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Elucidation of triacylglycerols in cod liver oil by liquid chromatography electrospray tandem ion-trap mass spectrometry

Ying-Xu Zeng[a,b,c], Pedro Araujo[a,*], Zhen-Yu Du[a], Thu-Thao Nguyen[a], Livar Frøyland[a], Bjørn Grung[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-5009 Bergen, Norway
[c] Faculty of Sciences and Technology, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

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ABSTRACT

Though liquid chromatography electrospray tandem mass spectrometry (LC–ESI-MS^2) has been widely used in the structural elucidation of triacylglycerols (TAG) in vegetable oils, its potentiality for the identification of TAG molecules in omega-3 rich oils remains unexplored till date. Hence, this article investigates the applicability of LC–ESI-MS^2 for the structural characterization of naturally occurring TAG in cod liver oil without the TAG fractionation during the sample preparation. A computational algorithm was developed to automatically interpret the mass spectra and elucidate the TAG structures respectively. The results were compared against the lipase benchmark method. A principal component analysis study revealed that it is possible to discriminate genuine from adulterated cod liver oil.

1. Introduction

Cod liver oil has attracted extensive interests due to the scientific evidence and consumer awareness of its nutritional advantages attributed to the abundant content of omega-3 (ω-3) fatty acids (FAs) such as eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) present in the form of triacylglycerols (TAG) [1–5].

Cod liver oil mainly contains TAG consisting of various esterified FAs at the three available stereospecific positions (*sn*-1, *sn*-2 and *sn*-3) of a glycerol molecule. Analysis of TAG in ω-3 rich oils is quite challenging due to the presence of a large number of positional and structural TAG isomers with very similar chemical and physical properties. Traditional chemical/enzymatic hydrolysis methods (Grignard reagent or lipases) [6–11] and sophisticated high resolution nuclear magnetic resonance spectrometry methods (^{13}C NMR or ^{1}H NMR) [12–14] have been used for the stereospecific analysis of TAG in ω-3 rich oils. In general, the titles of published articles on the analysis of TAG in ω-3 rich oils by these approaches seem to imply the elucidation of TAG structures. However, a close inspection of these articles demonstrated that they cannot provide any information regarding the structural elucidation of intact TAG not to mention positional isomers. Instead, they are mainly concerned with the quantification of the "total amount" of individual FAs at *sn*-1, *sn*-2 and *sn*-3 spatial positions. For instance, chemical hydrolysis [11], ^{13}C NMR [13] and ^{1}H NMR [14] have been implemented in the analysis of different fish oils (e.g. cod liver oil) for determining the amounts of esterified FAs at *sn*-1, *sn*-2 and *sn*-3, however the exact position of the various FAs on the backbone of the glycerol molecules was not determined. Traditional hydrolysis methods are characterized by laborious and time-consuming sample preparation protocols such as the cleavage of one or two FAs from intact TAG in order to produce the monoacylglycerols (MAG) or diacylglycerols (DAG); multiple extractions of the various free FAs, MAG or DAG; methylation of the various fractions prior to gas chromatography (GC); derivatization of the MAG and DAG fractions prior to high-performance liquid chromatography (HPLC) [6–11]. In addition, these steps are not always applicable since they are often accompanied by problems such as restrictions due to the intrinsic characteristics of the lipase, inaccuracies due to the incidence of acyl migration and hydrolysis selectivity [15–18]. Sophisticated NMR methods are affected by the presence of strongly overlapping signals, and the effect on chemical shift of the neighboring chains which in turn affect the carbonyl region by preventing the extraction of any qualitative or quantitative information in this region and rendering the C2 region (signal relative

* Corresponding author. Tel.: +47 95285039; fax: +47 55905299.
E-mail address: Pedro.Araujo@nifes.no (P. Araujo).

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to *sn*-2 position) unsuitable for the analysis of FAs composition [19,20].

The structural elucidation of the exact positioning of the various FAs on the glycerol molecules is essential for understanding the physiology of food processing. It has been demonstrated that FAs at *sn*-1 and *sn*-3 of the TAG are hydrolyzed during digestion and absorption of dietary oils while FAs at the *sn*-2 position remain intact [21]. Numerous studies have also shown that the positioning of FAs on the backbone of TAG molecules could affect many lipid properties such as physical and nutritional properties, oxidative stability, lipid absorption, metabolism and atherogenesis [21–24]. In addition, the determination of the stereospecific positioning of FAs on TAG (especially those at *sn*-2) could help to evaluate the quality and authenticity of nutritional ω-3 rich oils such as cod liver oil. Nowadays, the worldwide growing popularity of edible fish and ω-3 rich oils is acknowledged in rich and poor nations where they are making newspaper headlines due to their associated health benefits and also their adulteration [25,26]. For instance, the newspaper with the widest circulation in United States has recently regarded fish as the most frequently adulterated food in America [25]. In addition, it should be mentioned that the importance of developing techniques aiming at detecting adulteration of fish oils has been emphasized since the late 19th early 20th century when a great scarcity of cod liver oil accompanied by famine prices of the market brought about adulteration of genuine cod liver oil with low-grade shark oil [27,28].

For these reasons, national and international organisations have encouraged and supported the development of reliable methods for the analysis of ω-3 rich oils, such as cod liver oil, not only with the capacity to characterize quantitatively the FAs on the glycerol backbone but also to elucidate qualitatively the structures of intact TAG. The combination of these quantitative and qualitative results will assist in gaining a better knowledge of their various properties, nutritional values, commercial quality and the involvement of specific chemical structures in different human and animal physiological processes [29,30].

Several instrumental techniques such as GC, HPLC, silverion HPLC with mass spectrometry (MS), HPLC with fast atom bombardment-MS (FAB-MS), have been used for elucidating the structures of intact TAG in dietary ω-3 rich oils [31–33]. However, the commonly persistent limitation is the exclusive elucidation of TAG structures that can be resolved by chromatographic means and matched to commercially available TAG reference standards [33]. Such a limitation becomes a serious problem for the elucidation of TAG structures in ω-3 rich oils due the complexity of their naturally occurring TAG species. Other problems associated with these instrumental techniques are the tedious sample preparation protocols and the application of complex mathematical equations and models based on the specialized theories for identification purpose [31,32,34].

Liquid chromatography electrospray tandem MS (LC–ESI-MS^2) has been effectively used in the elucidation of TAG structures in a range of simple plant oils [35–39]. However, it is surprising the current literature on the elucidation of TAG structures in ω-3 rich oils has ignored its potentiality. The reason behind this lack of interest could be the enormous amount of time required by manual data analysis of the very complex chromatograms characteristic of ω-3 rich oils. It can be foreseen that the application of LC–ESI-MS^2 in conjunction with the automation of the interpretation process might offer a powerful means for elucidating TAG structures in cod liver oil.

The objective of the present study is to explore the capability of LC–ESI-MS^2 to identify the relative arrangement of the acyl groups on intact TAG molecules in cod liver oil. By using the basic structural features of a TAG molecule and its fragmentation mechanism, a computational algorithm is developed to assist the interpretation and prediction processes. The elucidated spatial positioning of the various acyl groups by LC–ESI-MS^2 was compared against the well-established lipase method. To our knowledge, this is the first study on structural elucidation of TAG molecules present in cod liver oil by LC–ESI-MS^2.

