A critical assessment of different transmethylation procedures commonly employed in the fatty acid analysis of aquatic organisms

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Abstract

Several transmethylation procedures have been used for fatty acid analysis of aquatic organisms although the suitability of the applied procedures has rarely been tested. The aim of this study was to demonstrate how different derivatization procedures can affect the result of fatty acid analysis. Different transmethylation procedures based on the acidic catalysts boron trifluoride, concentrated sulphuric acid and anhydrous hydrochloric acid were applied to cold-pressed copepod oil and Atlantic salmon flesh lipids rich in wax esters and triacylglycerols, respectively. The results show that 1) the use of unsuitable catalysts and/or incubation conditions may influence the data obtained which can lead to inaccurate conclusions about the presence of fatty acids in aquatic organisms/ecosystems 2) different derivatization procedures based on the same catalyst can produce diverging results and 3) the efficiency of a selected catalyst/procedure should be verified (e.g. by thin-layer chromatography) to ensure the complete transmethylation of fatty acids.

Abbreviations

ANOVA, analysis of variance; ASFL, Atlantic salmon flesh lipids; BHT, butylated hydroxyl toluene; CPCO, cold-pressed copepod oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; FFA, free fatty acid; GC, gas chromatography; HPTLC, high performance thin-layer chromatography; HUFA, highly unsaturated fatty acids; MUFA, monounsaturated fatty acids; NL, neutral lipids; n-3 PUFA, n-3 polyunsaturated fatty acids; n-6 PUFA, n-6 polyunsaturated fatty acids; PL, polar lipids; Rf, retention factor; SFA, saturated fatty acids; SNK, Student Newman Keuls Test; TAG, triacylglycerols; TLC, thin-layer chromatography; total PUFA, total polyunsaturated fatty acids; WE, wax esters;

Introduction

The volume of literature on lipids in aquatic organisms has expanded greatly during the last two decades showing an increasing interest in the fatty acid composition of marine and freshwater species (Bergé and Barnathan 2005; Tocher 2003; Arts and Wainman 1999). The fatty acids of biogenic lipids are esterified within various lipid classes and have to be transesterified to methyl esters prior to analysis by gas chromatography. This can be achieved by several derivatization procedures based on different acidic or basic catalyst reagents (e.g. boron trifluoride, sodium methoxide, hydrochloric and sulphuric acids). Common procedural deficiencies associated with ester preparation have been described. The incomplete conversion of fatty acids esterified within lipids to fatty acid methyl esters (FAME), the structural alteration of fatty acids during esterification and the formation of artefacts can all affect the quantitative yields of FAME and their relative proportions (Christie 1993; Shantha and Napolitano 1992). No single procedure is suitable for all types of lipids (Christie 2003) however, a clear picture about how the results of fatty acid analysis can vary, depending on the techniques applied, is missing.

Several derivatization techniques have been used in studies on aquatic organisms although the suitability of the applied procedures has rarely been tested. The aim of this study was to assess how the use of inappropriate derivatization procedures can affect the result of fatty acid analysis. Commonly used derivatization procedures based on different catalysts were applied to two samples of lipids from aquatic organisms characterized by high proportions of triacylglycerols (TAG, Atlantic salmon flesh) and wax esters (WE, copepod).

Materials and Procedures

Transmethylation procedures

Methanolic Sulphuric Acid (1%) FAME were prepared by acid-catalysed transesterification of total lipid as described by Christie (2003). Lipids (1mg) were resuspended in 1mL toluene prior to derivatization. Two mL of methanolic H_2SO_4 (1% v/v) were added and the vials sealed with nitrogen. The reaction mixture was heated in a stoppered tube at 50ºC overnight (16 h). Water (2mL) containing potassium bicarbonate (2%; w/v) was added and the transmethylated fatty acids extracted with hexane/diethylether (1:1, by vol; 2*5mL) containing 0.01% (w/v) butylated hydroxyl toluene (BHT).

Methanolic Boron Trifluoride (14%) Fatty acids were derivatized by a modified procedure originally described by Morrison and Smith (1964). Lipids (1mg) were resuspended in 2mL hexane prior to derivatization. Two mL of BF_3-methanol (14% w/w, Supelco B1252) were added and the reactivials® sealed with nitrogen. The mixture was refluxed at 70ºC for 2 h. After the sample had cooled down one mL of ultrapure water was added to the vial and the hexane layer containing FAME was carefully removed. The derivatization mixture was then washed twice with one mL hexane to extract the remaining FAME.

Hydrochloric acid in methanol (3M) Fatty acids were transesterified with a modified version of the procedure described by Mason and Waller (1964). The lipid samples (1mg) were resuspended in 3mL of methanolic HCl (3M; Supelco, 33050-U) and subsequently incubated at 60ºC for 15 min in a stoppered vial sealed with nitrogen. FAME were extracted two times with 3mL of hexane.

Methanolic Boron Trifluoride (12.5%) after saponification Free fatty acids (FFA) were prepared according to the AOAC-IUPAC Method 969.33 (AOAC, 1990). Lipid samples (1mg) were incubated at 100ºC for 10 min after addition of 2mL of methanolic NaOH solution (0.5N). Following saponification, samples were cooled down to room temperature. 2mL of BF_3-methanol (14% w/w, Supelco B1252 diluted with water-free methanol to 12.5%) were added to the reactivials® and the samples reheated for 2 min (100ºC). Samples were cooled down again and 1mL of n-heptane added. All samples were vortexed and reheated to 100ºC for 1 min. Finally, one mL of saturated NaCl solution was added to guarantee the complete separation and transfer of the heptane phase containing the methylated fatty acids.

Hydrochloric acid in methanol (5%, w/v) Lipids (1mg) were transesterified with 5% (w/v) anhydrous methanolic HCl as described by Christie (2003). The procedure is in accordance with the method described above using methanolic sulphuric acid (1%). All solvents used in this study were HPLC grade. Extracts of FAME prepared by the different procedures were evaporated to dryness under nitrogen gas and resuspended in 0.1mL chloroform/methanol (2:1).

