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Analytical Methods

A critical assessment of transmethylation procedures for n-3 long-chain polyunsaturated fatty acid quantification of lipid classes

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ABSTRACT

Lipid transmethylation methods described in the literature are not always evaluated with care so to insure that the methods are effective, especially on food matrix or biological samples containing polyunsaturated fatty acid (PUFA). The aim of the present study was to select a method suitable for all lipid species rich in long chain n-3 PUFA. Three published methods were adapted and applied on individual lipid classes. Lipid (trans)methylation efficiency was characterized in terms of reaction yield and gas chromatography (GC) analysis. The acid-catalyzed method was unable to convert triglycerides and sterol esters, while the method using an incubation at a moderate temperature was ineffective on phospholipids and sterol esters. On the whole only the method using sodium methoxide and sulfuric acid was effective on lipid classes taken individually or in a complex medium. This study highlighted the use of an appropriate (trans)methylation method for insuring an accurate fatty acid composition.

1. Introduction

The technique of gas chromatography (GC) is the most commonly used to determine the fatty acid (FA) composition of lipids in food matrices and biological samples. FA are analyzed by GC as fatty acid methyl esters (FAME) in order to prevent potential hydrogen bonding between the FA carboxyl group and the fused silica of the GC column. Moreover, FAME are more volatile compounds than FA and more easily separated based on their boiling point, allowing the reliable identification according to their chemical structure. Esterification of free FA (FFA) and transesterification of esterified lipids generally occur in a one-step reaction, in presence of acid catalysts (for FFA and esterified lipids) or basic catalysts (for esterified lipids, only) and a methanolic phase in excess (Christie, 1993). The choice of the (trans)methylation method is highly dependent on the lipid classes present in the sample and on the FA nature, i.e. the chain length, the double bound configuration and the unsaturation degree. Base-catalyzed transesterification is rapid (1–10 min) (Christie, 1993; Marinetti, 1966; Santos Júnior et al., 2013) and operates at room or moderate temperatures (lower than 60 °C) (Christie, 1993; ISO 12966-2). However, using basic reagents such as potassium hydroxide (KOH) or sodium hydroxide (NaOH) increases the pH over the pKa of carboxyl group of FFA which generate sodium (or potassium) soaps rather than FAME (Christie, 1993). Moreover, sphingolipids are resistant to alkaline treatment due to the presence of an amide bound instead of an ester bound (MacGee & Williams, 1981). Thereby, basic catalysts also lead to an incomplete conversion of sphingolipid FA into FAME. In contrast, acid catalysts can be used to esterify FFA or to transesterify FA linked by ester bonds to glycerol (Christie, 1993). However, they are not suitable for cholesterol esters (Christie, 1993). Sphingolipids are transesterified by acid-catalyzed methanolysis but side-products may be formed as artefacts (Christie, 1993). Moreover, acid-catalyzed (trans)methylation often proceeds at high temperatures (higher than 80 °C), associated with long incubation times (30–90 min) (Christie, 1993; Klopfenstein, 1971; Kramer et al., 1997; Lepage & Roy, 1988; Morrison & Smith, 1964). Concerning the quantification of the FA composition of the lipid sample, because internal standards are not systematically used, any FA loss during the experimental steps ((trans)methylation or FAME recovery) potentially results in an underestimation and, thereby, to an overestimation of the other FA species. Therefore, the presence of short-chain FA requires specific precautions due to their volatility for a quantitative recovery (Christie, 1993). High temperatures used in acid-

Abbreviations: 2,7-DCF, 2,7-dichlorofluorescein; BF_3, boron trifluoride; CE 22:0, cholest-5-eω-3β-yl-docosanoate; CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl esters; FFA, free fatty acids; FFA 17:0, heptadecanoic acid; GC, gas chromatography; HP-TLC, high-performance thin layer chromatography; LC-PUFA, long chain polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; PC 15:0, 1,2-dipentadecanoyl-sn-glycero-3-phosphatidylcholine; PUFA, polyunsaturated fatty acids; SE, sterol esters; SFA, saturated fatty acids; TG, triglycerides; TG 17:0, 1,2,3-triheptadecanoyl-sn-glycerol; TLC, thin layer chromatography

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catalyzed reactions may induce isomerization of thermolabile FA such as conjugated linoleic acid (CLA) and conjugated dienes (Castro-Gómez, Fontecha, & Rodríguez-Alcalá, 2014; Kramer et al., 1997; Murrieta, Hess, & Rule, 2003). Recently, a method combining the use of sodium methoxide and sulfuric acid was proposed (Castro-Gómez et al., 2014). It appeared to be an effective method for the derivatization of all lipid classes, in particular towards sterol esters (SE) and sensitive FA such as CLA. However, as other acid-catalyzed reactions, it has to be performed at high temperature (mostly 100 °C) (Christie, 1993; Kramer et al., 1997; Lepage & Roy, 1988; Morrison & Smith, 1964). Although, a good quantification of polyunsaturated FA (PUFA) up to 22:6 was observed, the amounts of eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) present in the reference samples were far below the values that can be found in marine products or biological samples analyzed after fish oil diets.

Thus, although several (trans)methylation methods are commonly used, they either mainly focused on α-linolenic acid (18:3) containing lipids as polyunsaturated species or do not consider all the lipid classes that may be present in a true sample. The purpose of this work was to assess the efficiency of three (trans)methylation methods: (1) the acid-catalyzed method of Morrison and Smith (1964), and the two sequentially base- and acid-catalyzed methods of (2) Castro-Gómez et al. (2014) and (3) Christie, Sébédio, and Juanéda (2001). These three methods were applied on various lipid classes, i.e. phospholipids (PL), triglycerides (TG), FFA, and SE, all coming from marine organisms and, thus, rich in ω-3 long-chain PUFA (ω-3 LC-PUFA). Because the analyzed samples were rich in EPA and DHA, it was necessary to increase the reaction times that may alter PUFA especially when high temperatures are applied. We showed that method 2, adapted with the correct reaction times to (trans)methylate all the lipid species, was the most efficient one. This method was then applied to total lipids extracted from Human serum and rat liver and plasma, these two last being obtained after diets enriched in ω-3 long-chain PUFA, as typical examples of complex lipid mixtures.