2. Experimental

2.1. Materials and reagents

1-Arachidin-2-Olein-3-Palmitin-glycerol (AOP), 1-Arachidin-2-Palmitin-3-Olein-glycerol (APO), 1-Palmitin-2-Arachidin-3-Olein-glycerol (PAO), 1-Arachidin-2-Linolein-3-Olein-glycerol (ALO), and 1-Palmitin-2-Olein-3-Linolein-glycerol (POL) were from Larodan Fine Chemicals (Malmö, Sweden). 1,2,3-α-Linolenoyl-glycerol (LnLnLn) and butylated hydroxytoluene (BHT) were from Sigma–Aldrich Corporation (St. Louis, MO, USA). Mixtures of the TAG standards were prepared in a chloroform:methanol (2:1, v/v) solution. Cod liver oil was from Peter Möller (Lysaker, Norway). Linseed and rapeseed oils were from Kinsarvik Naturkost (Bergen, Norway), soy oil was from Mills DA (Sofienberg, Norway) and seal oil was from Rieber Skinn A/S (Bergen, Norway). All solvents were HPLC grade. Lipase from *Rhizopus arrhizus* was obtained from Sigma–Aldrich (Schnelldorf, Germany). Fatty acid methyl ester (FAME) pure standards and also model mixture standards 2A and 2B ($C_{18:0}$, $C_{18:1n-9}$, $C_{18:2n-6}$, $C_{18:3n-3}$, $C_{20:4n-6}$), 3A ($C_{18:2n-6}$, $C_{18:3n-3}$, $C_{20:4n-6}$, $C_{22:6n-3}$), 4A ($C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$), 6A ($C_{16:0}$, $C_{18:0}$, $C_{20:0}$, $C_{22:0}$, $C_{24:0}$), 7A ($C_{16:1n-7}$, $C_{16:1n-9}$, $C_{20:1n-9}$, $C_{22:1n-11}$, $C_{24:1n-9}$) and 14A ($C_{13:0}$, $C_{15:0}$, $C_{17:0}$, $C_{19:0}$, $C_{21:0}$) were purchased from Nu-Chek Prep (Elysian, MN). Nonadecanoic acid methyl ester ($C_{19:0}$) internal standard and formic acid were from Fluka (Buchs, Switzerland).

2.2. Sample protocols

2.2.1. Lipase method

The protocol was slightly modified from the procedure described elsewhere [40]. Briefly, 1 ml of Tris–HCl buffer (40 mM, pH 7.2) containing 50 mM of sodium borate was added to a nitrogen-dried oil sample (1 ml) and the mixture sonicated for 10 min. 60 μl of lipase (150 units) were added to the sonicated mixture and incubated at 22 °C for up to 60 min with continuous shaking. The reaction was stopped by adding 0.8 ml of acetic acid (0.1 M) and the total lipids exacted by adding 3 ml of chloroform/methanol (2:1, v/v). The lipid solution was divided into two equal portions (I and II), dried under nitrogen and methylated for 30 and 2 min at room temperature and in a microwave oven by using 1 ml methanolic solutions of NaOH (0.1 N) and HCl (0.2 N) for portion I and II respectively. The FAME in each methylation reactor were extracted into hexane after the addition of 0.2 ml of water to the reaction mixture. The hexane extracts of the NaOH reaction were washed once with water to remove any trace of NaOH before drying under nitrogen. The dried FAME extracts were redissolved in hexane and analyzed by GC. The FAME were estimated quantitatively by using $C_{19:0}$ internal standard. The lipase method was also applied to the TAG standards dissolved in chloroform:methanol (2:1, v/v). It must be mentioned that the acidic reaction allows the methylation of both DAG and FAs generated by the lipase procedure, while the basic reaction allows exclusively the methylation of DAG. The difference between both methylations (acidic and basic) will indicate which particular FAs were released from the *sn*-2 position and consequently those in the terminal positions. The calculation, the positional distribution determination and the data enhancement were based on a protocol described in the literature [40].

2.2.2. Sample preparation for LC–ESI-MS^2 analysis

An aliquot of cod liver oil (2 ml) was dissolved in 2 ml of chloroform:methanol (2:1, v/v), 2 ml of hexane and vortex-mixed for 30 s. The hexane phase was collected and dried under a gentle stream of nitrogen at room temperature. The dried residue was redissolved into 0.5 ml of acetonitrile:acetone (2:1, v/v). The final product was submitted to LC–ESI-MS^2 analysis. This procedure was also applied to TAG standards dissolved in chloroform:methanol (2:1, v/v).

2.3. Instrumentation

2.3.1. Gas chromatography

The GC analysis of the FAME prepared by the lipase method was performed on a Perkin-Elmer AutoSystem XL gas chromatograph (Perkin-Elmer, Norwalk, Connecticut) equipped with a liquid autosampler and a flame ionization detector. The FAME samples were analyzed on a CP-Sil 88 capillary column (50 m × 0.32 mm i.d. 0.2 μm film thickness, Varian, Courtaboeuf, France). Data collection was performed by the Perkin-Elmer TotalChrom Data System Software version 6.3. The temperature program was as follows: the oven temperature was held at 60 °C for 1 min, ramped to 160 °C at 25 °C/min, held at 160 °C for 28 min, ramped to 190 °C at 25 °C/min, held at 190 °C for 17 min, ramped to 220 °C at 25 °C/min and finally held at 220 °C for 10 min. Direct on-column injection was used. The injector port temperature was ramped instantaneously from 50 to 250 °C and the detector temperature was 250 °C. The carrier gas was ultra-pure helium at a pressure of 82 kPa. The analysis time was 60 min. This time interval was sufficient to detect FAME with chains from 10 to 24 carbons in length. The FAME peaks were identified by comparison of their retention times with the retention times of highly purified FAME standards.

2.3.2. Liquid chromatography ion-trap mass spectrometry

The LC–ESI-MS^2 used in this study was 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. The reversed phase Ultrasphere® 5 μm Spherical 80 Å pore C-18 analytical column (250 mm × 4.6 mm i.d., Beckman Coulter, Kolbotn, Norway) was kept in the column compartment at 30 °C and the solvent system in gradient mode consisted of isopropanol: (10 mM) ammonium acetate (90:10, v/v) (A), acetone (B) and acetonitrile (C) at a flow rate of 0.8 ml/min and UV detection at 254 nm. After testing different delivered LC solvent programs, the following gradient was selected: an initial 5 min condition 90% A and 10% C that was ramped in 5 min to 65% A and 5% C and returned to the initial condition in 15 min and subsequently ramped in 5 min to 65% A and 5% C and returned to the initial condition in 30 min where it was held for 30 min.

By using this gradient program, reproducible retention times and peak areas from sample to sample were monitored. Nitrogen was used as nebulizing (50 psi) and drying gas (8 l/min) at 350 °C. The ESI source was operated in positive ion mode and the ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option with a resolution of 13000 m/z/s (FWHM/m/z = 0.6–0.7). Auto MS/MS full scan mode for 90 min in the scan range of 200–1500 m/z without dividing the acquisition program into time segments was used. The most intense ions eluting in each of the ESI-MS spectrum are automatically selected as the precursor ions for the following auto MS/MS experiments using helium as the collision gas. The product ions in ESI-MS^2 spectra are recorded and the resulting MS^2 chromatograms represent the sums of product ions from the precursor ions. Complete system control, data acquisition and processing were done using the ChemStation for LC/MSD version 4.2 from Agilent.

2.4. Computation

The identification of TAG structures in complex oils (e.g. ω-3 rich oils) is regarded as the bottleneck of LC–ESI-MS^2 analysis due to tedious and time-consuming manual calculations during the interpretation process [41,42]. To address this issue, a computational algorithm was developed to assist automatically the elucidation process.

The algorithm for the automatic interpretation of TAG molecules from LC–ESI-MS^2 data was developed by using MATLAB 7.9 [43] and the corresponding computation was performed on a Microsoft Windows XP® 2003 operating system (Microsoft Corporation, Redmond, WA, USA). The total LC + MS data (chromatograms + spectra) were exported to netCDF file and ASCII file by DataAnalysis for LC/MSD Trap Version 3.3, and were then used as the input files for the algorithm, which could automatically give the elucidation results of TAG structures without manually introducing data into the algorithm.