Lipid class analysis

The separation of FAME from other lipid classes was performed by high performance thin-layer chromatography (HPTLC). Two µL of FAME extract diluted in100µL chloroform/methanol (1:1) were loaded as a 2 mm streak on HPTLC plates (10 cm x 10 cm x 0.15 mm), precoated with silica gel 60 (Merck, Darmstadt, Germany). Plates without fluorescent indicators were used. The plates were developed to two-thirds distance with methyl acetate/isopropanol/chloroform/methanol/ 0.25% aqueous KCl

(25:25:25:10:9, by vol.). After desiccation, the plate was fully developed with isohexane/diethyl ether/acetic acid (85:15:1, by vol.). The classes were quantified by charring at 160ºC for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and quantified by densitometry using a Camag 3 TLC Scanner (Camag, Muttenz, Switzerland) and winCATS software (Henderson and Tocher 1992). The identities of individual lipid classes were confirmed by comparison with reference to the Rf values of authentic standards run alongside samples on HPTLC plates and developed in the above solvent systems. The lipid class composition of the original lipid samples and the FAME extracts obtained after derivatization were compared.

Fatty acid analysis

Transesterified fatty acids were purified by thin-layer chromatography (TLC) prior to GC analysis. Samples were applied as 2 cm streaks to TLC plates (20 cm x 20 cm x 0.15 mm) precoated with silica gel 60 (Merck, Darmstadt, Germany) and FAME separated from non-derivatized lipid classes using hexane/diethyl ether/acetic acid (90:10:1, by vol.) as developing solvent. FAME were separated and quantified by gas-liquid chromatography (Fisons GC8600, Fisons Ltd., Crawley, U.K) using a 30 m x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak Ltd., London, U.K.). The GC was equipped with on-column injection and a flame ionisation detector (FID). Hydrogen was used as carrier gas and temperature programming was from 50^{o}C to 150^{o}C at 40^{o}C min^{-1} and then to 230^{o}C at 2.0^{o}C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman 1980; Tocher and

Harvie 1988). Data were collected and processed using the Chromcard for Windows (Version 1.19) computer package (Thermoquest Italia S.p.A., Milan, Italy).

Methylation of free fatty acids

Saponification Ten mg of a FAME standard mix (Supelco, UK 1859) were hydrolysed by refluxing in a 1M solution of potassium hydroxide in 95% ethanol as described by Christie (2003). The solvent was evaporated by a stream of oxygen-free nitrogen and the FFA redissolved in 1mL of chloroform/methanol (2:1, by vol.).

Re-methylation Aliquots of 100µL were dried down under nitrogen and the FFA re-methylated with different derivatization procedures (3 replicates). The methylation efficiency was verified by HPTLC and FAME analysed by gas chromatography (GC) without further purification steps.

Statistical analysis

Data recorded as percentages were arcsine-transformed to ensure a normal distribution and subjected to analysis of variance (ANOVA). Student Newman Keuls Test (SNK) was used to identify differences among treatment means ($p<0.05$) (SigmaStat 3.1 software).

Assessment

Critical assessment of different transmethylation procedures (Experiment 1) Three methods commonly cited in the literature for the derivatization of fatty acids were selected to assess how different procedures can affect the result of fatty acid analysis. The selected methods were based on the acidic catalyst reagents methanolic sulphuric acid (H_2SO_4, 1%), methanolic boron trifluoride (BF_3, 14%) and methanolic hydrochloric acid

(HCl, 3M). To test their derivatization efficiency, all procedures were applied to two types of lipids from aquatic organisms which were characterized by different lipid class compositions (Figure 1). Cold-pressed copepod oil (CPCO) from *Calanus finmarchius* contained a high proportion of wax esters (WE, 77.1%) and a minor fraction of triglycerols (TAG, 2.9%). Due to the extraction procedure, this oil was free of polar lipids (PL). Lipids extracted from Atlantic salmon flesh (ASFL) by the procedure of Folch et al. (1957) were rich in TAG (79%). The lipid extract further contained a low proportion of FFA (4.4%) and several PL classes including phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol adding up to 9.7%.

Lipid class proportions in transmethylated CPCO and ASFL were analyzed by HPTLC to investigate whether all lipid classes containing fatty acids were completely converted to FAME. All derivatization procedures were tested in triplicate. WE consist of fatty acids esterified to long-chain fatty alcohols. Fatty acids in wax esters of CPCO were largely transmethylated with H_2SO_4 in contrast to the BF_3 and HCl based procedures, which resulted in a ratio of wax esters to FAME of about 2:1 (Figure 1A). Samples derivatized with these procedures also contained residual proportions of TAG (1-1.5%). The overall higher proportions of FAME in comparison to free fatty alcohols after transmethylation may be due to the lipid class determination method. The FAME which are rich in PUFA are known to stain much more intensely than the free fatty alcohols which are predominantly saturated and monounsaturated (Henderson and Tocher 1992).

The H_2SO_4 catalysed transmethylation of ASFL resulted in a complete derivatization of TAG and all PL classes. However, a small fraction of FFA (1.6%) and sterol esters (0.4%) could be determined (Figure 1B). The lipid class compositions of

samples transmethylated with the BF_3 and HCl based procedures were characterized by high proportions of TAG with 58.9% and 55.3%, respectively. FAME represented only a smaller fraction of 33.5% (BF_3) and 38.1% (HCl). In contrast to the neutral lipid (NL) fraction, which further consisted of 1.6% (BF_3) and 1.4% (HCl) underivatized sterol esters, all PL of these samples were completely transmethylated.

