in fractions T_S and H_S (Table 2). A 93% of identified TAG molecules in Table 2 (142 out of 153 structures) revealed an increase in their signals after washing the initial hexane layer with methanol and water as reflected in their ratios $\alpha/\beta > 1$. Remarkable increases ($\alpha/\beta \geq 1.5$) were observed in 80% of identified TAG molecules while 7% of detected TAG molecules were characterized by α/β ratios in the range of $0.82 < \alpha/\beta < 1$. The results for salmon liver show a consistent increase in the majority of the TAG signals after implementing the LLE protocol (Table 2), confirming that washing the hexane phase with methanol and water is a simple an effective strategy for overcoming the suppression of TAG ions by PL ions in MS experiments. The small fraction of molecules (11 out of 153 structures) characterized by α/β ratios in the range of $0.82 < \alpha/\beta < 1$ could indicate a possible decrease in the TAG signals after the proposed washing procedure. However, a t-test ($p=0.05$, $n=3$) revealed no differences between the TAG structures in the aforementioned range and a nominal value of 1, suggesting that the signals for these 11 structures were unaffected by the presence of PL. It must be noted, that the detrimental effect of PL on TAG has been explained as competition for charge by its quaternary ammonium group [10,47]. In this process, the various analyte and matrix ions (formed from charged matrix/analyte clusters) coexist and compete for the limited number of net charges available [47].

Similar suppression differences to those observed in the present research have been reported elsewhere [10,12,47–50]. For instance, equal amounts of PtdEtn and tripalmitin glycerol (PPP) exhibited a peak ratio of 1 regardless of whether they were analyzed as individual or mixed lipids. However, ratios of 1:1 and 5:1 were recorded when PtdCho and PPP were analyzed individually and as mixed lipids respectively [10]. Other pair of individual and mixed lipids, in which PtdCho was present, has been studied with the expected supression in the mixture [10,12,47–50].

3.1.2.1. Effect of the volume of water on the extraction. The result of this study along with a working example showing how the statistical analysis was performed is presented in the Supplementary Table 1 (ST1). The results revealed the same number of identified TAG (160 structures) at the four tested volumes of water (0–3 mL). However, the recorded TAG signals without adding water were consistently lower than their counterpart between 1–3 mL of water (ST1). It was also observed that not adding water to the system hexane:methanol (containing 2 mL of hexane) causes the collection of a reduced volume of hexane phase (~0.6 mL) while adding a volume of water between 1 and 3 mL to the system methanol:hexane allows the collection of the originally added 2 mL of hexane (~1.9 mL was consistently collected in all instances). The TAG signals for 1–3 mL of water (ST1) were compared by means of an F-test at the 95% confidence level (the statistical evaluation is explained at the end of ST1). The results for 0 mL of water were excluded for comparison purposes due to their general low intensity compared to 1–3 mL of water. The statistical comparison revealed that the recorded TAG signals belong to the same population regardless of the added volume of water in the range of 1–3 mL. Based on the lack of statistical difference between TAG signals, a volume of 2 mL of water was selected for the final LLE protocol.

Our findings indicate that published LLE protocols where the hexane phase is extracted against methanol and without adding water (or any other polar solvent) are suspect. For example, Sun and coworkers [51] suggested a LLE protocol, to remove PL from TAG, where three aliquots of hexane (2 ml) were used to wash a methanol:water system containing the sample. After the washing step, the gathered hexane phases (~6 ml) were reduced to 2 ml and back extracted against 2 ml of methanol twice, the collected hexane phase was dried, redissolved and submitted to TAG analysis [51]. We implemented this procedure on a krill oil sample and after submitting the reduced hexane phase (2 ml) to the proposed back extraction with methanol, a paltry volume of hexane volume (0.5 ml) was recovered. No phase separation occurred after performing the second suggested back extraction with methanol. We cannot understand how Sun and collaborators [51] succeeded in preventing the formation of the well-documented methanol:hexane azeotropic mixture after performing twice the methanol extraction. Perhaps, their methanol contained something else (e.g. water?) that helped recovering the 2 ml of hexane. The authors, however, do not provide any indication of the purity or analytical quality of the solvents used in their extraction.

4. Conclusions

The proposed approach to remove PL from TAG in biological samples could be regarded as an important alternative especially in cases where the lack of chromatographic equipments is a limitation for performing analytical or preparative separations. In addition, the proposed protocol enables the regiospecific analysis of TAG by LC–MS/MS without suspicion of ion suppression by PL and in a more simple and economical way when compared to previously reported traditional or sophisticated strategies.