2.4.1. General algebraic expression for TAG elucidation

Different TAG molecules possess several common chemical groups as is shown in Scheme S1 (available in Supplementary material). For instance, (1) a common glycerol backbone (41 g/mol); (2) three methyl groups (3 × 15 g/mol); (3) three carboxylate groups (3 × 44 g/mol); (4) x, x' and x'' numbers of ethylene (–CH_2–CH_2–) groups (28 g/mol each) at *sn*-1, *sn*-2 and *sn*-3; (5) y, y' and y'' numbers of ethenyl (–CH=CH–) groups (26 g/mol each) at *sn*-1, *sn*-2 and *sn*-3 respectively. These common features are combined and used to generate a general algebraic expression for TAG elucidation.

$$[\mathrm{M}] = 41 + 3 \times 15 + 3 \times 44 + 28 \times (x + x' + x'') + 26 \times (y + y' + y'')$$

By representing the total number of ethylene and ethenyl groups as X and Y respectively,

$$X = x + x' + x'' \quad (1)$$

$$Y = y + y' + y' \quad (2)$$

it is possible to derive the general expression:

$$[\mathrm{M}] = 218 + 28 \times X + 26 \times Y \quad (3)$$

where [M] represents the TAG molecular weight (MW). It must be emphasized that X and Y should be always integral numbers (e.g. A TAG molecule containing 2.5 ethylene or 3.2 ethenyl groups does not exist). When LC–ESI-MS^2 in positive mode is used, under our experimental conditions, TAG adducts (e.g. $[M+NH_4]^+$) rather than protonated TAG molecules ($[M+H]^+$) are determined, in such a case the contribution of the ammonium (18 g/mol) should be added to Eq. (3), i.e.,

$$[\mathrm{M} + \mathrm{NH_4}]^+ = 236 + 28 \times X + 26 \times Y$$

$$X = \frac{[\mathrm{M} + \mathrm{NH_4}]^+ - 236 - 26 \times Y}{28} \quad (4)$$

By introducing the experimental m/z value of the precursor adduct ion $[M+NH_4]^+$ and substituting automatically only integral numbers of Y from 0 to 18 (the total possible range of double ethenyl bonds), it is possible to estimate X the total number of single ethylene bonds by using Eq. (4). It is important to highlight that Eq. (4) will yield a positive TAG identification if and only if Y (introduced as an integral number) is able to generate an integral X value. For example, when a TAG ammoniated adduct (m/z 890) containing three linolenic acids (18:3n) is analyzed, the only possible solution from Eq. (4) that yields Y and X integral values is 9 and 15 respectively (Scheme S1). Values such as 8 and 15.93 or 10 and 15.07 for Y and X are automatically rejected. The described approach is also

applicable for other types of TAG adducts. For instance, the presence of a sodiated TAG adduct $[M+Na]^+$ imply an additional contribution of the sodium (23 g/mol) to Eq. (3).

2.4.2. Computational theory for TAG interpretation

The computational theory was based on the fragmentation mechanism of TAG when using ESI-MS^2 as demonstrated in previous studies [44–46]. Briefly, the precursor adduct ions from the ESI-MS^2 mass spectrum of TAG produce very abundant DAG fragment ions due to the loss of fatty acyl moieties from the glycerol backbone. In view of the above information, the following rules were applied in the computation of TAG from the mass spectra.

1. All the observed adduct ions are of form $[M+NH_4]^+$ or $[M+Na]^+$.
2. The major product ions generated from $[M+NH_4]^+$ or $[M+Na]^+$ are DAG fragments in the form of $[M+NH_4-RCOONH_4]^+$ or $[M+Na-RCOOH]^+$ respectively, which correspond to the loss of particular FAs from the TAG backbone.
3. Only the product ions with m/z values exhibiting intensities higher than 10,000 icps (ions count per second) are screened and subjected to computation.
4. The positional distribution of the FAs on the TAG molecule is based on the relative intensities of its DAG fragments. The fatty acid which corresponds to the least abundant DAG fragment (lowest intensity) will be assigned in the *sn*-2 position on the TAG backbone. All the m/z values of possible DAG fragments observed from the mass spectrum are designated as $Frag_1, Frag_2, \ldots, Frag_i$, and the MW of corresponding FAs are designated as $FA_1, FA_2, \ldots, FA_i$.
5. The FA_i is calculated by subtracting $Frag_i$ from its observed precursor adduct (either $[M+NH_4]^+$ or $[M+Na]^+$) as follows:

   For $[M+NH_4]^+$ adducts:

$$FA_i = [M+NH_4]^+ - [M+NH_4-RCOONH_4]^+ - [NH_4]^+ + [H]^+$$

$$FA_i = [M+NH_4]^+ - Frag_i - 17 \quad (5)$$

   For $[M+Na]^+$ adducts:

$$FA_i = [M+Na]^+ - [M+Na-RCOOH]^+$$

$$FA_i = [M+Na]^+ - Frag_i \quad (6)$$

   The potential FAs identified by Eq. (5) or (6) are compared against their nominal MW with a tolerance of ± 0.5 m/z.
6. All the possible fatty acid candidates are combined on the TAG backbone and their theoretical X and Y values can be easily obtained by Eqs. (1) and (2) respectively. A positive TAG identification is achieved when the theoretical X and Y values are equal to those estimated from the experimental m/z value of the precursor adduct by Eq. (4).
7. The equivalent carbon number (ECN) of each identified TAG is calculated by the following equation:

$$ECN = CN - 2Y \quad (7)$$

   where CN is the total carbon number of a TAG molecule.

In summary, the user only needs to load the exported files (netCDF file and ASCII file) into the algorithm which in turn will determine all the possible TAG molecules in the whole chromatogram fulfilling the criteria defined above.

2.5. Chemometric discrimination analysis

To examine the discrimination between genuine and adulterated cod liver oils, two different kinds of oils (marine and vegetable) were used to adulterate pure cod liver oil. The adulterants were evaluated at two different concentration levels (25 and 50%). Duplicates samples were prepared only for pure and 25% adulterated cod liver oil. The discrimination of the various samples was performed by means of principal component analysis (PCA) using their total ion current (TIC) chromatograms. The chromatogram files (1442 data points) are firstly converted into netCDF files and subsequently into Matlab files. The m/z values were rounded up to integral numbers in order to reduce the amount and complexity of the data and to allow subsequent data analysis. These chromatograms files are subjected to PCA (coded in MATLAB 7.9) after normalization. The first three scores of PCA are used to make projection plots that provide the visual discrimination between the genuine and adulterated cod liver oils.

Table 1
Positional distribution (%) of FAs on TAG from cod liver oil.

FAs	FAs composition (%)[a]			Percentage (%)[b]	
	Total%	*sn*-1 + 3%	*sn*-2%	*sn*-1 + 3%	*sn*-2%
14:0	3.93	2.71	1.22	68.89	31.11
15:0	0.42	0.35	0.07	83.55	16.45
16:0	11.88	9.29	2.59	78.17	21.83
16:1*n*-7	7.94	6.29	1.65	79.17	20.83
16:1*n*-9	0.54	0.40	0.15	73.28	26.72
16:2*n*-4	0.48	0.33	0.15	68.23	31.77
16:3*n*-3	0.30	0.12	0.18	39.61	60.39
16:4*n*-3	0.59	0.45	0.13	77.38	22.62
17:0	0.38	0.01	0.37	3.40	96.60
18:0	3.34	3.27	0.07	97.84	2.16
18:1*n*-11	1.56	1.03	0.53	66.23	33.77
18:1*n*-7	5.17	4.61	0.56	89.13	10.87
18:1*n*-9	17.56	15.17	2.39	86.39	13.61
18:2*n*-6	2.47	2.00	0.47	80.88	19.12
18:3*n*-3	0.98	0.75	0.23	76.51	23.49
18:4*n*-3	1.92	0.42	1.50	21.88	78.12
20:1*n*-11	1.35	1.08	0.27	80.04	19.96
20:1*n*-7	0.42	0.33	0.09	78.76	21.24
20:1*n*-9	9.95	7.66	2.29	77.00	23.00
20:2*n*-6	0.31	0.22	0.09	71.40	28.60
20:4*n*-3	0.68	0.24	0.45	34.58	65.42
20:4*n*-6	0.54	0.16	0.38	29.64	70.36
EPA	8.54	2.11	6.43	24.72	75.28
22:1*n*-11	6.23	4.66	1.58	74.72	25.28
22:1*n*-9	0.89	0.80	0.10	89.16	10.84
DPA	1.30	0.31	0.99	24.19	75.81
DHA	9.55	0.40	9.18	4.07	96.04
24:0	0.18	0.11	0.07	60.37	39.63
24:1*n*-9	0.58	0.25	0.34	42.15	57.85

[a] Each value represents the mean value of duplicates (Total: total FAs on all the positions; *sn*-2%: FAs on *sn*-2 position; *sn*-1 + 3%: FAs on both *sn*-1 and *sn*-3 positions).
[b] *sn*-1 + 3% = (*sn*-1 + 3/Total) × 100%, *sn*-2% = (*sn*-2/Total) × 100%.