The fatty acid composition of CPCO transmethylated with the different procedures is presented in Table 1. The lowest proportions of total monounsaturated fatty acids (MUFA) were obtained for aliquots derivatized with methanolic HCl and BF_3 with 15.0% and 16.0%, respectively. CPCO transmethylated with methanolic H_2SO_4 showed a significantly higher proportion of MUFA (22.4%) but a lower percentage of n-3 polyunsaturated fatty acids (n-3 PUFA; 34.2%) in comparison to the other treatments (40.1-42.7%). No differences were observed for the proportions of saturated fatty acids (SFA, 38.5-40.1%) and n-6 polyunsaturated fatty acids (n-6 PUFA; 2.5-2.7%).

The transmethylation of ASFL was also leading to diverging fatty acid patterns (Table 2). In aliquots transmethylated with the H_2SO_4 procedure, monounsaturates represented the major group of fatty acids with 42.2% followed by SFA and total PUFA with around 29.0% each. In contrast, transmethylation with the other derivatization procedures resulted in relatively higher proportions of SFA (34.2-38.2%) and total polyunsaturated fatty acids (total PUFA; 30.5-32.0%) but in lower levels of monounsaturates (31.3-33.8%).

The results of this study indicate that the BF_3 and HCl based derivatization procedures are obviously not suitable to ensure the complete transesterification of fatty acids from all lipid classes. Christie (1993) stated that certain classes of simple lipids,

such as cholesterol esters and TAG, are not soluble in methanolic HCl and BF_3 alone and an inert solvent must be added to ensure their solution and derivatization. The HCl based procedure did not contain such a solvent. This might explain, together with the short reflux time (15 min) and the relatively low incubation temperature (60°C), the low transmethylation efficiency obtained by this method. The BF_3 based procedure involves hexane as inert solvent and is characterized by a relatively long reaction time of 2 h. The poor results obtained indicate that BF_3-methanol is of limited use as a transesterification reagent which is in accordance with Christie (2003) but in contradiction to Ackman (1998) who stated that BF_3-methanol alone seems to be suitable for the derivatization of fish oil.

It is evident that the differential derivatization of the CPCO and ASFL lipid classes had a clear effect on the resulting fatty acid patterns. The decreased proportions of MUFA observed for ASFL derivatized with methanolic HCl and BF_3 for instance can be explained with the incomplete conversion of NL-fatty acids which are relatively rich in MUFA (Sargent et al. 2002) in comparison to PL-fatty acids which were completely transmethylated.

Only CPCO aliquots transmethylated by the H_2SO_4 based procedure, part of which is a 16 h incubation period at 50°C, were free of WE residues. The fatty acid moieties in WE of *Calanus finmarchicus* consist of a range of fatty acids including SFA, MUFA and PUFA (Table 1). The lowest MUFA levels were estimated for CPCO aliquots derivatized by the BF_3 and HCl procedure indicating that different WE were transmethylated at different rates.

However, it cannot be excluded that differences in the fatty acid patterns were also caused by the destruction of certain fatty acids during derivatization. In the second part of this study we therefore investigated the effect of the three different derivatization procedures on the recovery of fatty acids after methylation.

Suitability of acidic catalyst for the esterification of fatty acids (Experiment 2) A FAME standard mix (Table 3) which comprised different saturated, monounsaturated and PUFA including highly unsaturated fatty acids (HUFA, e.g. EPA and DHA) was saponified and equal aliquots of the FFA re-methylated with the different derivatization procedures.

HPTLC analysis confirmed that the saponified fatty acids were completely re-methylated to FAME by all procedures tested in this study. Re-methylation with the H_2SO_4 and HCl based procedures resulted in similar fatty acid compositions (Table 3). However, slightly decreased EPA and DHA proportions in comparison to the original FAME standard mix were estimated. These differences were probably caused by the partial destruction of these fatty acids during the saponification procedure since both catalysts are unlikely to produce differential losses of specific fatty acids during esterification (Christie 1993) under the conditions applied.

In contrast, the BF_3 catalysed methylation of the saponified fatty acids resulted in reduced proportions of all HUFA. This might be explained by the relatively long incubation period of 2 h at 70ºC. BF_3-methanol (14%) is a rapid and powerful catalyst for the esterification of fatty acids and the reflux time could be reduced to a few minutes (Christie 1993). However, the application of the full transesterification protocol gave us the opportunity to investigate which fatty acids are prone to destruction under such

incubation conditions. The addition of methanol across the double bonds may be one explanation for the observed loss of polyunsaturated fatty acids (Christie 2003).

Critical assessment of different transmethylation procedures (Experiment 3) The first assessment study showed that the use of different derivatization procedures can lead to diverging results regarding the fatty acid composition of lipid samples. However, it would be wrong to draw general conclusions about the suitability of the three catalyst reagents used based on a single investigation since there is evidence from other studies (Christie 1993) that the derivatization conditions may have a significant effect on the data obtained. Therefore, the aim of the third part of this study was to examine how modifications to the transmethylation procedures may affect the results of fatty acid analysis. Two alternative transmethylation procedures based on HCl-methanol and BF_3-methanol were selected and applied to CPCO and ASFL. Aliquots of both lipids were also transmethylated by the methanolic H_2SO_4 (1%) based procedure applied during the first study to make the results of both studies comparable. The second methanolic HCl based protocol was characterized by a lower catalyst concentration (5%) and a longer reflux time (16 h). The second methanolic BF_3 (12.5%) based protocol included a saponification step and was characterized by a clearly reduced incubation time of 3 min at 100°C.

Differences in the lipid class composition of CPCO and ASFL analyzed during the first and third study can be explained by the use of different sample material. As in the first study, aliquots of the derivatized samples were separated by HPTLC to check the transmethylation efficiency of the applied procedures. NL of CPCO were completely derivatized by all methods (Figure 2), apart from minor amounts of TAG (0.7%) and WE

(0.2%) found after BF_3 catalysed derivatization and 1.3% FFA in the aliquots transmethylated with the H_2SO_4 based procedure. Also TAG and PL, as the major lipid classes of ASFL, were completely derivatized by all procedures. However, small proportions of FFA (2.8%) and sterol esters (0.7%) were still found after H_2SO_4 catalysed transmethylation. Minor proportions of sterol esters were also found in samples derivatized with BF_3 (0.8%) and HCl (0.5%).