Acknowledgments

ET is grateful for financial support from the Erasmus Mundus programme EMQAL (Grant Number 2008-0095) (European Joint Master in Quality in Analytical Laboratories).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.11.016.

References

[1] L. Tang, P. Kebarle, Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution, Anal. Chem. 65 (1993) 3654–3668.
[2] A.A. Zoerner, S. Batkai, M.T. Suchy, F.M. Gutzki, S. Engeli, J. Jordan, D. Tsikas, Simultaneous UPLC–MS/MS quantification of the endocannabinoids 2-arachidonoyl glycerol (2AG), 1-arachidonoyl glycerol (1AG), and anandamide in human plasma: minimization of matrix-effects, 2AG/1AG isomerization and degradation by toluene solvent extraction, J. Chromatogr. B 883 (2012) 161–171.
[3] P.J. Taylor, Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry, Clin.

Biochem. 38 (2005) 328–334.
[4] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on variability of the electrospray ionization response for model drug compounds, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185.
[5] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, Mechanistic investigation of ionization suppression in electrospray ionization, J. Am. Soc. Mass Spectrom. 11 (2000) 942–950.
[6] A. Schieber, Introduction to food authentication in modern techniques for food authentication, in: D.-W. Sun (Ed.), Modern Techniques for Food Authentication, Academic Press, New York, 2008.
[7] P. Damiani, L. Cossignani, M.S. Simonetti, F. Blasi, T. Petrosino, A. Neri, Identification of cocoa butter equivalents added to cocoa butter. III. Stereospecific analysis of triacylglycerol fraction and some its subfraction, Eur. Food Res. Technol. 223 (2006) 645–648.
[8] P.L. Zock, J.H. de Vries, N.J. de Fouw, M.B. Katan, Positional distribution of fatty acids in dietary triglycerides: effects on fasting blood lipoprotein concentrations in humans, Am. J. Clin. Nutr. 61 (1995) 48–55.
[9] J. Schiller, R. Süss, J. Arnhold, B. Fuchs, J. Lessig, M. Müller, M. Petković, H. Spalteholz, O. Zschörnig, K. Arnold, Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry in lipid and phospholipid research, Prog. Lipid Res. 43 (2004) 449–488.
[10] B. Emerson, J. Gidden Jr, J.O. Lay, B. Durham, A rapid separation technique for overcoming suppression of triacylglycerols by phosphatidylcholine using MALDI-TOF MS, J. Lipid Res. 51 (2010) 2428–2434.
[11] B. Fuchs, J. Schiller, R. Süss, M. Schürenberg, D. Suckau, A direct and simple method of coupling matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) to thin-layer chromatography (TLC) for the analysis of phospholipids from egg yolk, Anal. Bioanal. Chem. 389 (2007) 827–834.
[12] R.A. Johanson, R. Buccafusca, J.N. Quong, M.A. Shaw, G.T. Berry, Phosphatidylcholine removal from brain lipid extracts expands lipid detection and enhances phosphoinositide quantification by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, Anal. Biochem. 362 (2007) 155–167.
[13] C. Cheng, M.L. Gross, E. Pittenauer, Complete structural elucidation of triacylglycerols by tandem sector mass spectrometry, Anal. Chem. 70 (1998) 4417–4426.
[14] L.A. Marzilli, L.B. Fay, F. Dionisi, P. Vouros, Structural characterization of triacylglycerols using electrospray ionization-MSn ion-trap MS, J. Am. Oil Chem. Soc. 80 (2003) 195–202.
[15] K. Shoji, Y. Kazutaka, N. Masatoshi, Rapid discrimination of fatty acid composition in fats and oils by electrospray ionization mass spectrometry, Anal. Sci. 21 (2005) 1457–1465.
[16] C. Beermann, N. Winterling, A. Green, M. Möbius, J. Schmitt, G. Boehm, Comparison of the structures of triacylglycerols from native and transgenic medium-chain fatty acid-enriched rape seed oil by liquid chromatography–atmospheric pressure chemical ionization ion-trap mass spectrometry (LC–APCIITMS), Lipids 42 (2007) 383–394.