3. Results and discussion

3.1. Lipase stereospecific analysis

The positional distribution of FAs in the TAG of cod liver oil obtained by the benchmark lipase method is shown in Table 1. The total FAs composition analysis indicated that cod liver oil is principally characterized by 18:1*n*-9 (17.56%), 16:0 (11.88%), 20:1*n*-9 (9.95%), DHA (9.55%) and EPA (8.54%). In addition, the results in Table 1 showed that ω-3 FAs such as DHA (96.04%), 18:4*n*-3 (78.12%), DPA (75.81%), EPA (75.28%), 20:4*n*-3 (65.42%) and 16:3*n*-3 (60.39%) are mainly located at the *sn*-2 position of TAG species. A published stereospecific analysis of cod liver oil of the same brand used in the present article and by ^{13}C NMR [13] failed to detect 20:4*n*-3 and DPA. In addition, this reported study found that EPA and 18:4*n*-3 were equally distributed on the three stereospecific positions of TAG species. The only result in agreement with the present lipase method (Table 1) was DHA primarily at the *sn*-2 position.

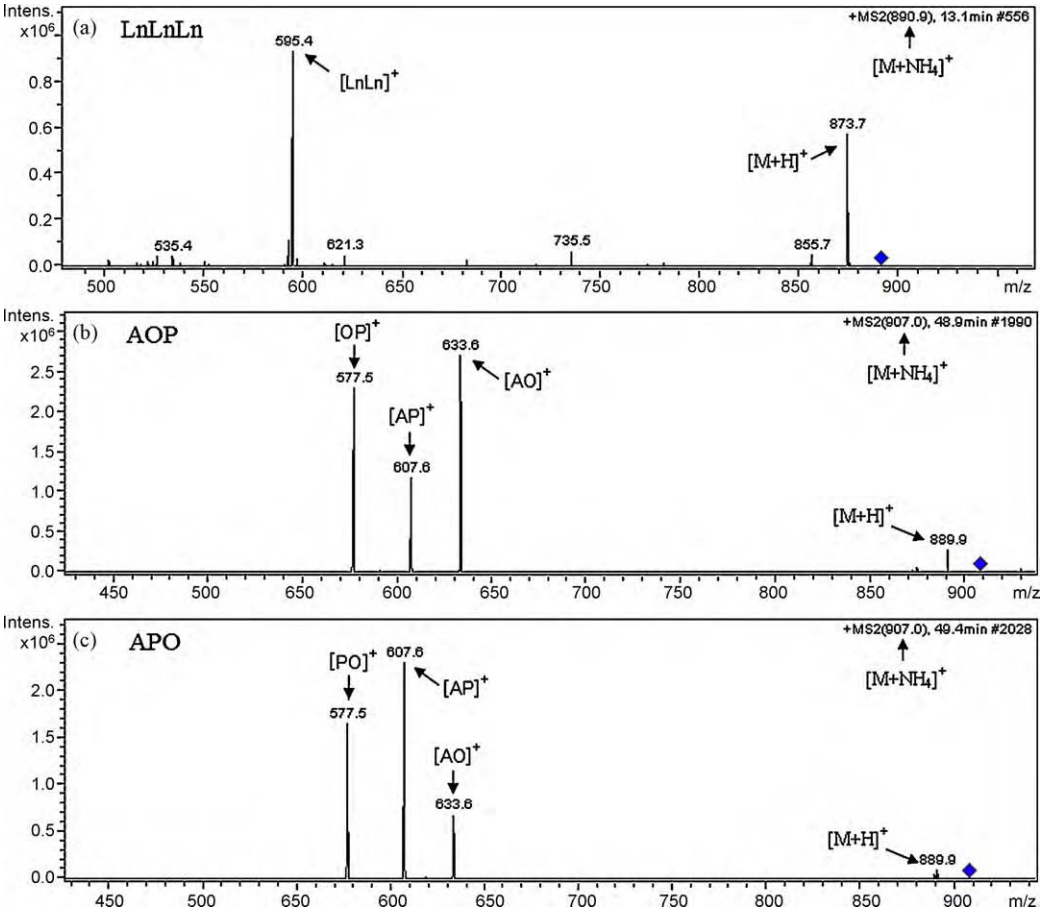

Fig. 1. ESI-MS^2 spectra of the ammoniated TAG standards: (a) LnLnLn, (b) AOP and (c) APO.

3.2. Elucidation of TAG in standards and vegetable oils by LC–ESI-MS^2

The performance of the developed TAG elucidation algorithm was firstly tested by using TAG standards. It is important to mention that the preferential cleavage fragmentation mechanisms by ESI-MS^2 to be discussed below have been demonstrated previously [44–46] and incorporated in the algorithm. The following examples will illustrate the interpretation function as well as the behaviour of TAG mass spectra.

A TAG molecule with the same fatty acid on its backbone, such as LnLnLn, exhibits a very simple mass spectrum (Fig. 1a) with only a single DAG fragment ion ($[LnLn]^+$ at m/z 595.4) resulting from the dissociation of linolenic acid (18:3n, Ln) from the LnLnLn. A different pattern arises from a TAG molecule containing three different acyl groups such as AOP. The AOP ammoniated precursor $[M+NH_4]^+$ at m/z 907 (Fig. 1b) gives rise to three DAG fragments $[OP]^+$, $[AP]^+$ and $[AO]^+$ at m/z 577.5, 607.6 and 633.6 respectively. The least abundant DAG fragment ion, at m/z 607.6, corresponds to the loss of oleic acid (18:1n, O) from the middle position (*sn*-2), indicating that the cleavage from this particular position is energetically less favoured than the outer positions (*sn*-1 and *sn*-3). Similarly, the mass spectrum of APO (Fig. 1c) displays the same three DAG fragment ions observed in the mass spectrum of its stereoisomer AOP, however the relative intensities of the generated DAG fragments are different in both spectra. In the case of APO (Fig. 1c), the DAG fragment $[AO]^+$ at m/z 633.6 displays the lowest intensity, indicating the loss of palmitic acid (16:0, P) from the *sn*-2 position. The observed ESI-MS^2 preferential cleavage of the FAs from the outer positions and the relative low intensity at the middle position of the DAG fragments which enables assigning a particular fatty acid to the *sn*-2 position have been generally investigated by means of TAG standards [44–46].

The elucidation capability of the proposed algorithm was also tested by using commercial linseed and rapeseed oils. It must be said that published reports on the elucidation of TAG species of these particular oils by LC atmospheric pressure chemical ionization single MS (LC–APCI-MS) are generally based on the above described preferential cleavage [47,48]. The elucidated TAG structures by using the developed algorithm for linseed and rapeseed oils were in accordance with those reported elsewhere [47,49–51]. The positional distribution of FAs in TAG and the elucidated TAG species of these vegetable oils are listed in the Supplementary material.

3.3. Elucidation of TAG in cod liver oil by LC–ESI-MS^2

The TAG species in the cod liver oil are identified by exporting simultaneously the total LC + MS data (chromatograms + spectra) into the developed algorithm where the mass spectra are elucidated and associated automatically to specific retention times.

The TIC chromatogram of cod liver oil and associated ECN values is shown in Fig. 2. The various elucidated TAG structures described in Table 2 are listed in increasing order of ECN along with their *sn*-2 and *sn*-1/3 positions (no distinction is made between the outer positions). Table 2 revealed that the FAs exhibiting the highest relative concentrations in Table 1 (lipase method) namely, 16:0, 16:1n, 18:1n, 20:1n, 22:1n, EPA and DHA were the most frequent detected in the various TAG structures.

Several examples for the identification of TAG species in cod liver oil are given to illustrate the interpretation process of the algorithm.

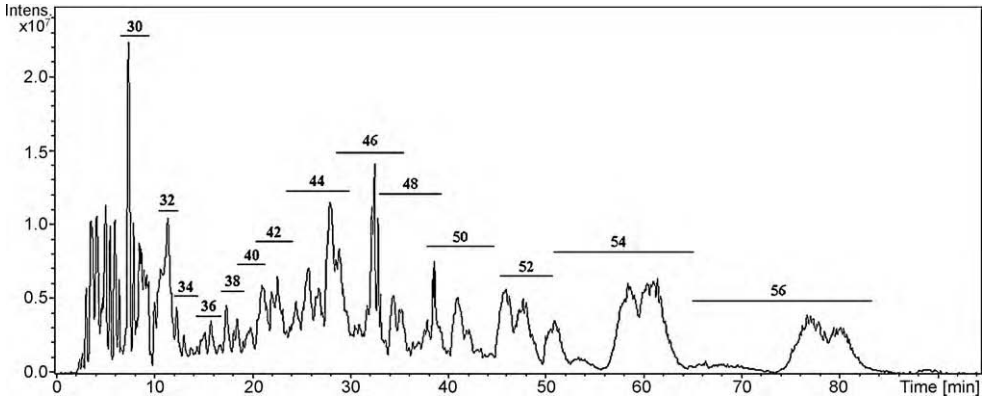

Fig. 2. TIC chromatogram of cod liver oil with the associated ECN values.

3.3.1. Elucidation of single TAG structures in cod liver oil

The ESI-MS^2 spectrum of an ammoniated TAG adduct obtained at 16.3 min is shown in Fig. 3a. The precursor ion $[M+NH_4]^+$ at *m*/*z* 968.9 produces six possible DAG fragments which can be easily visualized in the mass spectrum. The algorithm firstly, arranges the potential DAG fragments in descending order of intensity, namely *m*/*z* 649.5, 623.5, 631.4, 621.5, 669.4, 606.9 (Fig. 3b) and after performing the various computation rules previously described it indicates that four out of six fragments, specifically *m*/*z* 649.5, 623.4, 669.4 and 621.5 result from the loss of EPA, DHA, 18:1*n* and DPA from potential TAG ammoniated precursors respectively, while the masses at *m*/*z* 320.49 and 344.99 estimated from the fragments at *m*/*z* 631.4 and 606.9 respectively do not match any saturated or unsaturated FAs containing between 14 and 35 carbon molecules. The algorithm identified the combination EPA, DHA and 18:1*n* as a TAG molecule. This combination fulfils all the requirements described in Section 2.4. In addition, the algorithm assigned the *sn*-2 position to 18:1*n* as a result of the low intensity of the corresponding fragment at *m*/*z* 669.4. Although fragment D (*m*/*z* 621.5) (Fig. 3a) seems to correspond with the loss of DPA, this particular fatty acid does not comply with the general requirements for a positive TAG identification described in Section 2.4. The calculation of the total number of ethylene (*X*) and ethenyl (*Y*) group excludes automatically DPA from the precursor ion $[M+NH_4]^+$ at *m*/*z* 968.9. All the combinations containing DPA cannot yield the integral numbers 15 and 12 for *X* and *Y* respectively. The presence of the fragment at *m*/*z* 621.5 might be due to the interference from other TAG fractions.

3.3.2. Elucidation of TAG positional and structural isomers in cod liver oil

The analysis of complex mixtures, such as cod liver oil, by LC–ESI-MS^2 brings about the presence of overlapping chromatographic peaks corresponding to positional or structural isomers. For instance, the extracted ion chromatogram (EIC) of the sodiated precursor ion at *m*/*z* 927.9 (Fig. 4) exhibits two chromatographic peaks overlapping at 22.6 and 22.8 min. Although the mass spectra of these peaks display similar fragmentation patterns at *m*/*z* 577.5, 599.5, 623.4, 645.4, 671.5 and 699.5, their relative intensities are different, indicating the presence of stereoisomers. The algorithm revealed that only the combination of 16:0, 18:1*n* and DHA constitutes a positive TAG molecule in both spectra (Fig. 4a and b) and that 16:0 and DHA (the least intense fragments) are located in the *sn*-2 position of the identified TAG positional isomers at 22.6 and 22.8 min respectively. It is important to mention that the sodiated adducts observed in Fig. 4 might be ascribed to some sodium impurities in the solvents which have been reported elsewhere [52–54].

The LC–ESI-MS^2 analysis of cod liver oil also revealed the presence of structural isomers. For instance, although the EIC at *m*/*z* 877.0 exhibits one chromatographic peak at 32.8 min (Fig. 5a), the algorithm shows firstly, that the four DAG fragment ions (*m*/*z* 577.5, 603.5, 605.6 and 549.5) derived from the precursor ion $[M+NH_4]^+$ at *m*/*z* 877 (Fig. 5a) result from the loss of 18:1*n*, 16:0, 16:1*n* and 20:1*n* from TAG molecules and secondly that with these identified FAs only two TAG species fulfil the algorithm criteria, namely 18:1*n*/16:0/18:1*n* and 16:0/20:1*n*/16:1*n* (*sn*-2 positions are underlined). Similarly, the ability of the algorithm to identify co-eluting sodiated TAG isomers from a single chromatographic peak is showed in Fig. 5b where the two TAG molecules fulfilling the algorithm criteria are 18:1*n*/DHA/20:1*n* and 16:1*n*/22:1*n*/DHA.

3.4. Comparison with other LC–ESI-MS^2 studies

Although plant oils are the most studied samples by LC–ESI-MS^2, little information is given regarding how the reported TAG species were identified [35–39,55]. For instance, Svensson and Adlercreutz [55] identified 12 TAG species in the transesterified blend of rapeseed and butter oils, however, the identification of TAG was not explained. Complex samples have been also studied by LC–ESI-MS^2 [41,42]. For instance, Kalo et al. [41] reported the determination of TAG in butterfat by normal-phase LC–ESI-MS^2, where they analyzed four fractions of butterfat separated by solid phase extraction and subsequently identified 450 TAG species in total. However, the details regarding the identification of TAG species were not sufficiently illustrated. Our investigation explains the derivation of the rules for TAG elucidation by LC–ESI-MS^2 in conjunction with the proposed algorithm, based on TAG structural features and fragmentation mechanisms. Typical examples for the elucidation of positional and structural isomers of TAG structures are also provided, which gives a full overview of the interpretation of intact TAG molecules determined by LC–ESI-MS^2.

3.5. Chemometric detection of adulteration

The converted data points of the TIC chromatograms were studied by PCA to evaluate if the TAG information contained in the TIC chromatograms enables the discrimination of pure from adulterated cod liver oil. The 3D score plot (Fig. 6) explains 75.4% of the total data variation and provides a clear differentiation between genuine and adulterated cod liver oils. The pure cod liver oil samples (designated as CLO) are clustered together and clearly separated from cod liver oil adulterated with soy oil (CLO/SOY) or seal oil (CLO/SEAL) at the two levels of impurities added in this study (25 and 50%). In general, the CLO/SEAL samples in Fig. 6 are closer to pure CLO samples compared to CLO/SOY. This behaviour could

Table 2
TAG species identified by LC–ESI-MS^2 in cod liver oil. Note that no distinction is made between *sn*-1 and *sn*-3 positions.