CPCO transmethylated with the H_2SO_4 and HCl based procedures showed comparable fatty acid patterns (Table 4) which is in accordance with Christie (1993). In contrast to these treatments the BF_3 catalysed derivatization produced significantly lower proportions of total n-3 PUFA and increased percentage values for SFA.

ASFL transmethylated with the three different procedures resulted in overall comparable fatty acid patterns (Table 5). Differences were only observed for 20:5n-3, 18:4n-3 and total C16 polyunsaturated fatty acids (C16 PUFA) with the lowest proportions again observed for the aliquots derivatized with methanolic BF_3. The results obtained are another indication for the destruction of fatty acids by BF_3-methanol as catalyst reagent and are thus in accordance with the results of the re-methylation study described before. BF_3-methanol has a limited shelf-life and there is some evidence that especially the use of old reagent may have adverse effects on the esterification of sensitive fatty acids (Ackman, 1998, Christie 2003). However, fresh BF_3-methanol reagent was used for the first derivatization study and the reagent was kept refrigerated in a tightly closed bottle until the second study was carried out only two weeks later. Generally, for all studies dry reagents and glassware were used to avoid the presence of

water as this would affect the acid-catalysed esterification of fatty acids as well as the transesterification of lipids.

In summary we can say that 1) the use of unsuitable catalysts and/or derivatization conditions (e.g., time and temperature) may significantly influence the fatty acid data obtained and 2) different derivatization procedures based on the same catalyst can produce diverging results.

Discussion

No single derivatization procedure is suitable for all types of lipids (Christie 1993, 2003). This study demonstrates how the use of unsuitable methods chosen for transmethylation can affect the result of fatty acid analysis. Fatty acid data are usually presented on a percentage or absolute basis. It is clear that the incomplete derivatization of a lipid sample has a direct impact on the estimated amount of fatty acids. In contrast, a procedure with a low transmethylation efficiency can principally still produce accurate proportional data given that all lipid samples are derivatized at the same rate. However, this was not confirmed by the results of this study.

Individual methods applied in this assessment study (e.g., HCl, 5%; H_2SO_4, 1%) appear to have a higher suitability for the derivatization of lipids than others however, the results are based on the analysis of only two different types of lipids and thus cannot be generalized. That means also that a method which turned out to be unsuitable for the derivatization of CPCO and ASFL [e.g., methanolic BF_3 (14%) without saponification] might be still appropriate for the transmethylation of lipid samples characterized by a different lipid class and fatty acid composition. The fact that no single procedure is

available for the derivatization of all types of lipids requires that the suitability of transmethylation procedures should be always critically evaluated especially when new types of lipid samples have to be analyzed. According to the literature all methods tested in this study are commonly used for fatty acid analysis of a broad range of lipids from marine and freshwater species. It can be only speculated how often inappropriate derivatization procedures are applied leading to inaccurate conclusions about the presence of fatty acids in aquatic organisms/ecosystems.

Comments and recommendations

The results of this study emphasize that the transmethylation of fatty acids should be always verified and optimized if required to achieve good quantitative results. A simple TLC separation of derivatized lipid samples can give important information about the efficiency of the transmethylation procedure applied. In brief, a small volume (5-10µl) of FAME sample is spotted along with a TLC reference standard (e.g. NU-Chek-Prep 18-4) on a precoated TLC plate (20cm x 20cm x 0.15 mm) and developed in the solvent system hexane/diethyl ether/acetic acid (85:15:1, by vol.). The plate is taken out of the TLC chamber when the solvent front has reached the top of the plate. The plate should dry in air and then be sprayed with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid. After charring the plate at 160°C for 15min the pattern of lipid composition and thus the completeness of FAME preparation can be checked.

Several reviews about derivatization techniques (see references) have been published in the past containing a wealth of methodological information, which can guide the analyst to optimize established procedures or to adopt improved methods of analysis.

The participation in inter-laboratory trials and/or proficiency schemes may help to recognize suitable and unsuitable analytical practice.

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Figures

Figure 1**.** Lipid class composition of cold pressed copepod oil (Fig.1A) and Atlantic salmon flesh lipids (Fig.1B) after transmethylation with different derivatization procedures. Mean values ± SD of the same lipid class with a different superscript letter are significantly different (n=3; Student-Newman-Keuls test; p=0.05); ASFL, Atlantic salmon flesh lipids (underivatized); CPCO, cold pressed copepod oil (underivatized); FAME, fatty acid methyl esters

Figure 2. Lipid class composition of cold pressed copepod oil (Fig. 2A) and Atlantic salmon flesh lipids (Fig. 2B) after transmethylation with different derivatization procedures. Mean values ± SD of the same lipid class with a different superscript letter are significantly different (n=3; Student-Newman-Keuls test; p=0.05); ASFL, Atlantic salmon flesh lipids (underivatized) ; CPCO, cold pressed copepod oil (underivatized); FAME, fatty acid methyl esters

Figure 1.