2. Materials and method

2.1. Materials

Lecithin from herring roe (Lecimarin F50) was kindly provided by Novastell (Etrépagny, France). Fish oil was supplied by BIPEA (Paris, France). Dihydrate calcium chloride ($CaCl_2$, PubChem CID: 5284359) was purchased from VWR (Radnor, Pennsylvania, USA). (Tris)-buffered saline, lipase from porcine pancreas (PubChem CID: 54603431), sodium déoxycholate (PubChem CID: 5283834), boron trifluoride-methanol solution (BF_3; 14%, w/v, PubChem CID: 6356), sodium methoxide solution (0.5 N, PubChem CID: 10942334), serum from Human male AB plasma (USA origin; product number H4522), 2,7-dichlorofluorescein (2,7-DCF, PubChem CID: 64944), primuline (PubChem CID: 24852417) and FAME mix C4-C24 were obtained from Sigma-Aldrich (Saint-Louis, Missouri, USA). Anhydrous sodium carbonate (PubChem CID: 10340) and orthophosphoric acid (PubChem CID: 1004) were supplied by Merck KGaA (Darmstadt, Germany). Copper sulfate pentahydrate (PubChem CID: 24463) was purchased from Pancreatic (Barcelona, Spain). Acetic acid (PubChem CID: 176), sulfuric acid (PubChem CID: 1118), and potassium chloride (KCl, PubChem CID: 4873) were provided by Thermo Fisher Scientific (Strasbourg, France). They also supplied the organic solvents used (analytical or HPLC grades). Internal standards for HP-TLC analysis were: 1,2-diheptadecanoyl-sn-glycero-3-phosphatidylethanolamine (PE 17:0), 1-monooleyl-rac-glycerol (MG 18:1n-9), Cholesterol (Chol), 1,3-di-11-eicosenoin (DG 20:1n-11), α-linolenic acid (ALA), 1,2,3-triheptadecanoyl-*sn*-glycerol (TG 17:0), cis-8,11,14-eicosatrienoic acid methyl ester (FAME 20:3n-6), cholest-5-en-3β-yl-octadec-9-oate (cholesterol ester 18:1n-9; CE) all purchased from Avanti Polar Lipids INC (Alabaster, Alabama, USA). Internal standards for GC analysis were: 1,2-dipentadecanoyl-sn-glycero-3-phosphatidylcholine (PC 15:0, PubChem CID: 134308), 1,2,3-triheptadecanoyl-sn-glycerol (TG 17:0, PubChem CID: 3625612), heptadecanoic acid (FFA 17:0, PubChem CID: 10465) and cholest-5-eω-3β-yl-docosanoate (CE 22:0, PubChm CID: 53477887) all purchased from Avanti Polar Lipids INC (Alabaster, Alabama, USA). Rat liver and plasma samples were issued from a nutritional study in which rats were fed with diet enriched in n-3 LC-PUFA for 8 weeks kindly provided by ITERG (Institut des Corps Gras, Pessac, France).

2.2. Lipid class preparation and purification

Fish oil was used as the source of marine TG without further purification. In order to get purified PL and SE extracts, purification steps performed on the herring roe lecithin and the total lipids extracted from *Pecten maximus* gonads, respectively. FFA were prepared from fish oil enzymatic hydrolysis.

PL were isolated from herring roe lecithin by solvent fractionation using cold acetone. Briefly, 1 mL of a mixture composed of chloroform/methanol (2:1, v/v) was added to 1 g of marine lecithin and maintained at room temperature until complete dissolution of sample. After addition of 20 mL of cold acetone (−20 °C), the sample was briefly homogenized by vortex (30 s) and centrifuged (1050*g*, 5 min, 4 °C; Sorvall ST-40R, Thermo Scientific, Waltham, Massachusetts, USA). The supernatant, containing the neutral lipids, was removed and the pellet, containing the polar lipids, was extracted twice and stored at −20 °C.

Total lipids from *Pecten maximus* gonads were extracted with chloroform/methanol (2:1, v/v) according to the method of Folch, Lees, and Sloane Stanley (1957). SE were purified from *Pecten maximus* lipids by liquid chromatography (Silica gel 63–200 μm (Sigma-Aldrich), glass column 15 × 45 cm). Hydrocarbons were first eluted using a large volume of hexane. Then, SE were separated from the other lipids using a mixture of hexane/diethyl ether (99:1, v/v) (ISO 12873:2017). Purified SE were stored in chloroform/methanol (2:1, v/v) at - 20 °C until use.

FFA were obtained by lipase hydrolysis of fish oil according to Desnuelle (1961) and Entressangles, Sari, and Desnuelle (1966) method. Succinctly, after homogenization of 15 mg of fish oil in 200 μL of isooctane, the fish oil was added to 2 mL of pancreatic lipase solution (0.5 g/mL in 1 M of Tris HCl, pH 8), 0.5 mL of sodium deoxycholate (1 g/L in distillated water), and 0.2 mL of a calcium chloride solution (220 g/L in distilled water), maintained 5 min at 40 °C and 30 min at room temperature. Total lipids were extracted by addition of 1 mL of HCl 6 N and 10 mL of diethyl ether followed by homogenization and centrifugation (1050*g*, 5 min, 4 °C; Sorvall ST-40R, Thermo Scientific). Lipids were dried under nitrogen and dissolved in an accurate volume of a mixture composed of chloroform/methanol (2:1, v/v). FFA were separated from non-hydrolyzed TG and reaction products, i.e. mono- and di-glycerides, by thin-layer chromatography (TLC) (glass plates 20 × 20 cm pre-coated with silica gel 60H (Merck KGaA)) using a solvent mixture composed of hexane, diethyl ether and acetic acid (70:30:1, v/v/v). Spots were visualized under UV-light after vaporization of 2,7-DCF in ethanol solution (0.2%, m/v). FFA were extracted from the silica gel by addition of 2.5 mL of chloroform/methanol (2:1, v/v). After homogenization and centrifugation of the scrapped sample (1050*g*, 5 min, 20 °C; Sorvall ST-40R, Thermo Scientific), the organic phase was collected. Distilled water (100 μL) and 2 mL of chloroform/methanol (2:1, v/v) were added to the silica gel phase. The extraction step was repeated once again and the organic phase was collected. Lipid extraction from silica gel ended by addition of 2 mL of methanol to the silica gel phase, homogenization and centrifugation. The organic phases were collected. Finally, 2,7-DCF was removed using 0.4 mL of a potassium chloride solution (0.8% in distilled water, w/v) and 2 mL of chloroform/methanol (2:1, v/v) to the lipid solution. The organic phase was washed twice by addition of 0.8 mL of a mixture composed of chloroform/methanol/potassium chloride 0.8% in distilled water (15:240:235, v/v/v). FFA were pooled, dissolved in chloroform/methanol (2:1, v/v) and stored at −20 °C.

Total lipids from Human serum (Sigma-Aldrich) and rat liver and plasma were extracted according to the method of Folch et al. (Folch et al., 1957). Lipid extracted were stored in chloroform/ methanol (2:1, v/v) at −20 °C until use.

2.3. Transmethylation procedures

FAME from individual lipid class (PL, TG, FFA and SE) were prepared according to three methods with modified incubation times. Indeed, the ISO 12966-2 standard (ISO 12966-2) recommends to increase the incubation time for oils rich in LC PUFA.

Method 1: FA were (trans)methylated according to the acid-catalyzed method adapted from Morrison & Smith (Morrison & Smith, 1964). 2 mg of lipids were mixed with 1 mL of BF_3 in methanol solution in a glass tube. Isooctane (0.5 mL) was added as co-solvent when (trans) methylation was performed on FFA, TG and SE classes. Samples were incubated at 100 °C, under constant agitation, for times varying from 15 min for FFA, 60 min for TG and 90 min for PL and SE. After addition of 2 mL of isooctane followed by 1 mL of distilled water in the case of FFA, TG and SE classes, or 5 N sodium hydroxide for PL, samples were centrifuged (1050*g*, 10 min, 4 °C; Sorvall ST-40R, Thermo Scientific). The organic phase containing the FAME was collected. The extraction step was repeated three times by addition of 2 mL of isooctane. FAME were pooled, concentrated by evaporation of the solvent under nitrogen, washed with 1 mL of distilled water and stored in isooctane at 4 °C until analysis.