ECN	Identified TAG species							
30	EEE*	StDE*	EDE*	DStD				
32	ELnE*							
34	MDSt*	PEHt	PoRE	PoStSt*	PoStD	PoESt*	PoDR	PoDE*
	HtPD	HtDO	OHtE	LDSt	LDE*	LnLnSt	LnLnD	StME
	StPoE	StPoD	StLSt	StLE	ArLnE	ArArE	EME*	EMD
	EPoE*	EPoD	ELE*	ELD	DPoD			
36	MLnD	MStDo	MArE	MArD	MDoSt	MDoE	PStE	PStD
	PEE*	PDR	PDSt*	PDE*	PoLnE	PoLnD	PoStAr	PoArE
	PoArD	PoDPR	PoDoSt	PoDLn*	ROE	RDO	HtArO	ORD
	ORE	OStSt	OStE*	OStD	OESt	ODSt	ODE*	LArSt
	LnPoD	LnHDo	LnLE	LnLnLn	LnLnAr	LnArLn	LnArAr	StPD
	StPE	StPoDo	StOSt	StOD	StOE	StLAr	ArPoE	ArArAr
	EMDo	EPE*	EPD	EOE*	EOD	EEcE	DPD	DOD
38	MLE	MLD	MLnLn	MEPo*	MDPo*	PRLn	PArD	PDoE
	PDAr	PoME*	PoMD	PoPoE*	PoLE	PoLnLn	PoStPo	PoEPo*
	PoDPo*	RLnO	HtOL	SStD	OLnD	OArE	ODLn	LLSt
	LLD	GRD	ArPD	ArOE	ArLAr	EPDo	ESE	
40	MOE*	MOD	MArPo	MArL	MEO*	MDoM	MDoPo	MDoL
	MDP*	MDO*	PME*	PPoSt	PPoE*	PPoD	PLE	PLD
	PLnLn	PStPo	PStL	PArAr	PDPo*	PDL*	PoPO*	PoPE*
	PoPD	PoRO	PoOE*	PoOD	PoStO	PoArPo	PoArL	PoEO*
	PoDoPo	PoDoL	PoDO*	HSSt	HGD	RAE	OMSt	OME*
	OMD	OPoE*	OPoD	OHDo	OHtO	OLSt	OLE*	OLnLn
	OStL	OEL*	ODL*	LPE*	LPD	LOSt	LLLn	LLnL
	LArL	LEG*	LDoL	AHtAr	GPoD			
42	MSE	MSD	MGSt	MGE*	MGD	MArO	MES	MDoP
	MDoO	PtPtDo	PMDo	PPoAr	POSt	POE	PLAr	PStO
	PGHt	PEcE	PArPo	PEP*	PEO*	PDoPo	PDoL	PDP*
	PDO*	PoPDo	PoSSt	PoSD	PoLL	PoStG	PoGSt	PoGE*
	PoGD	PoEcAr	PoArO	PoDoO	HHG	HArG	SME*	SMD
	SPoSt	SPoD	SLnLn	SArLn	OMAr	OMDo	OPSt	OPE*
	OPD	OPoLn	OHtG	OStO	OArL	OEO*	ODO*	LLL
	LnGLn	StMG	StPoG	GME*	GMD	GPoD		
44	MAD	MGAr	MErSt	MErD	MDoS	MDoG	PSD	POAr
	PStG	PGSt	PGE*	PGD	PArO	PES*	PEG*	PDoP
	PDpO	PDS*	PDG*	PoSDo	PoOPo*	PoStEr	PoGAr	PoArG
	PoErSt	PoErD	PoDoS	MaMaD	SPD	SPoAr	SPoDo	SHtG
	SOSt	SOE*	SOD	SStO	SEO*	SDO*	OPAr	OPDo
	ORG	OHtEr	OSSt	OSE	OLnO	OStG	OGSt	OGD
	OArO	OEG*	ODS*	ODG*	LnLnEr	LnALn	StPoEr	AMD
	GMAr	GMDo	GPE*	GPD	GPoAr	GHtG	GOD	EMEr
	ErMD	ErPoD						
46	MHEr	MGPo*	MGL*	MEcO	MBD	MErH	MErDo	MNE
	MDPEr	PPoO*	PHG	PSLn	POL*	PLO	PLnS	PAD
	PGH	PGAr	PGDo	PEcPo	PArG	PEEr	PErSt	PErE
	PErD	PDoS	PDoG	PDEr	PoPO*	PoPoG*	PoSPo	PoSL
	PoADo	PoGPo*	PoArEr	PoErAr	PoND	HSO	SPoL*	SHO
	SODo	SGSt	SGE*	SGD	SEcAr	SEG*	SDoO	SDS*
	OMO*	OPL*	OPoO*	OSDo	OStEr	OAE*	OArG	OErSt
	OErD	ODEr	LMG*	LnNLn	StSG	StGG	APD	AOE*
	GPDo	GHtEr	GSE	GSD	GStG	GGD	GEG*	GDG*
	EPEr	BMD	ErMDo	ErPD				
48	MErPo	PPoG*	PHEr	PEcO	PDoEr	PNE	PND	PoMEr
	PoAL*	PoGO*	PoEcS	PoErPo	SHG	SOL*	SLO	SLnS
	SEEr	SErD	SDoG	SDEr	OMG*	OPO*	OPoG*	OSL
	OOO*	OArEr	ONSt	OND	LMEr	LPG*	StErG	AGD
	GMEc	GHtN	GSDo	GStEr	GAD	GArG	GErD	GDEr
	ArPEr	ArOEr	ErPDo	ErHtEr	ErSD	ErGD	DPMN	DPN
	DON							
50	MEcEr	MErO	PMEr	PPoEr	PSO	POS*	PLEr	PGP*
	PGO*	PGEc	PErPo	PErL	PDoN	PNDo	PoMG*	PoSG
	PoOEr	PoAEc	PoGG*	PoErO	PoNPo	SMG*	SPoG*	SHEr
	SOEc	SGL*	SDPEr	OMEr	OPG*	OPoEr	OSO	OOG*
	OAL*	OGO*	LMN	LPEr	LSG	LOA*	StGN	StNG
	AAD	GMG*	GPoG*	GArEr	GND	ArON	ArGEr	ErStEr
	ErErD	ErDEr	DPPN	DGN				
52	MAG*	MErG	MNO	PMN	PPoN	POEr	PAO*	PGS*
	PGG*	PErP	PErO	PErG	PoLiPo	SMEr	SOS*	OPEr
	OPoN	OSG	OGG*	OErO	GMEr	GPG*	GPoEr	GOG*
	ErDN	ErND						
54	MAEr	MGB*	POB*	PON	PGA*	PErS	PErG	PErO
	PNO	SPEr	SOEr	SAO*	SGS*	OSEr	OGEr	OErG
	ONO	GMN	GPEr	GSG	GOEr	GGG*	ErMEr	ErPoEr
56	MNEr	PGN	PNG	PoNEr	ONG	ONEr	GSEr	GGEr
	GErG	GNG	ErPEr	ErPoN	ErOEr	ErGS	ErGEr	ErON

Note: *major TAG species.
Abbreviations: M: 14:0; Pt: 15:0; P: 16:0; Po: 16:1*n*; H: 16:2*n*; R: 16:3*n*; Ht: 16:4*n*; Ma: 17:0; S: 18:0; O: 18:1*n*; L: 18:2*n*; Ln: 18:3*n*; St: 18:4*n*; A: 20:0; G: 20:1*n*; Ec: 20:2*n*; Ar: 20:4*n*; E: EPA; B: 22:0; Er: 22:1*n*; DPA: Do; DHA: D; Li: 24:0; N: 24:1*n*.

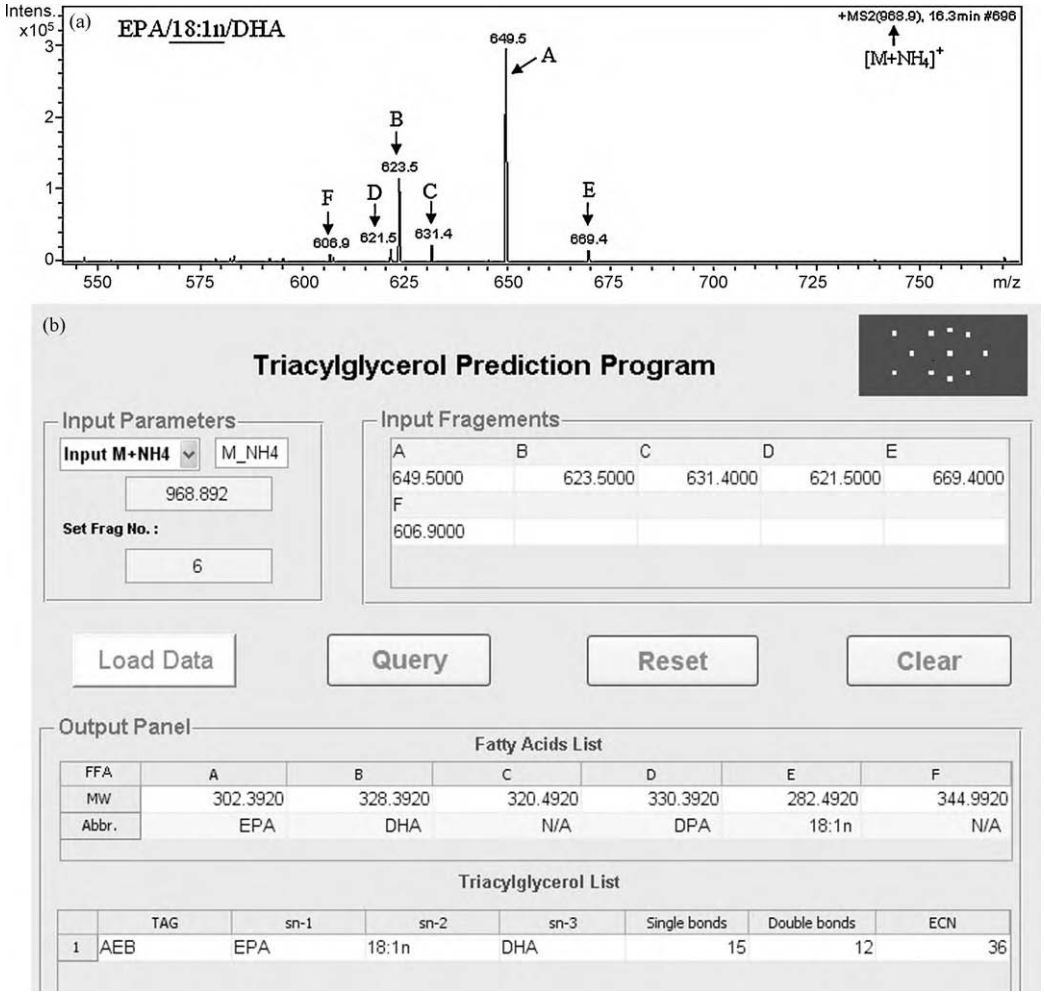

Fig. 3. (a) ESI-MS^2 spectrum of the ammoniated EPA/18:1*n*/DHA (*m*/*z* 968.9) obtained at 16.3 min of cod liver oil. (b) Algorithm outcomes of the above data at 16.3 min.