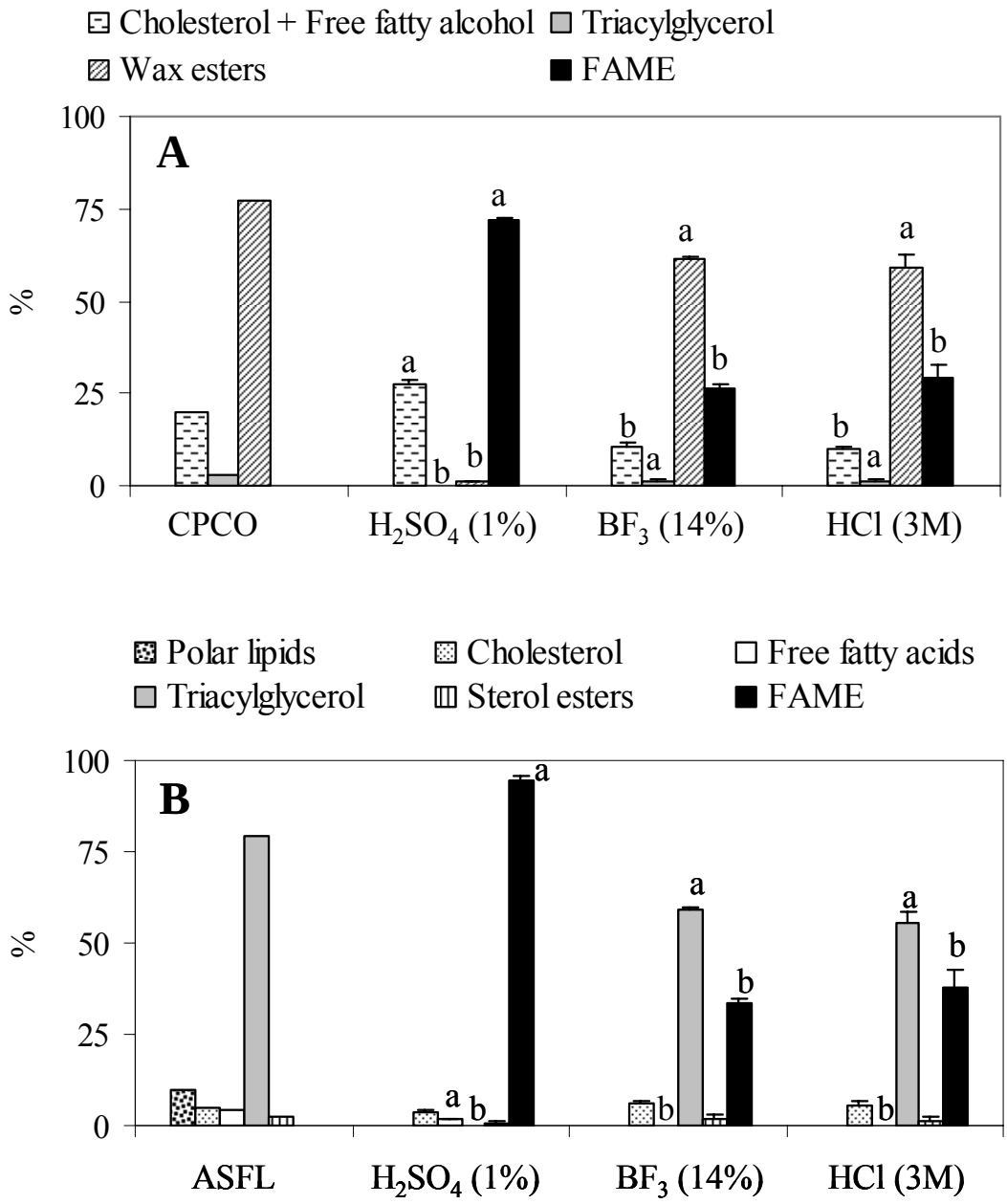
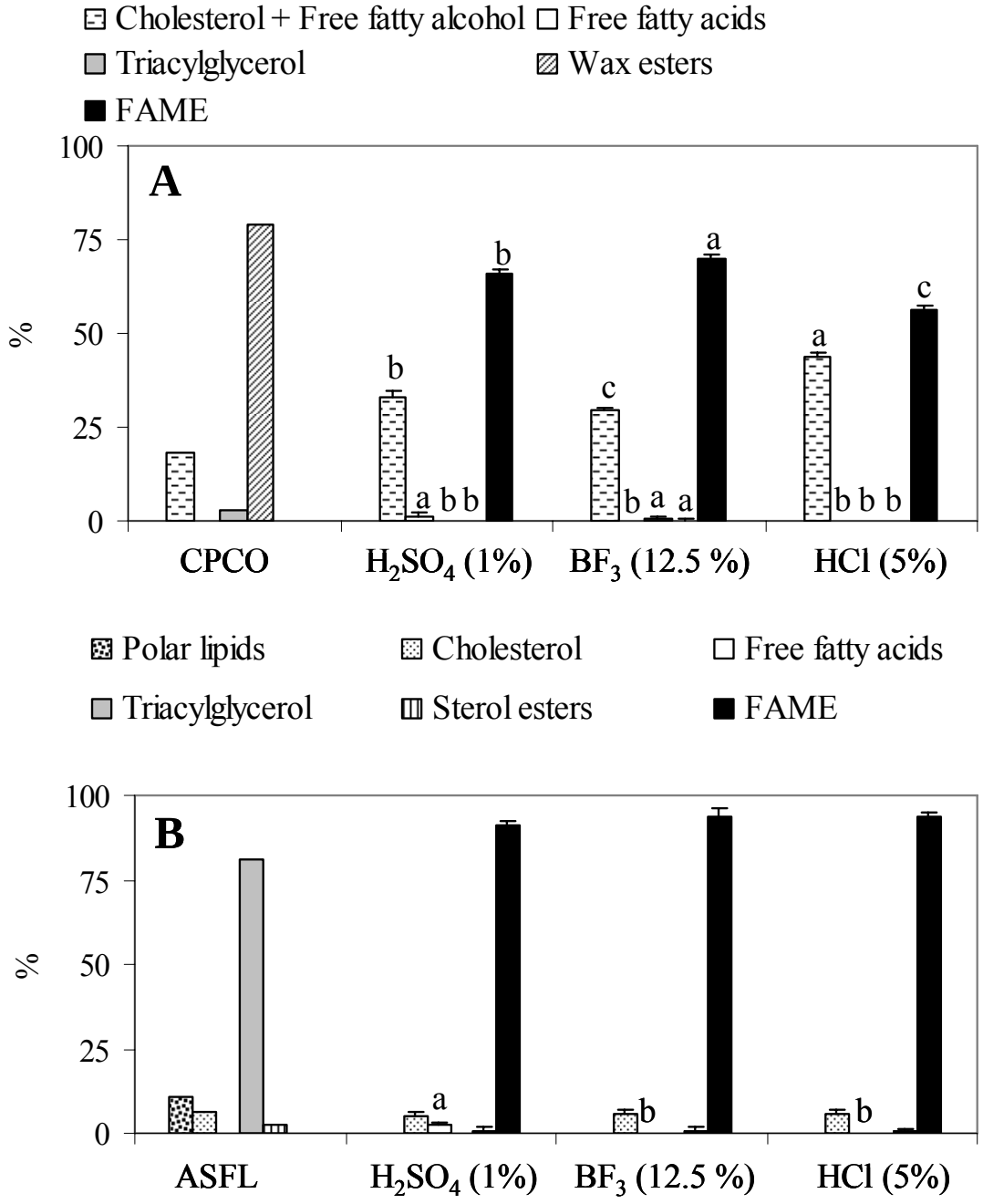