[17] P. Sandra, A. Medvedovici, Y. Zhao, F. David, Characterization of triglycerides in vegetable oils by silver-ion packed-column supercritical fluid chromatography coupled to mass spectroscopy with atmospheric pressure chemical ionization and coordination ion spray, J. Chromatogr. A 974 (2002) 231–241.
[18] H. Leskinen, J.P. Suomela, J. Pinta, H. Kallio, Regioisomeric structure determination of alpha- and beta linolenoyl dilinoleoyl glycerol in blackcurrant seed oil by silver ion high-performance liquid chromatography and mass spectrometry, Anal. Chem. 80 (2008) 5788–5793.
[19] P. Araujo, H. Zhu, J.F. Breivik, J.I. Hjelle, Y. Zeng, Determination and structural elucidation of triacylglycerols in krill oil by chromatographic techniques, Lipids 49 (2014) 163–172.
[20] Y. Zeng, P. Araujo, Z.Y. Du, T.T. Nguyen, L. Frøyland, B. Grung, Elucidation of triacylglycerols in cod liver oil by liquid chromatography electrospray tandem ion-trap mass spectrometry, Talanta 82 (2010) 1261–1270.
[21] E. Hvattum, Analysis of triacylglycerols with non-aqueous reversed-phase liquid chromatography and positive ion electrospray tandem mass spectrometry, Rapid Commun. Mass Spectrom. 15 (2001) 187–190.
[22] M. Malone, J.J. Evan, Determining the relative amounts of positional isomers in complex mixtures of triglycerides using reversed-phase high-performance liquid chromatography–tandem mass spectrometry, Lipids 39 (2004) 273–284.
[23] J.M. Rocha, P.J. Kalo, V. Ollilainen, F.X. Malcata, Separation and identification of neutral cereal lipids by normal phase high-performance liquid chromatography using evaporative light-scattering and electrospray mass spectrometry for detection, J. Chromatogr. A 1217 (2010) 3013–3025.
[24] P. Kalo, A. Kemppinen, V. Ollilainen, Determination of triacylglycerols in butterfat by normal-phase HPLC and electrospray-tandem mass spectrometry, Lipids 44 (2009) 169–195.
[25] P. Kalo, A. Kemppinen, V. Ollilainen, A. Kuksis, Regiospecific determination of short-chain triacylglycerols in butterfat by normal-phase HPLC with on-line electrospray-tandem mass spectrometry, Lipids 39 (2004) 915–928.
[26] P.J. Kalo, V. Ollilainen, J.M. Rocha, F.X. Malcata, Identification of molecular species of simple lipids by normal phase liquid chromatography–positive electrospray tandem mass spectrometry and application of developed methods in comprehensive analysis of low erucic acid rapeseed oil lipids, Int. J. Mass Spectrom. 254 (2006) 106–121.
[27] X. Han, R.W. Gross, Quantitative analysis and molecular species fingerprinting of triacylglyceride molecular species directly from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry, Anal. Biochem. 295 (2001) 88–100.
[28] V. Pucci, S. Di Palma, A. Alfieri, F. Bonelli, E. Monteagudo, A novel strategy for reducing phospholipids-based matrix effect in LC-ESI-MS bioanalysis by means of HybridSPE, J. Pharm. Biomed. Anal. 50 (2009) 867–871.
[29] J. Son, G. Lee, S. Cha, Direct analysis of triacylglycerols from crude lipid mixtures by gold nanoparticle-assisted laser desorption/ionization mass spectrometry, J. Am. Soc. Mass Spectrom. 25 (2014) 891–894.
[30] C.A. Demopoulos, S. Antonopoulou, N.K. Andrikopoulos, V.M. Kapoulas, Isolation and complete separation of lipids from natural sources, J. Liquid Chromatogr. Relat. Technol. 19 (1996) 521–535.
[31] J.C. Touchstone, Thin-layer chromatographic procedures for lipid separation, J. Chromatogr. B 671 (1995) 169–195.
[32] B. Fuchs, R. Süß, K. Teuber, M. Eibisch, J. Schiller, Lipid analysis by thin-layer chromatography—a review of the current state, J. Chromatogr. A 1218 (2011) 2754–2774.
[33] M.A. Kaluzny, L.A. Duncan, M.V. Merritt, D.E. Epps, Rapid separation of lipid classes in high yield and purity using bonded phase columns, J. Lipid Res. 26 (1985) 135–140.
[34] J. Giacometti, A. Milošević, Č. Milin, Gas chromatographic determination of fatty acids contained in different lipid classes after their separation by solid-phase extraction, J. Chromatogr. A 976 (2002) 47–54.
[35] C. Hoischen, K. Gura, C. Luge, J. Gumpert, Lipid and fatty acid composition of cytoplasmic membranes from streptomyces hygroscopicus and its stable protoplast-type L form, J. Bacteriol. 179 (1997) 3430–3436.
[36] S. Ahmad, H. Kalra, A. Gupta, B. Raut, A. Hussain, M.A. Rahman, HybridSPE: a novel technique to reduce phospholipid-based matrix effect in LC–ESI-MS Bioanalysis, J. Pharm. Bioallied Sci. 4 (2012) 267–275.
[37] S.T. Wu, D. Schoener, M. Jemal, Plasma phospholipids implicated in the matrix effect observed in liquid chromatography/tandem mass spectrometry bioanalysis: evaluation of the use of colloidal silica in combination with divalent or trivalent cations for the selective removal of phospholipids from plasma, Rapid Commun. Mass Spectrom. 22 (2008) 2873–2881.
[38] X. Han, H. Cheng, D.J. Mancuso, R.W. Gross, Caloric restriction results in phospholipid depletion, membrane remodeling, and triacylglycerol accumulation in murine myocardium, Biochemistry 43 (2004) 15584–15594.
[39] E. Kofroňová, J. Cvačka, V. Vrkoslav, R. Hanus, P. Jiroš, J. Kindl, O. Hovorka, I. Valterová, A comparison of HPLC/APCI-MS and MALDI-MS for characterising triacylglycerols in insects: species-specific composition of lipids in the fat bodies of bumblebee males, J. Chromatogr. B 877 (2009) 3878–3884.
[40] S.E. Ebeler, J.D. Ebeler, SPE methodologies for the separation of lipids, INFORM 7 (1996) 1094–1103.
[41] G.A. Junk, M.J. Avery, J.J. Richard, Interferences in solid-phase extraction using C-18 bonded porous silica cartridges, Anal. Chem. 60 (1988) 1347–1350.
[42] X.W. Crowley, V. Murugaiah, A. Nairn, R.W. Giese, Masking as a mechanism for evaporative loss of trace analyte especially after solid-phase extraction, J. Chromatogr. A 699 (1995) 395–402.
[43] G. Rouser, C. Kritchevsky, A. Yamamoto, Column chromatography and associated procedures for peparation and determination of phosphatides glycolipids, in: G.V. Marinetti (Ed.), Lipid Chromatographic Analysis, Marcel Dekker Inc., New York, 1967.
[44] I. Brondz, Development of fatty acid analysis by high-performance liquid chromatography gas chromatography and related techniques, Anal. Chim. Acta 465 (2002) 1–37.
[45] A. Reis, A. Rudnitskaya, G.J. Blackburn, N.M. Fauzi, A.R. Pitt, C.M. Spickett, A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL, J. Lipid Res. 54 (2013) 1812–1824.
[46] N. Hoem, Composition of Antarctic krill oil and methods for its harvesting, production and qualitative and quantitative analysis, in: Proceedings of Australasian Section of the American Oil Chemists' Society (AAOCS) Biennial Conference. ⟨http://www.aocs.org/Meetings/ConferenceArchivesDetail.cfm?ItemNumber=19156⟩, 2013 (accessed May 2015).
[47] X. Lou, J.L.J. van Dongen, J.A.J.M. Vekemans, E.W. Meijer, Matrix suppression and analyte suppression effects of quaternary ammonium salts in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: an investigation of suppression mechanism, Rapid Commun. Mass Spectrom. 23 (2009) 3077–3082.
[48] M. Petkovic, J. Schiller, M. Muller, S. Benard, S. Reichl, K. Arnold, J. Arnhold, Detection of individual phospholipids in lipid mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: phosphatidylcholine prevents the detection of further species, Anal. Biochem. 289 (2001) 202–216.
[49] B. Fuchs, J. Schiller, R. Süss, M. Schuerenberg, D. Suckau, A direct and simple method of coupling matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) to thin-layer chromatography (TLC) for the analysis of phospholipids from egg yolk, Anal. Bioanal. Chem. 389 (2007) 827–834.
[50] B. Fuchs, J. Schiller, R. Süss, M. Zscharnack, A. Bader, P. Mueller, M. Schuerenberg, M. Becker, D. Suckau, Analysis of stem cell lipids by offline HPTLC-MALDI-TOF MS, Anal. Bioanal. Chem. 392 (2008) 849–860.
[51] G. Sun, K. Yang, Z. Zhao, S. Guan, X. Han, R.W. Gross, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis of cellular glycerophospholipids enabled by multiplexed solvent dependent analyte-matrix interactions, Anal. Chem. 80 (2008) 7576–7585.