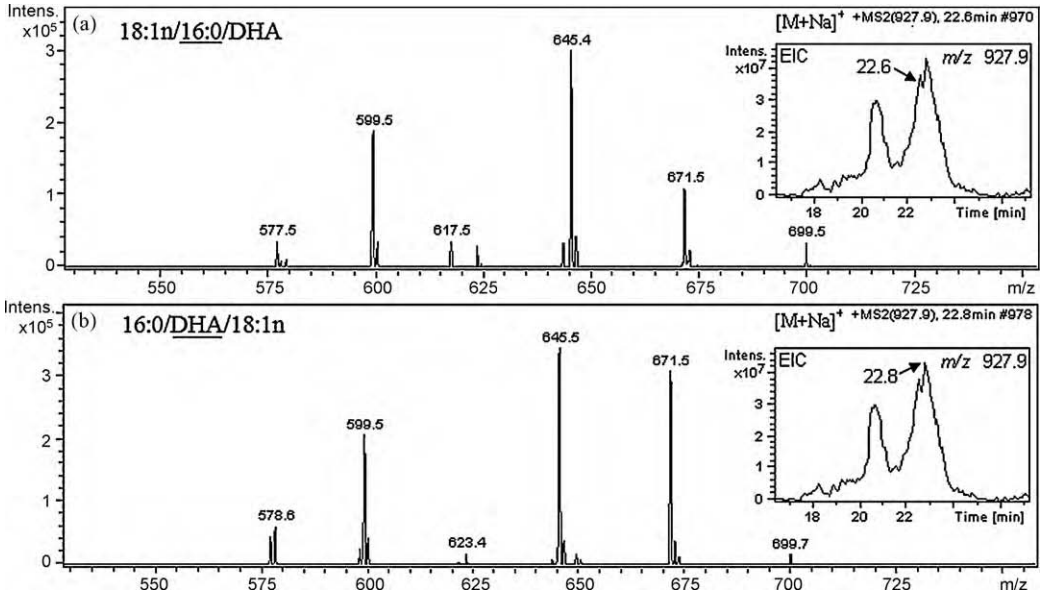

Fig. 4. ESI-MS^2 spectra of the sodiated adducts from cod liver oil: (a) 18:1*n*/16:0/DHA at 22.6 min and (b) 16:0/DHA/18:1*n* at 22.8 min and their corresponding embedded EIC at *m*/*z* 927.9.

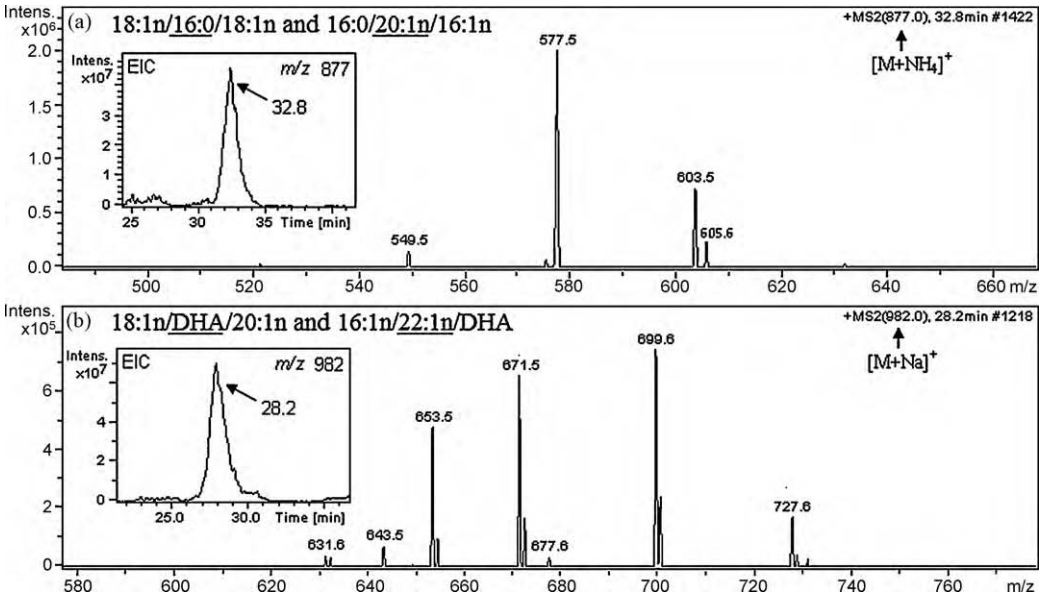

Fig. 5. (a) ESI-MS^2 spectrum of the ammoniated adducts from cod liver oil 18:1*n*/16:0/18:1*n* and 16:0/20:1*n*/16:1*n* at 32.8 min and their corresponding embedded EIC at *m*/*z* 877.0; (b) ESI-MS^2 spectrum of the sodiated adducts from cod liver oil 18:1*n*/DHA/20:1*n* and 16:1*n*/22:1*n*/DHA at 28.2 min and their corresponding embedded EIC at *m*/*z* 982.0.

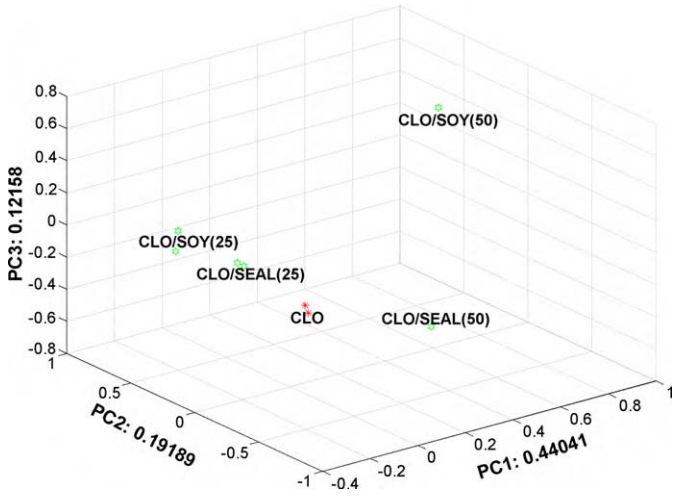

Fig. 6. PCA score plot of genuine and adulterated cod liver oil based on the LC–ESI-MS^2 analysis. (CLO: cod liver oil; SOY: soy oil; SEAL: seal oil. The numbers in bracket represent the concentrations of adulterant in cod liver oil.)

be ascribed to the lack of ω-3 polyunsaturated FAs (PUFAs) in soy oil. The detection of seal oil as adulterant of cod liver oil is regarded as exceedingly difficult due to their strong resemblance [56–59]. However, the developed algorithm, for elucidating TAG structures, revealed ω-3 PUFAs mainly located at the *sn*-2 position in pure cod liver oil, while for CLO/SEAL (25 or 50) the algorithm revealed ω-3 PUFAs not only at the *sn*-2 positions but also at the *sn*-1/3 positions which clearly indicated the presence of seal oil. It has been reported that ω-3 PUFAs are preferentially located at the terminal positions of TAG in seal oil [7,10]. The differences in TAG structures from CLO and CLO/SEAL samples elucidated by the algorithm were substantiated by the PCA discrimination study (Fig. 6).

4. Conclusion

A LC–ESI-MS^2 strategy was successfully established to directly identify the relative arrangement of the acyl groups on the glycerol backbone of cod liver oil. The developed computational algorithm facilitated the rapid structural elucidation of the TAG molecules in cod liver oil based on the information obtained from the LC–ESI-MS^2 data. The combined information from the lipase and LC–ESI-MS^2 approach enable a full examination not only on the total FAs composition but also on the specific positioning of FAs on intact TAG molecules in cod liver oil which represents a useful means to help the understanding of its properties and nutritional value as well as the detection of adulteration for these kinds of products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.06.055.