Cholesterol + Free fatty alcohol
Triacylglycerol
Wax esters
FAME
A
%
100
75
50
25
0
CPCO
H2SO4 (1%)
BF3 (14%)
HCl (3M)
Polar lipids
Cholesterol
Free fatty acids
Triacylglycerol
Sterol esters
FAME
B
%
ASFL
H2SO4 (1%)
BF3 (14%)
HCl (3M)

Figure 2.


Cholesterol + Free fatty alcohol
Free fatty acids
Triacylglycerol
Wax esters
FAME
A
%
100
75
50
25
0
b
a b b
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b a a
a
a
b b b
c
CPCO
H2SO4 (1%)
BF3 (12.5 %)
HCl (5%)
Polar lipids
Cholesterol
Free fatty acids
Triacylglycerol
Sterol esters
FAME
B
%
100
75
50
25
0
a
b
b
ASFL
H2SO4 (1%)
BF3 (12.5 %)
HCl (5%)

Table1. Fatty acid composition (percentage of total fatty acids) of copepod oil transmethylated with different derivatization procedures.

	H_2SO_4 (1%)		±SD	BF_3 (14%)		±SD	HCL (3M)		±SD
14:0	20.4	a	0.5	14.5	c	0.7	16.8	b	0.7
15:0	0.8	a	0.0	0.6	b	0.0	0.6	b	0.0
16:0	17.3	c	0.3	22.4	a	1.3	19.1	b	0.9
18:0	1.0	c	0.1	1.9	a	0.0	1.4	b	0.2
20:0	0.6		0.1	0.6		0.0	0.6		0.1
Total saturated	40.0		0.8	40.1		1.8	38.5		1.5
16:1n-9/n-7	4.1		0.4	4.3		0.1	4.1		0.3
18:1n-9	5.2		0.4	4.8		0.1	4.4		0.3
18:1n-7	0.7		0.1	0.7		0.1	0.7		0.0
20:1n-9	4.9	a	0.1	2.7	b	0.1	2.6	b	0.2
22:1n-11/n-9	7.2	a	0.6	2.9	b	0.0	2.9	b	0.1
24:1	0.4	b	0.0	0.5	a	0.0	0.3	b	0.0
Total monounsaturated	22.4	a	1.4	16.0	b	0.0	15.0	b	1.0
16:2	0.3	c	0.0	0.3	b	0.0	0.4	a	0.0
16:3	0.3	c	0.0	0.3	b	0.0	0.4	a	0.0
16:4	0.3	b	0.0	0.5	a	0.0	0.4	a	0.0
Total C16 PUFA	0.9	c	0.0	1.1	b	0.0	1.2	a	0.0
18:2n-6	1.9	b	0.1	2.2	a	0.0	2.1	a	0.1
18:3n-6	0.1		0.0	0.1		0.1	0.1		0.0
20:2n-6	0.3		0.0	0.2		0.0	0.3		0.0
20:4n-6	0.2		0.0	0.2		0.0	0.2		0.0
Total n-6 PUFA	2.5		0.1	2.7		0.1	2.6		0.1
18:3n-3	3.4	b	0.1	4.0	a	0.1	4.1	a	0.1
18:4n-3	17.6	b	0.4	18.0	b	0.8	20.6	a	0.5
20:3n-3	0.1		0.0	0.0		0.0	0.0		0.0
20:4n-3	1.3	b	0.0	1.3	b	0.0	1.4	a	0.0
20:5n-3	6.6	b	0.1	8.8	a	0.3	9.0	a	0.3
22:5n-3	0.3		0.0	0.3		0.0	0.4		0.0
22:6n-3	4.8	b	0.0	7.8	a	0.5	7.3	a	0.5
Total n-3 HUFA	13.2	b	0.1	18.2	a	0.9	18.0	a	0.7
Total n-3 PUFA	34.2	c	0.6	40.1	b	1.8	42.7	a	0.5
Total PUFA	37.6	c	0.6	43.9	b	1.9	46.5	a	0.6

Mean values within a row with a different superscript letter are significantly different (n=3; SNK test; p=0.05); PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids

Table 2. Fatty acid composition (percentage of total fatty acids) of salmon flesh lipids transmethylated with different derivatization procedures.