Method 2: This method proposed by Castro-Gómez et al. (2014) used sodium methoxide followed by sulfuric acid, in combination. Sodium methoxide solution (2.5 mL, 0.5 N) was added to 2 mg of lipids, in a glass tube and maintained at 80 °C under constant agitation for 10 min. After cooling in the ice, 3 mL of a sulfuric acid-methanol solution (1 M, pH 1) was added to the samples. The reaction was performed at 100 °C. Like in method 1, the incubation time varied with the type of lipids: 90 min for PL and 45 min for all the other lipid classes. FAME were extracted by addition of 2 mL of hexane and 7.5 mL of sodium carbonate (6% in distilled water, w/v) followed by a centrifugation step (1218*g*, 10 min, 4 °C; Sorvall ST-40R, Thermo Scientific). FAME were dried under nitrogen, dissolved in isooctane and stored at 4 °C until analysis.

Method 3: The Christie's method (Christie et al., 2001) was initially developed to (trans)methylate FA sensitive to temperature like CLA using a short incubation time (10 min) at moderate temperature (50 °C). Briefly, 0.5 mL of hexane and 0.2 mL of a sodium methoxide solution (2N) was added to 2 mg of lipids in a glass tube. The sample was maintained 10 min, at room temperature, under constant agitation. 1 mL of a BF_3 solution in methanol was added. Irrespectively of the lipid class, the sample was incubated at 50 °C, under constant agitation, for 30 min. FAME were extracted three times by addition of 0.5 mL of distilled water followed by 2 mL of isooctane. After centrifugation (1050*g*, 10 min, 4 °C; Sorvall ST-40R, Thermo Scientific), the organic phases containing the FAME were collected, pooled, concentrated by evaporating solvent under nitrogen, and washed with 1 mL of distilled water. FAME obtained were stored at - 20 °C until analysis.

Method 2 was then applied on biological lipid mixtures (i.e. total lipids from Human serum and from rat liver and plasma).

2.4. High-performance TLC (HP-TLC) analysis

High-performance TLC (HP-TLC) was used to assess lipid class purity (for SE and PL) and lipid (trans)esterification yields of the different lipid classes and of the lipid mixtures. Lipids were deposited by an automatic sampler (ATS4, Camag, Muttenz, Switzerland) on a HP-TLC plate (HP-TLC plate silica gel 60 F254 20x10 cm, Merck, Darmstadt, Germany) and separated in an automatic development chamber (ADC2, Camag, Muttenz, Switzerland). PL purity was assessed using a solvent mixture composed of diethyl ether/acetone (60:20, v/v). SE purity obtained from *Pecten maximus* gonads and FAME obtained after (trans)methylation of individual lipid classes and total lipids extracted from Human serum, rat liver and plasma were analyzed using an eluent composed of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). After lipid separation, the plate was dried and quickly immersed in a dye solution. Two different dyes were used for species detection, i.e. a primuline solution (detecting double bonds) or a copper sulfate solution (non specific detection). When using the primuline solution (0.06% in acetone/water (80:20, v/v)), the plate was dried at 30 °C for 5 min on a hotplate (TLC plate heater III, Camag, Muttenz, Switzrerland). Lipid classes were visualized under UV light (366 nm) in fluorescence mode using an automatic scanner (Scanner 4, Camag, Muttenz, Switzerland). When using the copper sulfate solution (20% in methanol/ sulfuric acid/ orthophosphoric acid (200:7.8:6.8, v/v/v)), the plate was dried at 140 °C for 15 min on a hotplate and the lipid classes were visualized at 720 nm using tungsten lamp in the automatic scanner. Primuline solution was used for PL purity assessment, analysis of total lipids from human serum, rat liver and plasma, and the corresponding FAME. The copper sulfate solution was used for SE purity assessment and FAME obtained after (trans)methylation of individual lipid classes.

Lipid classes were identified by comparing their frontal report with those of standard solutions of PL, TG, FFA, SE and FAME (1 mg/ml in chloroform/ methanol (2:1, v/v)) chromatographed on the same plate and in the same conditions and visualized at 366 nm or at 720 nm, according to the dye used.

2.5. Gas-chromatography analysis

FAME were analyzed on a gas chromatograph (TRACE GC, Thermo Scientific, Waltham, Massachusetts, USA) equipped with a flame-ionization detector and a split injector. A fused-silica capillary column (BPX 70, 60 m × 0.25 mm i.d., 0.25 µm film; SGE, France) was used with hydrogen as a carrier gas (inlet pressure: 120 kPa). The split ratio was 1:33. The column temperature program was as follow: from 160 °C, the temperature increased to 180 °C at 1.3 °C/min, and was maintained for 65 min before increasing at 25 °C/min until 230 °C for 15 min. The injection port and the detector were maintained at 250 °C and 280 °C, respectively. GC peaks were integrated using Chromquest software (Thermofinnigan, Courtaboeuf, France). The identification of individual FAME was performed by comparing their retention times with those of a FAME standard mixture (FAME mix C4-C24, Sigma-Aldrich) chromatographed in the same conditions and on the same chromatograph. In order to test both the recovery and efficiency of derivatization methods, an appropriate internal standard was added to the lipid samples at 10 wt% of total lipids, before the (trans)esterification experiment. More precisely, 200 µL of internal standard solution (1 mg/ml in chloroform/ methanol (2:1; v/v)) were added to 2 mg of lipids samples. The whole sample was dried under nitrogen in order to evaporate solvent traces (choloroform and methanol) and the (trans)esterification experiment performed. The internal standard was chosen for its representativeness of the lipid class and for the absence of its specific FA in the analyzed lipid sample. Thus, PC 15:0, TG 17:0, FFA 17:0, and CE 22:0 (1 mg/ ml in chloroform/ methanol (2:1; v/v)) were used as internal standard for FAME quantification from PL, TG, FFA and SE classes, respectively. TG 17:0 was used as internal standard for FAME quantification of total lipids extracted from Human serum and rat liver and plasma.