References

[1] H. Shimokawa, P.M. Vanhoutte, Circulation 78 (1988) 1421–1430.
[2] B. Osterud, E. Elvevoll, H. Barstad, J. Brox, H. Halvorsen, K. Lia, J.O. Olsen, R.L. Olsen, C. Sissener, O. Rekdal, E. Vognild, Lipids 30 (1995) 1111–1118.
[3] J. Gruenwald, H.J. Graubaum, A. Harde, Adv. Ther. 19 (2002) 101–107.
[4] L.A. Brunborg, T.M. Madland, R.A. Lind, G. Arslan, A. Berstad, L. Frøyland, Clin. Nutr. 27 (2008) 614–622.
[5] B. Galarraga, M. Ho, H.M. Youssef, A. Hill, H. McMahon, C. Hall, S. Ogston, G. Nuki, J.J.F. Belch, Rheumatology 47 (2008) 665–669.
[6] H. Brockerhoff, R.J. Hoyle, P.C. Hwang, C. Litchfield, Lipids 3 (1968) 24–29.
[7] Y. Ando, T. Ota, Y. Matsuhira, K. Yazawa, J. Am. Oil Chem. Soc. 73 (1996) 483–487.
[8] J.J. Myher, A. Kuksis, K. Geher, P.W. Park, D.A. DiersenSchade, Lipids 31 (1996) 207–215.
[9] C.V. Nwosu, L.C. Boyd, J. Food Lipids 4 (1997) 65–74.
[10] U.N. Wanasundara, F. Shahidi, J. Food Lipids 4 (1997) 51–64.
[11] Y. Ando, M. Satake, Y. Takahashi, Lipids 35 (2000) 579–582.
[12] F.D. Gunstone, S. Seth, Chem. Phys. Lipids 72 (1994) 119–126.
[13] M. Aursand, L. Jørgensen, H. Grasdalen, J. Am. Oil. Chem. Soc. 72 (1995) 293–297.

[14] M.D. Guillén, I. Carton, E. Goicoechea, P.S. Uriarte, J. Agric. Food Chem. 56 (2008) 9072–9079.
[15] F. Turon, F. Bonnot, Y.C.M. Pina, J. Graille, Chem. Phys. Lipids 125 (2003) 41–48.
[16] F. Turon, Oleag. Corps Gras Lipides 10 (2003) 144–149.
[17] H. Brockerhoff, Lipids 6 (1971) 942–956.
[18] M. Yurkowski, H. Brockerhoff, Biochim. Biophys. Acta 125 (1966) 55–59.
[19] G. Andreotti, E. Trivellone, R. Lamanna, A.D. Luccia, A. Motta, J. Dairy Sci. 83 (2000) 2432–2437.
[20] M.R.V. Calsteren, C. Barr, P. Angers, J. Arul, Bull. Magn. Reson. 18 (1996) 175–177.
[21] S. Kubow, J. Nutr. Biochem. 7 (1996) 530–541.
[22] W. Chakra, Food Sci. Technol. 20 (2008) 199–202.
[23] G. Nelson, R. Ackman, Lipids 23 (1988) 1005–1014.
[24] W. Neff, E. Selke, T. Mounts, W. Rinsch, E. Frankel, M. Zeitoun, J. Am. Oil Chem. Soc. 69 (1992) 111–118.
[25] E. Weise, USA Today, Retrieved from, 23 January 2009 (15.03.10) http://www.usatoday.com/news/health/2009-01-19-fake-foods_N.htm.
[26] Z. Hossain, The Daily Independent Bangladesh (Internet Edition), Retrieved from, 13 March 2005 (15.03.10) http://www.theindependent-bd.com/details.php?nid=162455.
[27] O.C.S. Carter, Am. Philos. Soc. 22 (1885) 296–299.
[28] E.J. Parry, Lancet 163 (1904) 378.
[29] M. Lees, Food Authenticity and Traceability, first ed., Woodhead Publishing Ltd., Cambridge, 2003.
[30] I. Martinez, D. James, H. Loréal, Application of Modern Analytical Techniques to Ensure Seafood Safety and Authenticity, FAO Fisheries Technical Paper 455, Rome, 2005.
[31] J.S. Perona, V. Ruiz-Gutiérrez, J. Liq. Chromatogr. Relat. Technol. 22 (1999) 1699–1714.
[32] M. Hori, Y. Sahashi, S. Koike, R. Yamaoka, M. Sato, Anal. Sci. 10 (1994) 719–724.
[33] A.S. Mcgill, C.F. Moffat, Lipids 27 (1992) 360–370.
[34] K. Takahashi, T. Hirano, M. Saito, Nippon Suisan Gakk 54 (1988) 523–528.
[35] S.D. Segall, W.E. Artz, D.S. Raslan, V. Ferraz, J.A. Takahashi, J. Am. Oil Chem. Soc. 81 (2004) 143–149.
[36] S.D. Segall, W.E. Artz, D.S. Raslan, G.N. Jham, J.A. Takahashi, J. Agric. Food Chem. 53 (2005) 9650–9655.
[37] S.D. Segall, W.E. Artz, D.S. Raslan, P.F. Vany, A.T. Jacqueline, J. Sci. Food Agric. 86 (2006) 445–452.
[38] J.L. Gómez-Ariza, A. Arias-Borrego, T. García-Barrera, R. Beltran, Talanta 70 (2006) 859–869.
[39] L. Heidi, S. Jukka-Pekka, K. Heikki, Rapid Commun. Mass Spectrom. 21 (2007) 2361–2373.
[40] J.P. Williams, M.U. Khan, D. Wong, J. Lipid Res. 36 (1995) 1407–1412.
[41] P. Kalo, A. Kemppinen, V. Ollilainen, Lipids 44 (2009) 169–195.
[42] K. Ikeda, Y. Oike, T. Shimizu, R. Taguchi, J. Chromatogr. B 877 (2009) 2639–2647.
[43] MATLAB 7.9 (R2009b), The Math Works Inc., 2009.
[44] C. Cheng, M.L. Gross, E. Pittenauer, Anal. Chem. 70 (1998) 4417–4426.
[45] E. Hvattum, Rapid Commun. Mass Spectrom. 15 (2001) 187–190.
[46] L. Marzilli, L. Fay, F. Dionisi, P. Vouros, J. Am. Oil Chem. Soc. 80 (2003) 195–202.
[47] M. Holčapek, P. Jandera, P. Zderadicka, L. Hrub, J. Chromatogr. A 1010 (2003) 195–215.
[48] H.R. Mottram, S.E. Woodbury, R.P. Evershed, Rapid Commun. Mass Spectrom. 11 (1997) 1240–1252.
[49] J.D.J. van den Berg, N.D. Vermist, L. Carlyle, M. Holcapek, J.J. Boon, J. Sep. Sci. 27 (2004) 181–199.
[50] R.M. Hazel, E.W. Simon, P.E. Richard, Rapid Commun. Mass Spectrom. 11 (1997) 1240–1252.
[51] M. Lísa, M. Holčapek, J. Chromatogr. A 1198 (2008) 115–130.
[52] K.L. Duffin, J.D. Henion, J.J. Shieh, Anal. Chem. 63 (1991) 1781–1788.
[53] K. Hartvigsen, A. Ravandi, K. Bukhave, G. Holmer, A. Kuksis, J. Mass Spectrom. 36 (2001) 1116–1124.
[54] S.D. Segall, W.E. Artz, D.S. Raslan, V.P. Ferraz, J.A. Takahashi, Food Res. Int. 38 (2005) 167–174.
[55] J. Svensson, P. Adlercreutz, Eur. J. Lipid Sci. Technol. 110 (2008) 1007–1013.
[56] H.G. Greenish, A Text Book of Materia Medica, Being an Account of the More Important Crude Drugs of Vegetable and Animal Origin, J. & A. Churchill, 1920.
[57] R. Benedikt, Chemical Analysis of Oils, Fats, Waxes, and of the Commercial Products Derived Therefrom, General Books, 2010.
[58] H. Richardson Procter, The Principles of Leather Manufacture, Nabu Press, 2010.
[59] J.C. Drummonda, S.S. Zilvaa, J. Goldinga, J. Agric. Sci. 13 (1923) 153–162.