	H_2SO_4 (1%)		±SD	BF_3 (14%)		±SD	HCL (3M)		±SD
14:0	6.0		0.0	6.6		0.4	6.9		0.3
15:0	0.5	b	0.0	0.6	a	0.0	0.6	a	0.0
16:0	19.0	c	0.1	25.2	a	0.3	22.3	b	0.5
18:0	3.3	b	1.0	5.6	a	0.5	4.3	ab	0.3
20:0	0.2		0.0	0.2		0.0	0.2		0.1
Total saturated	29.0	c	0.9	38.2	a	0.3	34.2	b	0.5
16:1n-9	0.1		0.1	0.0		0.0	0.1		0.0
16:1n-7	6.9		0.0	6.2		0.1	6.9		0.5
18:1n-9	18.8	a	0.2	14.7	b	0.8	15.7	b	0.6
18:1n-7	3.5		0.0	3.5		0.2	3.3		0.1
20:1n-9	5.7	a	0.0	3.4	c	0.1	3.9	b	0.1
20:1n-7	0.3	a	0.0	0.0	b	0.1	0.2	a	0.0
22:1n-11/n-9	6.2	a	0.1	3.1	b	0.3	3.3	b	0.2
24:1	0.7	a	0.0	0.4	b	0.1	0.3	b	0.1
Total monounsaturated	42.2	a	0.3	31.3	c	1.1	33.8	b	1.3
16:2	0.5	b	0.0	0.5	b	0.0	0.6	a	0.0
16:3	0.3	b	0.0	0.3	b	0.0	0.4	a	0.0
16:4	0.5	c	0.0	0.6	b	0.0	0.6	a	0.0
Total C16 PUFA	1.3	b	0.0	1.4	b	0.0	1.6	a	0.1
18:2n-6	7.0	a	0.2	6.4	b	0.2	7.1	a	0.2
18:3n-6	0.2		0.1	0.2		0.0	0.2		0.1
20:2n-6	0.5		0.0	0.6		0.0	0.6		0.0
20:3n-6	0.1		0.0	0.1		0.0	0.1		0.0
20:4n-6	0.5	b	0.0	0.7	a	0.1	0.7	a	0.0
22:5n-6	0.2		0.0	0.2		0.1	0.2		0.0
Total (n-6) PUFA	8.5	ab	0.2	8.2	b	0.2	8.8	a	0.2
18:3n-3	1.4		0.0	1.5		0.2	1.5		0.0
18:4n-3	1.2	b	0.0	1.2	b	0.0	1.4	a	0.1
20:3n-3	0.1		0.0	0.1		0.0	0.1		0.0
20:4n-3	1.0	a	0.0	1.0	b	0.0	1.1	a	0.0
20:5n-3	5.5	b	0.2	5.7	b	0.3	6.2	a	0.1
22:5n-3	2.0	a	0.0	1.8	b	0.1	1.9	a	0.1
22:6n-3	7.5	b	0.2	9.6	a	0.7	9.5	a	0.9
Total (n-3) HUFA	16.2	b	0.4	18.2	a	1.0	18.8	a	1.1
Total (n-3) PUFA	18.9	b	0.5	20.9	a	1.0	21.7	a	1.0
Total PUFA	28.8	b	0.7	30.5	a	0.8	32.0	a	0.9

Mean values within a row with a different superscript letter are significantly different (n=3; SNK test; p=0.05); PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids

Table 3. Fatty acid composition (percentage of total fatty acids) of a FAME standard mixture before and after saponification and remethylation with different derivatization procedures.

	FAME - Standard	H_2SO_4 (1%)	±SD	BF_3 (14%)	±SD	HCL (3M)	±SD
14:0	8.3	7.9 [b]	0.1	8.6 [a]	0.1	8.0 [b]	0.1
16:0	8.4	8.9 [b]	0.1	9.7 [a]	0.1	8.9 [b]	0.1
18:0	7.6	7.5 [b]	0.0	8.2 [a]	0.1	7.5 [b]	0.0
Total saturated	24.4	24.3 [b]	0.1	26.5 [a]	0.1	24.4 [b]	0.1
16:1n-7	8.5	8.5 [b]	0.1	9.1 [a]	0.0	8.5 [b]	0.1
18:1n-9/7	17.3	18.1 [b]	0.1	19.1 [a]	0.1	18.1 [b]	0.0
20:1n-9	8.7	8.9 [b]	0.0	9.3 [a]	0.0	8.9 [b]	0.0
22:1n-9	8.8	9.0 [b]	0.1	9.4 [a]	0.1	9.0 [b]	0.0
Total monounsaturated	43.2	44.5 [b]	0.1	46.9 [a]	0.1	44.6 [b]	0.1
18:2n-6	8.6	8.9	0.0	8.8	0.0	8.9	0.0
20:5n-3	8.5	7.8 [a]	0.1	6.6 [b]	0.0	7.7 [a]	0.0
22:5n-3	8.4	8.1 [a]	0.1	6.4 [b]	0.1	8.0 [a]	0.0
22:6n-3	7.0	6.5 [a]	0.1	4.8 [b]	0.1	6.4 [a]	0.1
Total PUFA	32.4	31.2 [a]	0.2	26.6 [b]	0.2	31.0 [a]	0.1

Mean values within a row with a different superscript letter are significantly different (n=3; SNK test; p=0.05); FAME, fatty acid methyl esters; PUFA, polyunsaturated fatty acids

Table 4. Fatty acid composition (percentage of total fatty acids) of copepod oil transmethylated with different derivatization procedures.