2.6. Statistical analysis

Data were expressed as mean values with standard deviation (mean ± SD). Methods 1, 2 and 3 were repeated six times (n = 6) each on each individual lipid class (PL, TG, FFA, SE) and on the three lipid mixtures (Human serum, rat liver and plasma). Intergroup comparisons were made on the basis of their respective mean. Data were analyzed by a Kruskal-Wallis test followed by a Dunns post hoc test. P values lower

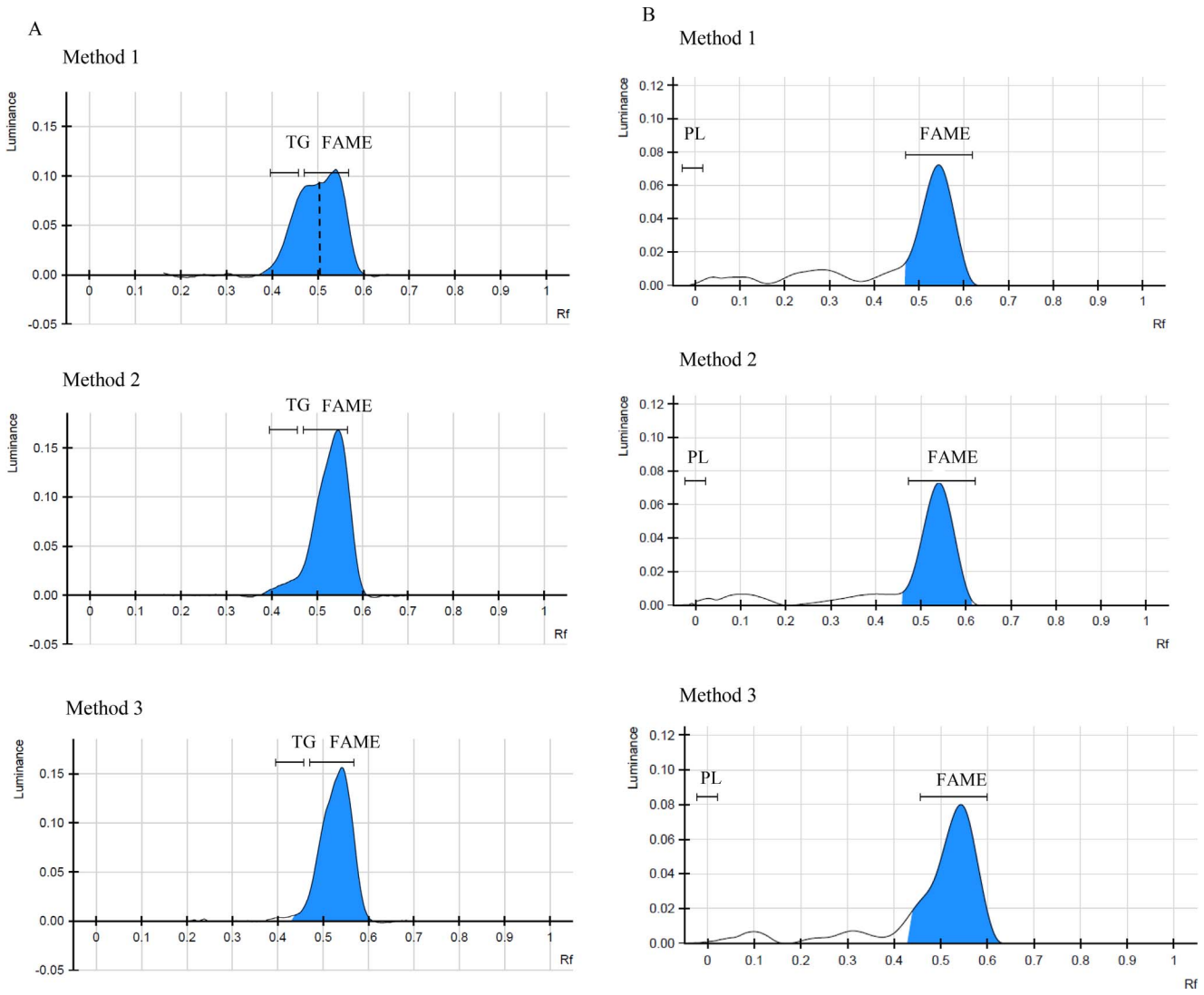

Fig. 1. HP-TLC of individual lipid classes subjected to the three methylation methods. (A) TG from fish oil; (B) PL purified from a marine lecithin; (C) FFA obtained by enzymatic lipolysis of a fish oil; (D) SE purified from the gonads of *Pecten maximus*. Methods 1, 2 and 3 were adapted from Morrison and Smith (1964), Castro-Gómez et al. (2014), and Christie et al. (2001), respectively. Method 1 consisted in an acid catalysis with an incubation at 100 °C. Methods 2 and 3 used a sequential basic and then acid catalysis with an incubation at 100 °C and 50 °C, respectively. FAME: fatty acid methyl esters, FFA: free fatty acids, PL: phospholipids, Rf: frontal report; SE: sterol esters, TG: triglycerides.

than 0.05 were considered to be statistically significant.

3. Results and discussion

Acid-catalyzed reactions were mostly used to convert FA into FAME. However, basic-catalyzed reactions were reported to be more rapid and more suitable on some lipid classes like SE although they did not properly convert FFA and sphingolipids (Castro-Gómez et al., 2014; Christie, 1993; Liu, 1994). Moreover, numerous methods were performed at high temperatures (mostly 100 °C) possibly resulting in a degradation of FA of interest (CLA, PUFA). Some authors have also speculated that BF_3 solution was unstable, leading to artefacts when high concentrations or aging reagents were used (Christie, 1993; Fulk & Shorb, 1970; Klopfenstein, 1971; Park, Albright, Cai, & Pariza, 2001). In order to overcome these problems, several (trans)methylation methods based on sequential basic-, acid-catalyzed reactions have been proposed.

The yield of (trans)esterification of individual lipid classes was qualitatively determined by HP-TLC with the presence of unreacted species. Fig. 1A-D present the yield of the different lipid classes (trans) methylated by the three methods analyzed by HP-TLC. Fig. 1A clearly pointed out that TG were not totally transesterified when method 1 was used unlike the two other methods since unreacted TG were recorded on the HP-TLC plate with these method. This was confirmed by the partial transmethylation of the TG 17:0 standard added to fish oil before the (trans)methylation procedure (9.3 ± 0.2, 11.8 ± 0.1 and 11.5 ± 0.3 wt% for methods 1, 2 and 3, respectively) observed by GC analysis. Because TG 17:0 could not be subjected to oxidative process, it could be assumed that about 21% of 17:0 from internal standard were not transmethylated using method 1. This result was in agreement with data obtained previously on pure TG 13:0 when acid-catalyzed methods were used (Castro-Gómez et al., 2014). Indeed, about 23% of TG remained unesterified when BF_3 or acetylchloride/ methanol were used, while a basic catalyst (KOH or sodium methoxide), followed or not by an acid catalysis, resulted in total TG transmethylation. Complete transmethylation of TG from Atlantic salmon using methanolic sulfuric acid solution (1%) has also been reported by Schlechtriem, Henderson, and Tocher (2008). Irrespectively of the method, PL and FFA rich in ω-3 LC-PUFA were totally (trans)methylated as shown by the absence of unreacted lipids on HP-TLC plates and the only presence of FAME (Fig. 1B and C, respectively). These results were confirmed by the GC quantification of the standards added to samples before the (trans)

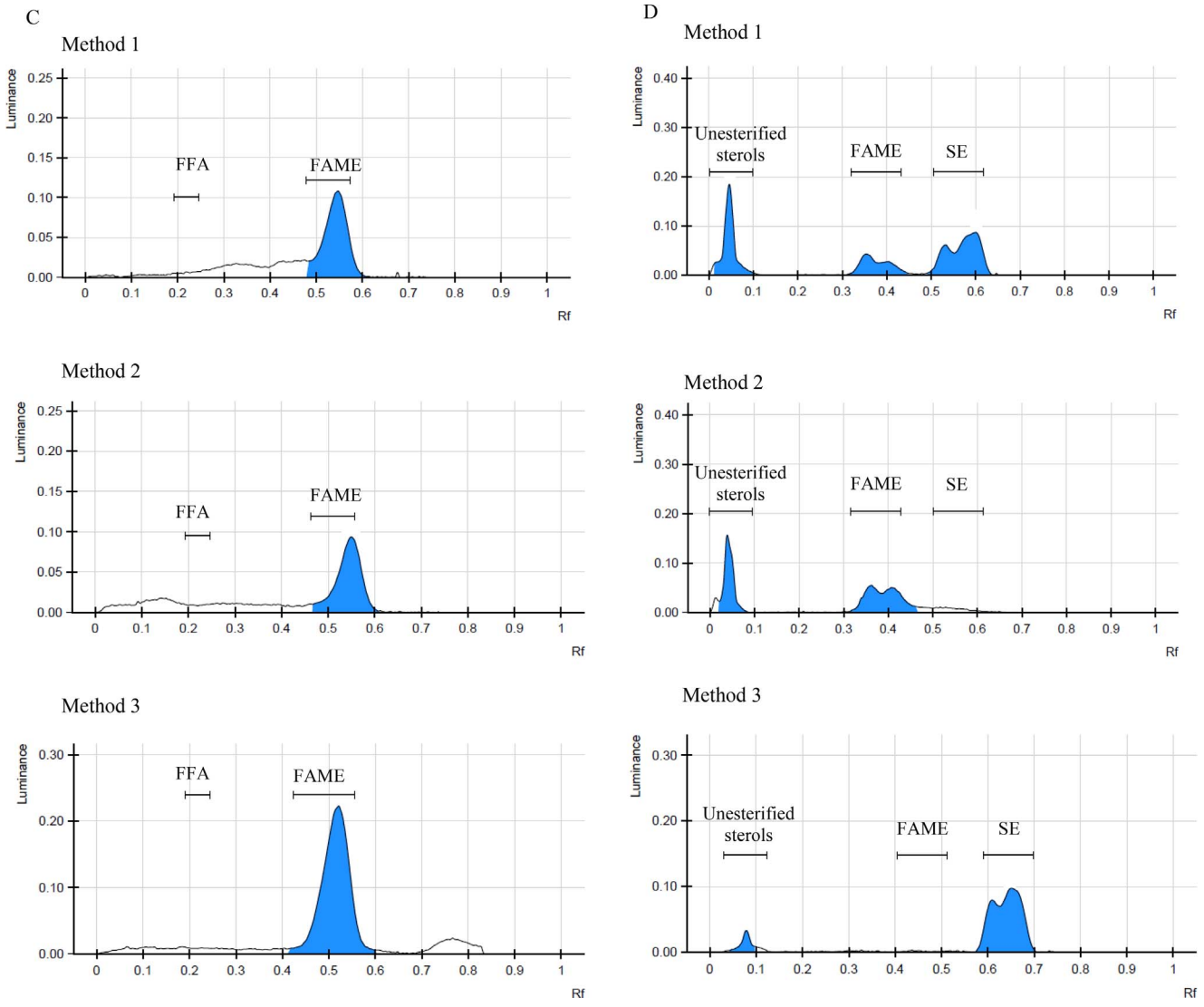

Fig. 1. (*continued*)

methylation procedure for the three methods (PC 15:0; 15.1 ± 0.7, 15.4 ± 0.7 and 14.8 ± 0.8 wt% for methods 1, 2 and 3, respectively and FFA 17:0; 13.1 ± 0.1, 13.0 ± 0.1 and 12.7 ± 0.1 wt% for methods 1, 2 and 3, respectively). Moreover, it appeared that the saponification step included in methods 2 and 3 was not necessary for total FFA esterification. With regard to SE, only method 2 led to the complete transmethylation (Fig. 1D) since no trace of unreacted SE and only the presence of FAME and free sterols were recorded on the HP-TLC plate contrary to methods 1 and 3. Otherwise, transmethylation of SE at moderate temperature (method 3) seemed to be the less efficient since FAME quantity were too low to be detected after HP-TLC of transmethylated samples. This qualitative observation was confirmed by GC recovery of CE 22:0 standard (12.1 ± 0.7, 18.8 ± 0.3 and 11.9 ± 0.4 wt% for methods 1, 2 and 3, respectively). It has been described that using basic catalysts or acid catalysts alone was inefficient for a complete SE transesterification (Castro-Gómez et al., 2014; Christie, 1993; Tuckey & Stevenson, 1979). For example, only 56% and 40% SE were transesterified when potassium hydroxide or BF_3 in anhydrous methanol were used, respectively (Castro-Gómez et al., 2014). In contrast, almost all SE were transesterified when saponification followed by an acid-catalyzed step were applied (Tuckey & Stevenson, 1979). Similar yield of transmethylation was obtained for cholesteryl ester (Castro-Gómez et al., 2014). Our results were in agreement with the finding that sequential basic and acid catalysis are required to transmethylate efficiently SE, but also an incubation at high temperature (100 °C) was fundamental.

Table 1 presents total FA quantification for TG from fish oil, transmethylated using the three methods. Quantification of FA issued from method 1 led to a higher content FA by 27% compared with the two other methods. We conclude that this overestimated value might partly result from the partial transmethylation of the internal standard (20%) but also to a slight loss of FA, presumably PUFA prone to oxidation. This incomplete transmethylation observed with method 1 significantly modified the FA profile, with a lower monounsaturated FA (MUFA) proportion by 5% and a higher content of ω-3 PUFA by 6% when compared with methods 2 and 3. In contrast, methods 2 and 3 led to similar results both in FAME quantification and proportions. Thus, the experimental conditions applied in method 2 (100 °C for 45 min) were not deleterious for ω-3 PUFA quantification. These results were in agreement with that obtained on aquatic organisms showing that the BF_3 method induced a loss of PUFA presumably due to addition of methanol across the double bonds (Schlechtriem et al., 2008).

Results for FA quantification of the marine PL are presented in Table 2. Only method 3 seemed to alter evaluation of FA composition of marine PL, compared with the two others, with a lower content of MUFA, accompanied by a higher content of ω-3 PUFA, notably EPA (20:5 ω-3) and DHA (22:6 ω-3). These results could account by the absence of nervonic acid (24:1n-9) in FA composition obtained with

Table 1
FA amount and composition of fish oil TG according to three transmethylation methods.

FA (g/100 g of total fatty acid)[1]	Method 1[2]	Method 2[2]	Method 3[2]
ΣSFA	27.8 ± 1.0	27.1 ± 0.6	26.7 ± 0.7
14:0	7.2 ± 0.8	6.8 ± 0.5	6.5 ± 0.6
16:0	16.6 ± 0.2^{a}	16.1 ± 0.2^{b}	16.1 ± 0.1^{b}
18:0	3.0 ± 0.0^{a}	3.1 ± 0.0^{b}	3.1 ± 0.0^{b}
Σ MUFA	39.1 ± 0.5^{a}	41.4 ± 0.3^{b}	41.4 ± 0.3^{b}
16:1n-7	7.9 ± 0.2^{a}	7.3 ± 0.1^{b}	7.3 ± 0.1^{b}
18:1n-9	13.0 ± 0.3^{a}	13.4 ± 0.2^{ab}	13.5 ± 0.3^{b}
18:1n-7	3.2 ± 0.0	3.2 ± 0.0	3.2 ± 0.0
20:1n-9	5.1 ± 0.2^{a}	6.0 ± 0.1^{b}	5.9 ± 0.1^{ab}
Σ PUFA	33.0 ± 0.6^{a}	31.3 ± 0.5^{b}	31.7 ± 0.9^{ab}
Σ ω-6 PUFA	4.4 ± 0.1	4.3 ± 0.0	4.4 ± 0.1
18:2n-6	2.8 ± 0.1	2.7 ± 0.0	2.7 ± 0.0
20:4n-6	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
Σ ω-3 PUFA	28.7 ± 0.5^{a}	27.2 ± 0.5^{b}	27.5 ± 0.9^{b}
18:3n-3	1.1 ± 0.0^{a}	1.0 ± 0.0^{b}	1.0 ± 0.0^{b}
20:5n-3	10.5 ± 0.1^{a}	9.5 ± 0.2^{b}	9.7 ± 0.3^{b}
22:5n-3	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.0
22:6n-3	9.1 ± 0.4	9.2 ± 0.2	9.4 ± 0.4
Total FA (μg/mg of TG)	1027.2 ± 51.6^{a}	806.0 ± 21.1^{b}	835.3 ± 52.0^{b}

Means in a line with superscripts without a common letter differ, $p < .05$.
Σ: sum of FA, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.
[1] Values are mean ± SD, n = 6.
[2] Methods 1, 2 and 3 were adapted from Morrison and Smith (1964), Castro-Gómez et al. (2014), and Christie et al. (2001), respectively. Method 1 consisted in an acid catalysis with an incubation at 100 °C. Methods 2 and 3 used a sequential basic and then acid catalysis with an incubation at 100 °C and 50 °C, respectively.

Table 2
FA amount and composition of PL purified from a marine lecithin according to three transmethylation methods.

FA (g/100 g of total fatty acid)[1]	Method 1[2]	Method 2[2]	Method 3[2]
Σ SFA	30.3 ± 0.6	30.5 ± 0.4	29.8 ± 0.5
14:0	2.9 ± 0.1^{ab}	3.0 ± 0.1^{a}	2.6 ± 0.2^{b}
16:0	24.2 ± 0.5	24.2 ± 0.2	24.0 ± 0.3
18:0	2.4 ± 0.1^{a}	2.4 ± 0.1^{a}	2.3 ± 0.0^{b}
Σ MUFA	16.2 ± 0.5^{a}	16.0 ± 0.3^{a}	15.4 ± 0.1^{b}
16:1n-7	2.6 ± 0.0	2.6 ± 0.0	2.6 ± 0.0
18:1n-7	3.7 ± 0.0^{a}	3.7 ± 0.0^{a}	3.8 ± 0.0^{b}
18:1n-9	4.5 ± 0.6	4.1 ± 0.0	4.1 ± 0.0
20:1n-9	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0
24:1n-9	0.6 ± 0.2^{a}	0.7 ± 0.3^{a}	0.0 ± 0.0^{b}
Σ PUFA	53.3 ± 0.6^{a}	53.3 ± 0.2^{a}	54.7 ± 0.5^{b}
Σ ω-6 PUFA	1.7 ± 0.0	1.7 ± 0.1	1.7 ± 0.1
18:2n-6	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
20:4n-6	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Σ ω-3 PUFA	51.8 ± 0.6^{a}	51.8 ± 0.2^{a}	53.1 ± 0.4^{b}
18:3n-3	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:5n-3	13.6 ± 0.1^{a}	13.6 ± 0.0^{a}	14.0 ± 0.1^{b}
22:5n-3	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.0
22:6n-3	35.5 ± 0.4^{a}	35.5 ± 0.2^{a}	36.5 ± 0.3^{b}
Total FA (μg/mg of PL)	459.7 ± 49.0	452.0 ± 48.5	471.8 ± 60.2

Means in a line with superscripts without a common letter differ, $p < .05$.
Σ: sum of FA, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.
[1] Values are mean ± SD, n = 6.
[2] Methods 1, 2 and 3 were adapted from Morrison and Smith, 1964, Castro-Gómez et al. 2014, and Christie et al. (2001), respectively. Method 1 consisted in an acid catalysis with an incubation at 100 °C. Methods 2 and 3 used a sequential basic and then acid catalysis with an incubation at 100 °C and 50 °C, respectively.

Table 3
FA amount and composition of FFA obtained by enzymatic lipolysis of fish oil according to three transmethylation methods.

FA (g/100 g of total fatty acid)[1]	Method 1[2]	Method 2[2]	Method 3[2]
Σ SFA	33.3 ± 0.1	33.3 ± 0.1	33.2 ± 0.0
14:0	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0
16:0	17.8 ± 0.1	17.7 ± 0.0	17.6 ± 0.0
18:0	12.8 ± 0.0^{ab}	12.9 ± 0.0^{a}	12.8 ± 0.0^{b}
Σ MUFA	45.8 ± 0.1	45.9 ± 0.1	45.9 ± 0.0
16:1n-7	2.2 ± 0.0^{a}	2.1 ± 0.0^{b}	2.2 ± 0.0^{ab}
18:1n-7	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0
18:1n-9	30.9 ± 0.0	31.0 ± 0.0	30.9 ± 0.0
20:1n-9	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0
Σ PUFA	20.9 ± 0.0	20.9 ± 0.0	20.9 ± 0.0
Σ ω-6 PUFA	12.1 ± 0.0	12.0 ± 0.0	12.1 ± 0.0
18:2n-6	10.5 ± 0.0	10.4 ± 0.0	10.4 ± 0.0
20:4n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Σ ω-3 PUFA	8.8 ± 0.0	8.8 ± 0.0	8.8 ± 0.0
18:3n-3	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0
20:5n-3	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0
22:5n-3	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
22:6n-3	1.7 ± 0.0	1.8 ± 0.0	1.8 ± 0.0
Total FA (μg/mg of FFA)	765.9 ± 17.3	767.3 ± 14.1	786.7 ± 8.4

Means in a line with superscripts without a common letter differ, $p < .05$.
Σ: sum of FA, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.
[1] Values are mean ± SD, n = 6.
[2] Methods 1, 2 and 3 were adapted from Morrison and Smith, 1964, Castro-Gómez et al. (2014), and Christie et al. (2001), respectively. Method 1 consisted in an acid catalysis with an incubation at 100 °C. Methods 2 and 3 used a sequential basic and then acid catalysis with an incubation at 100 °C and 50 °C, respectively.

Table 4
FA amount and composition of sterol esters from the gonads of *Pecten maximus* according to three transmethylation methods.

FA (g/100 g of total fatty acid)[1]	Method 1[2]	Method 2[2]	Method 3[2]
Σ SFA	16.8 ± 0.2^{a}	18.6 ± 1.7^{a}	22.7 ± 2.4^{b}
14:0	1.3 ± 0.1^{ab}	1.5 ± 0.1^{a}	0.8 ± 0.3^{b}
16:0	10.3 ± 0.2^{a}	10.5 ± 0.6^{ab}	12.4 ± 2.0^{b}
18:0	2.4 ± 0.1^{a}	3.7 ± 0.8^{ab}	5.5 ± 0.3^{b}
Σ MUFA	17.3 ± 0.7^{a}	18.2 ± 0.2^{ab}	20.2 ± 2.2^{b}
16:1n-7	3.5 ± 0.2^{a}	3.3 ± 0.1^{ab}	2.2 ± 0.3^{b}
18:1n-7	3.6 ± 0.1^{ab}	3.8 ± 0.1^{a}	2.5 ± 0.1^{b}
18:1n-9	1.7 ± 0.2^{a}	2.0 ± 0.5^{ab}	4.5 ± 2.4^{b}
20:1n-9	2.1 ± 0.1^{ab}	2.5 ± 0.1^{a}	1.5 ± 0.3^{b}
Σ PUFA	64.7 ± 0.7^{a}	61.9 ± 1.8^{ab}	57.2 ± 2.8^{b}
Σ ω-6 PUFA	7.5 ± 0.2^{ab}	8.0 ± 0.3^{a}	4.3 ± 0.8^{b}
18:2n-6	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.3
20:4n-6	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.4
Σ ω-3 PUFA	58.2 ± 0.7^{a}	55.2 ± 1.6^{ab}	52.8 ± 2.4^{b}
18:3n-3	0.9 ± 0.1^{a}	0.8 ± 0.0^{ab}	0.6 ± 0.1^{b}
20:5n-3	26.2 ± 0.5^{a}	24.4 ± 0.6^{ab}	22.7 ± 1.6^{b}
22:5n-3	1.4 ± 0.1^{a}	1.4 ± 0.1^{a}	0.0 ± 0.0^{b}
22:6n-3	22.9 ± 0.2	22.1 ± 0.7	23.6 ± 1.4
Total FA (μg/mg of SE)	447.1 ± 62.1^{a}	285.3 ± 12.5^{b}	452.3 ± 35.7^{a}

Means in a line with superscripts without a common letter differ, $p < .05$.
Σ: sum of FA, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.
[1] Values are mean ± SD, n = 6.
[2] Methods 1, 2 and 3 were adapted from Morrison and Smith (1964), Castro-Gómez et al. (2014), and Christie et al. (2001), respectively. Method 1 consisted in an acid catalysis with an incubation at 100 °C. Methods 2 and 3 used a sequential basic and then acid catalysis with an incubation at 100 °C and 50 °C, respectively.

method 3. Because the marine lecithin used in this work contained sphingomyelin (SM) with about 42% of 24:1n-9, this suggested that method 3 was ineffective to transmethylate this specific PL. Our results were in agreement with others showing that basic catalysis is not suitable for lipids such as sphingolipids in which FA are joined by an amide *N*-acyl bound to the base unless using very long incubation periods (up to 15 days for complete transesterification) which could create artefacts like *O*-methyl ethers or PUFA degradation (Christie, 1993; MacGee & Williams, 1981). In conclusion, it appeared that a method combining an acid catalysis and 90 min incubation time, at

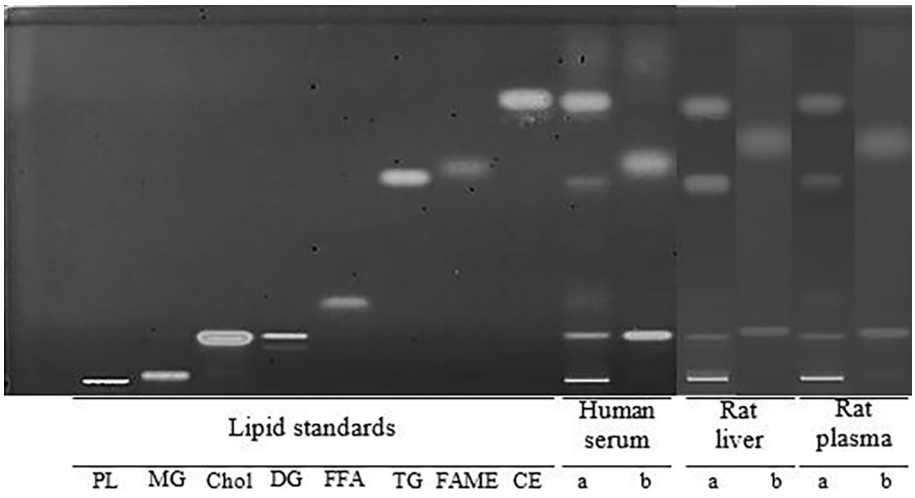

Fig. 2. HP-TLC plate picture of total lipids extracted from human serum, rat liver and plasma, (traces a) and their corresponding FAME obtained after (trans)methylation procedure using method 2 (Castro-Gómez et al., 2014) (traces b). Traces 1 to 8: lipid standards; 1: 1,2-diheptadecanoyl-sn-glycero-3-phosphatidylethanolamine (PE 17:0); 2: 1-monooleyl-rac-glycerol (MG 18:1n-9); 3: Cholesterol (Chol); 4: 1,3-di-11-eicosenoin (DG 20:1n-11); 5: α-linolenic acid (ALA; FFA); 6: 1,2,3-triheptadecanoyl-sn-glycerol (TG 17:0); 7: cis-8,11,14-eicosatrienoic acid methyl ester (FAME 20:3n-6); 8: cholest-5-en-3β-yl-octadec-9-oate (cholesterol ester 18:1n-9; CE). Traces 9 and 10: total lipids extracted from human serum (a) and the corresponding FAME (b). Traces 11 and 12: total lipids extracted from rat liver (a) and the corresponding FAME (b). Traces 13 and 14: total lipids extracted from rat plasma (a) and the corresponding FAME (b).

100 °C, as proposed in methods 1 and 2, could be considered as suitable methods for FA quantification of PL, even when sphingolipids are present.

Results for FFA prepared from fish oil lipolysis are presented in Table 3. No significant difference was observed concerning FAME quantification, in agreement with HP-TLC observations and internal standard quantification. Thus, all the three (trans)methylation methods led to a complete derivatization of FFA. Concerning FA composition, no difference was pointed out between results issued from the three methods. Thereby, all the three tested methods led to a correct assessment of FA quantification and composition of a FFA class composed of ω-3 PUFA. Moreover, ω-3 PUFA in their free form were not prone to oxidation during 45 min at 100 °C as applied in method 2.

Table 4 presents FA quantification for purified SE rich in ω-3 LC-PUFA, transmethylated using the three methods. Methods 1 and 3 led to a higher FA content by 57–59% compared with method 2. These overestimated values might partly result from the partial transmethylation of the internal standard (36–37%) but also to a loss of FA. No difference on FA composition was observed between methods 1 and 2, suggesting that the partial transmethylation of SE obtained with method 1 occurred independently of the FA nature esterified on the sterol. However, significant differences were observed between methods 1 and 3 on saturated FA (SFA), MUFA, and total ω-3 PUFA amounts. Notably, it appeared that 22:5n-3 esterified on sterols was not transmethylated with method 3. Thus, the temperature and the incubation time had a greater impact on the SE derivatization efficiency than the nature of catalyst. The three tested methods had not the same transmethylation yield and seemed to altered FA composition, thus it was not possible to conclude about the impact of the incubation temperature on oxidation of PUFA.

Since method 2 appeared to be the most efficient (trans)methylation method on all lipid classes rich in LC n-3 PUFA, it was applied on three different samples, i.e. total lipids extracted from Human serum and rat liver and plasma. These samples were chosen because they are classically analyzed in nutritional Human and animal studies and they contain different lipid classes, i.e. PL, TG, CE and FFA, in different proportions (Morris and Courtice, 1955; Yaqoob et al., 1995). (Trans) methylation efficiency was evaluated qualitatively by HP-TLC (Fig. 2). FA composition obtained by GC is presented in Supplementary data. In agreement with the results obtained on individual lipid classes, HP-TLC analysis after (trans)methylation of the three total lipid extracts confirmed that method 2 resulted in total (trans)methylation of all lipid classes present in the samples, with no unreacted species. Only free cholesterol and FAME were recorded. This suggested that (trans)methylation from the method 2 allowed correct FAME quantification irrespectively of the complexity of the lipid matrix rich in LC n-3 PUFA.

4. Conclusion

In order to ensure the reliability of the results concerning the FA composition and quantification, a (trans)methylation method adapted to all lipid classes analyzed in lipidomics, taken individually as well as in a mixture, should be selected. We demonstrated that the sequential basic/acid catalysis method with an incubation at 100 °C, as described by Castro-Gómez et al. (2014) could also be applied to purified lipids rich in PUFA. Because no significant difference was observed between methods 2 and 3 on total, n-6 and n-3 PUFA in FFA and TG classes, this suggested that an incubation of 100 °C did not induce a noticeable PUFA oxidation, at least in these two lipid classes. Our study also highlighted the importance of the choice of the lipid derivatization method. This may be of peculiar importance when an accurate fatty acid composition of a food matrix is required, in the case of n-3 PUFA supplementation for example. The incomplete (trans)methylation of some lipid classes can alter FA composition of total lipids. Unfortunately, this may not be overcome by the use of a single internal standard, because its proportion may also be under- or over-estimated depending on the method used. On the whole, FA compositions have to be interpreted with care within a study and, even more, when comparing studies using different (trans)methylation methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2018.01.060.

References

Castro-Gómez, P., Fontecha, J., & Rodríguez-Alcalá, L. M. (2014). A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples. *Talanta, 128*, 518–523. http://dx.doi.org/10.1016/j.talanta.2014.05.051.

Christie, W. W. (1993). Preparation of ester derivatives of fatty acids for chromatographic analysis. In W. W. Christie (Ed.). *Advances in lipid methodology – Two* (pp. 69–111). Dundee: Oily Press.

Christie, W. W., Sébédio, J. L., & Juanéda, P. (2001). A practical guide to the analysis of conjugated linoleic acid (CLA). *Inform, 12*, 147–152.

Desnuelle, P. (1961). Pancreatic lipase. *Advances in Enzymology and Related Subjects of Biochemistry, 23*, 129–161.

Entressangles, B., Sari, H., & Desnuelle, P. (1966). On the positional specificity of pancreatic lipase. *Biochimica Biophysica Acta, 125*(3), 597–600.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry, 226*(1), 497–509.

Fulk, W. K., & Shorb, M. S. (1970). Production of an artifact during methanolysis of lipids by boron trifluoride-methanol. *Journal of Lipid Research, 11*(3), 276–277.

ISO 12966-2:2011 - Animal and vegetable fats and oils – Gas chromatography of fatty acid methyl esters – Part 2: Preparation of methyl esters of fatty acids. Retrieved November 22, 2016.

ISO 12873:2017 – Olive oils and olive-pomace oils – Determination of wax content by capillary gas chromatography. June 2017.

Klopfenstein, W. E. (1971). On methylation of unsaturated acids using boron trihalide-methanol reagents. *Journal of Lipid Research, 12*(6), 773–776.

Kramer, J. K. G., Fellner, V., Dugan, M. E. R., Sauer, F. D., Mossoba, M. M., & Yurawecz, M. P. (1997). Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. *Lipids, 32*(11), 1219–1228. http://dx.doi.org/10.1007/s11745-997-0156-3.

Lepage, G., & Roy, C. C. (1988). Specific methylation of plasma nonesterified fatty acids in a one-step reaction. *Journal of Lipid Research, 29*(2), 227–235.

Liu, K.-S. (1994). Preparation of fatty acid methyl esters for gas-chromatographic analysis of lipids in biological materials. *Journal of the American Oil Chemists' Society, 71*(11), 1179–1187. http://dx.doi.org/10.1007/BF02540534.

MacGee, J., & Williams, M. G. (1981). Preparation of sphingolipid fatty acid methyl esters for determination by gas-liquid chromatography. *Journal of Chromatography, 205*(2), 281–288.

Marinetti, G. V. (1966). Low temperature partial alcoholysis of triglycerides. *Journal of Lipid Research, 7*(6), 786–788.

Morris, B., & Courtice, F. C. (1955). The protein and lipid composition of the plasma of different animal species determined by zone electrophoresis and chemical analysis. *Quartely Journal of Experimental Physiology and Cognate Medical Sciences, 40*(2), 127–137.

Morrison, W. R., & Smith, L. M. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *Journal of Lipid Research, 5*, 600–608.

Murrieta, C. M., Hess, B. W., & Rule, D. C. (2003). Comparison of acidic and alkaline catalysts for preparation of fatty acid methyl esters from ovine muscle with emphasis on conjugated linoleic acid. *Meat Science, 65*(1), 523–529. http://dx.doi.org/10.1016/S0309-1740(02)00244-9.

Park, Y., Albright, K. J., Cai, Z. Y., & Pariza, M. W. (2001). Comparison of methylation procedures for conjugated linoleic acid and artifact formation by commercial (trimethylsilyl) diazomethane. *Journal of Agricultural and Food Chemistry, 49*(3), 1158–1164.

Santos Júnior, O. O., Montanher, P. F., Bonfaé, E. G., Maruyama, S. A., Carbonera, F., Alberici, R. M., et al. (2013). Efficiencies of acid and base-catalyzed methylation of vegetable oils by ambient mass spectrometry. *Journal of the Brazilian Chemical Society, 24*(11), 1764–1771. http://dx.doi.org/10.5935/0103-5053.20130221.

Schlechtriem, C., Henderson, R. J., & Tocher, D. R. (2008). A critical assessment of different transmethylation procedures commonly employed in the fatty acid analysis of aquatic organisms: Fatty acid derivatization. *Limnology and Oceanography, Methods, 6*(10), 523–531. http://dx.doi.org/10.4319/lom.2008.6.523.

Tuckey, R. C., & Stevenson, P. M. (1979). Methanolysis of cholesteryl esters: conditions for quantitative preparation of methyl esters. *Analytical Biochemistry, 94*(2), 402–408.

Yaqoob, R., Sherrington, E. J., Jeffery, N. M., Sanderson, P., Harvey, D. J., Newsholme, E. A., & Calder, P. C. (1995). Comparison of the effects of a range of dietary lipids upon serum and tissue lipid composition in the rat. *The International Journal of Biochemistry and Cell Biology, 27*(3), 297–310.