	H_2SO_4 (1%)	±SD	BF_3 (12.5%)	±SD	HCL (5%)	±SD
14:0	15.1 [b]	0.9	17.8 [a]	1.8	13.8 [b]	1.0
15:0	0.6 [b]	0.0	0.8 [a]	0.1	0.6 [b]	0.0
16:0	13.5 [b]	0.9	17.3 [a]	1.6	12.8 [b]	1.0
18:0	0.8 [b]	0.1	1.0 [a]	0.1	0.8 [b]	0.1
20:0	0.1 [b]	0.0	0.2 [a]	0.0	0.1 [b]	0.0
22:0	nd [b]		0.1 [a]	0.1	nd [b]	
Total saturated	30.1 [b]	2.0	37.1 [a]	3.6	28.1 [b]	2.2
16:1n-9/n-7	4.3 [a]	0.2	3.7 [b]	0.3	4.3 [a]	0.3
18:1n-9	4.9	0.3	4.3	0.4	5.0	0.3
18:1n-7	0.6	0.0	0.6	0.0	0.6	0.0
20:1n-9	4.6 [b]	0.3	4.6 [b]	0.1	5.3 [a]	0.3
20:1n-7	nd		0.1	0.0	nd	
22:1n-11/n-9	7.0	0.4	6.8	0.6	7.2	0.7
24:1	0.4	0.0	0.4	0.1	0.4	0.0
Total monounsaturated	21.8	1.2	20.4	1.4	22.8	1.5
16:2	0.6	0.3	0.3	0.0	0.4	0.0
16:3	0.3	0.0	0.3	0.0	0.3	0.1
16:4	0.2	0.1	0.2	0.1	0.4	0.0
Total C16 PUFA	1.1 [a]	0.2	0.8 [b]	0.1	1.1 [a]	0.0
18:2n-6	2.1	0.1	2.0	0.2	2.1	0.1
18:3n-6	0.3 [a]	0.1	0.2 [b]	0.0	0.1 [b]	0.0
20:2n-6	0.3	0.1	0.2	0.0	0.2	0.0
20:3n-6	0.0	0.1	0.1	0.0	nd	
20:4n-6	0.3	0.0	0.2	0.0	0.3	0.0
22:5n-6	nd		0.1	0.0	nd	
Total n-6 PUFA	2.9	0.2	2.8	0.2	2.7	0.1
18:3n-3	4.0 [a]	0.1	3.7 [b]	0.1	4.0 [a]	0.1
18:4n-3	20.2 [b]	1.2	18.2 [c]	0.3	21.7 [a]	0.0
20:3n-3	0.1	0.1	0.1	0.0	0.1	0.0
20:4n-3	1.7	0.0	1.6	0.1	1.7	0.0
20:5n-3	9.2 [a]	0.2	7.9 [b]	0.7	9.4 [a]	0.2
22:5n-3	1.3	0.5	0.7	0.1	0.6	0.0
22:6n-3	7.7	0.1	6.7	0.8	7.8	0.3
Total n-3 HUFA	20.0 [a]	0.3	17.0 [b]	1.5	19.6 [a]	0.5
Total n-3 PUFA	44.1 [a]	1.0	38.9 [b]	2.0	45.3 [a]	0.6
Total PUFA	48.1 [a]	1.1	42.4 [b]	2.2	49.1 [a]	0.7

Mean values within a row with a different superscript letter are significantly different (n=3; SNK test; p=0.05); PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids

Table 5. Fatty acid composition (percentage of total fatty acids) of salmon flesh lipids transmethylated with different derivatization procedures.

	H_2SO_4 (1%)		±SD	BF_3 (12.5%)		±SD	HCL (5%)		±SD
14:0	4.8		0.5	5.1		1.2	5.0		0.4
15:0	0.4		0.0	0.4		0.1	0.4		0.0
16:0	17.2		1.4	18.1		1.5	17.2		1.2
18:0	3.5		0.3	6.0		1.9	3.5		0.2
20:0	0.2		0.0	0.2		0.0	0.2		0.0
22:0	0.1		0.0	0.1		0.1	0.0		0.0
Total saturated	26.2		2.3	30.0		4.5	26.4		1.9
16:1n-9	0.2		0.0	0.0		0.0	0.1		0.1
16:1n-7	6.0		0.0	5.9		0.3	6.2		0.2
18:1n-9	17.0		0.1	17.1		0.7	17.3		0.3
18:1n-7	3.1		0.0	2.8		0.5	3.0		0.1
20:1n-9	5.2		0.0	5.9		0.7	5.3		0.2
20:1n-7	0.3		0.0	0.3		0.0	0.3		0.0
22:1n-11/n-9	5.7		0.1	6.4		0.8	5.8		0.4
24:1	0.6		0.0	0.6		0.1	0.7		0.1
Total monounsaturated	38.1		0.1	38.9		2.0	38.6		1.0
16:2	0.5	a	0.0	0.4	b	0.1	0.5	a	0.0
16:3	0.4	a	0.0	0.2	b	0.1	0.4	a	0.0
16:4	0.4		0.0	0.2		0.1	0.5		0.0
Total C16 PUFA	1.3	a	0.0	0.8	b	0.2	1.4	a	0.0
18:2n-6	7.7		0.2	7.5		0.2	7.8		0.2
18:3n-6	0.1		0.0	0.1		0.0	0.1		0.0
20:2n-6	0.6		0.0	0.5		0.1	0.6		0.0
20:3n-6	0.1		0.0	0.1		0.0	0.1		0.0
20:4n-6	0.6		0.1	0.5		0.2	0.6		0.0
22:4n-6	0.1		0.0	0.1		0.0	0.1		0.0
22:5n-6	0.2		0.0	0.2		0.1	0.2		0.0
Total n-6 PUFA	9.4		0.3	8.9		0.5	9.4		0.3
18:3n-3	1.6		0.1	1.2		0.3	1.6		0.1
18:4n-3	1.5	a	0.1	1.0	b	0.3	1.4	a	0.0
20:3n-3	0.2		0.0	0.1		0.0	0.2		0.0
20:4n-3	1.3		0.1	1.1		0.3	1.3		0.0
20:5n-3	7.0	a	0.5	5.9	b	0.4	6.9	a	0.1
22:5n-3	2.9		0.2	3.3		0.9	2.8		0.1
22:6n-3	10.5		1.0	8.8		1.8	10.1		0.4
Total n-3 HUFA	21.9		1.8	19.2		2.0	21.3		0.6
Total n-3 PUFA	24.9		1.9	21.4		2.5	24.2		0.7
Total PUFA	35.6		2.2	31.2		3.2	35.0		0.9

Mean values within a row with a different superscript letter are significantly different (n=3; SNK test; p=0.05); PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids