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Determination of Origin of Atlantic Salmon (*Salmo salar*): The Use of Multiprobe and Multielement Isotopic Analyses in Combination with Fatty Acid Composition To Assess Wild or Farmed Origin

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Variability within the stable isotope ratios in various lipidic fractions and the fatty acid composition of muscle oil has been analyzed for a large sample (171 fish) of wild and farmed Atlantic salmon (*Salmo salar*) from 32 origins within Europe, North America, and Tasmania. Sampling was extended over all seasons in 2 consecutive years and included fish raised by different practices, in order to maximize the range of variation present. It is shown that two readily measured parameters, δ^{15}N measured on choline and δ^{18}O measured on total oil, can be successfully used to discriminate between fish of authentic wild and farmed origin. However, the certainty of identification of mislabeling in market-derived fish is strengthened by including the percentage of linoleic acid C18:2n-6 in the lipidic fraction. Thus, several apparent misidentifications were found. The combination of these three analytical parameters and the size of the database generated makes the method practical for implementation in official laboratories as a tool of labeling verification.

KEYWORDS: Atlantic salmon (*Salmo salar*); stable isotopes; IRMS; authenticity; salmon; pyrolysis; fatty acids

INTRODUCTION

With marine resources becoming evermore depleted, the EC is increasingly required to impose fishing quotas for species of fish of commercial interest. On the other hand, government health services encourage the consumption of fish, and most consumers are well aware of the beneficial roles of omega-3 and omega-6 fatty acids in helping combat the onset of cardiovascular diseases. Despite a European regulation in 2001 imposing more detailed labeling of fish and fish products (*1*) with a particular concern for the label to indicate whether the fish are of wild or farmed origin, there is to date still no official methodology to monitor whether these labeling requirements are respected.

Various means to discriminate between fish of dissimilar origins have been investigated in several previous studies. These have used a range of intrinsic parameters, notably the compositional analysis of fatty acids, the nature of the xanthin pigments present, and isotopic ratios in body tissue and/or bone. In all cases, the aim has been to define criteria that can differentiate between fish derived from different production areas and/or regimes. Distinguishing between wild and farmed fish is relatively straightforward, as there are significant differences in their diets that lead to distinct compositional characteristics.

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For example, the fatty acid composition of cultured and wild sturgeon, determined by gas chromatography and subjected to statistical analysis, was successfully used to distinguish between individuals from one or the other of these two populations (*2*). Similarly, flesh coloration in salmonids is highly dependent on the carotenoids present in their diet. Because the xanthins accumulated in the fish muscle are not the same chemical species in wild and culture populations (*3*) (the pigment found in wild fish is astaxanthin, whereas the diet of most farmed salmons is supplemented with canthaxanthin), it should be relatively easy to distinguish between these populations. However, the maximum permitted level of canthaxanthin (25 mg/kg of feed) is not sufficient to give an attractive coloration to the flesh, and the fish diet frequently also contains astaxanthin, both from natural and synthetic origins. Since chiral chromatography can be used to differentiate natural from synthetic astaxanthin [the *meso* form (3*R*,3′*S*) does not occur naturally], a high level of this isomer is a good indicator of a farmed origin (*4, 5*). However, much of the astaxanthin used in fish feed is produced from cultured microalgae or from krill, meaning that virtually any ratio of the astaxanthin stereoisomers may be manufactured by modern techniques (*5*). Volatile compounds have also been investigated as a way to differentiate cultured and wild gilthead sea bream (*6*). However, this analysis may be biased by different storage conditions. The major problem with all of these compositional analyses is that they are diet-dependent, and it is now relatively straightforward for fish feed producers to adjust the composition of the diet.

An alternative approach that has proved very successful in tracing the biological and geographical origin of food matrices is to determine a range of isotopic parameters in a number of different chemicals or tissues (*7*). Isotopic composition is primarily determined by diet, which itself is influenced by the environment, and by subsequent metabolism of primary dietary inputs (*8*). In the first study to apply these techniques to aquaculture in the context of authentication, Aursand et al. (*9*) showed that the stable isotope content found in salmon reflects both the environment in which it is grown and the composition of the diet consumed and, when combined with fatty acid composition, could distinguish wild from farmed salmon (*9*). Similarly, characteristic isotopic signatures for flesh $\delta^{13}C$ and $\delta^{15}N$ values differentiated wild and farmed Atlantic salmon (*Salmo salar*) from Newfoundland (*10*), while gilthead sea bream (*Sparus aurata*) from four origins could be separated into wild and farmed populations on these same parameters (*11*). However, a similar study on Atlantic salmon (*S. salar*) with the principal objective of identifying organic/conventional farmed fish and a secondary interest in farmed/wild fish concluded that isotopic techniques alone were insufficient (*12*). Nevertheless, by combining both isotopic and fatty acid compositional analyses with artificial neural network statistical treatment of the data, it proved possible to categorize the studied samples into their three categories. However, in this study only global $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ were measured on lyophilized muscle tissue. Focusing on specific groups of compounds should improve the robustness of the method, as whole flesh values are more subject to environmental fluctuations. The muscle lipids, notably the fatty acids, have proved to be a sound isotopic probe (*9*) ,but the xanthins, although able to show some discrimination (*3, 13*), are probably too susceptible to manipulation of the source.

Hence it is evident that isotopic signatures, either alone or combined with chemical compositional analysis, are a realistic approach to the problem of origin identity in fish. However, those studies detailed above either tested relatively limited ranges of fish or focused on one or two specific parameters. In none of the reports to date has a general methodology been assessed, nor has its robustness been fully tested. In order to fill this void, in 2001 we initiated with funding from the European Commission a large-scale analysis sampling in total 225 salmon from 32 origins, with the objective of establishing traceability criteria for the salmon found in the marketplace in Europe. The study covered all major European origins of cultivated and wild salmon as well as including fish from the southern hemisphere. Samples were subjected to a wide range of analyses, including triacylglyceride structure and fatty acid composition, global $\delta^{13}C$ and $\delta^{15}N$ values on muscle and other body tissue, global $\delta^{18}O$ on muscle flesh, lipids, and body water, and ^{2}H analysis of the glycerol and fatty acids of the muscle lipids. This paper reports the analyses of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values on muscle components ($\delta^{13}C_{oil}$, $\delta^{18}O_{oil}$, $\delta^{13}C_{glycerol}$, $\delta^{15}N_{choline}$, $\delta^{18}O_{glycerol}$, $\delta^{13}C_{fatty\ acids}$, $\delta^{13}C_{muscle}$, $\delta^{15}N_{muscle}$, $\delta^{18}O_{water}$) and fatty acid composition and shows that from these a method that effectively can verify the farmed/wild salmon obtained over a wide geographical range can be derived.

MATERIALS AND METHODS

Samples. The reference group was composed of 171 salmon collected from 32 different geographical origins covering the major producing countries: Norway (northern and southern), Scotland, Ireland, Iceland, the Faeroe Islands, Canada, and Tasmania (**Figure 1**). Each sample set was composed of at least five individual fish (six to eight for some wild origin acquisitions), taken at random from a larger population. Salmon were collected from several farms having different farming practices, and fishermen were asked to collect wild salmon in different regions.

A further sample set composed of 54 salmon was collected by the scientific partners in markets or supermarkets in France, Italy, Norway, or the United Kingdom. This independent sample set was used to test and validate the methodology.

Salmon were collected from 7 January 2002 to 21 August 2003. Each fish was coded, and its details were logged in an ACCESS2000 database. The following parameters were recorded: species, exact wild/farmed origin, geographical location, season and date of collection, age, sex, weight, length, water temperature, type of feed for farmed fish, and additional information.

Water samples were taken at the same location as the fish samples. These were logged in the database.

Feed samples from different farming regimes were collected as appropriate and logged in the database.

Sample Handling and Preparation. At sampling, fish were immediately chilled and frozen at −20 °C and then, as required, transferred to −80 °C for storage. Frozen fish at −80 °C were transported in polystyrene boxes with ice packs and a 48 h delivery requirement. Under these conditions, fish sent from one partner to another arrived frozen.

Following defrosting to ice-cold temperature, each salmon carcass was divided into the following fractions: muscle, head, bones, otoliths, vertebrae, and scales. Unless otherwise indicated, these fractions were stored at −20 °C or dispatched to appropriate partners under the same conditions as for whole fish.

Muscle Oil Extraction. Oil was extracted from flesh muscle which had not been refrozen. Two laboratories were involved in this process. Following a preliminary analysis to establish the optimal extraction procedure (see Results and Discussion) a modified Bligh and Dyer method (*14*) was adopted. All operations were carried out at 4 °C. Essentially, salmon muscle (400 g taken from the region of the dorsal fin) was diced and homogenized in a blender. To this was added 1200 mL of ice-cold $CHCl_3$/MeOH (1:2), and the mixture was homogenized in an Ultraturrax (1 min/1 min rest/1 min). The homogenate was filtered under vacuum through Whatman No. 1 filter paper. The vessels and residue were further washed with $CHCl_3$ (400 mL). To the

Figure 1. Salmon sites explored in this study.

combined filtrate was added 400 mL of 0.88% KCl, and the whole mixture was shaken vigorously for ~1 min before being left to stand to allow phase separation (~20–30 min). The organic (lower) phase was recovered and solvent removed by rotary evaporation *in vacuo* (40 °C). The residue was transferred to a desiccator and dried to constant weight *in vacuo*. The samples were stored under nitrogen in sealed, Teflon-capped, glass containers at −70 °C prior to analysis. For shipping, samples at −70 °C were packed in polystyrene boxes with ice packs and shipped in 48 h delivery. No antioxidant was added to the lipid samples.

Preparation of the Glycerol and Choline Fraction by Saponification. To 15 g of fish oil in a 500 mL round-bottomed flask fitted with a condenser was added with stirring 150 mL of 1 M KOH/EtOH solution (10 g of KOH in 150 mL of EtOH). The mixture was refluxed for 2 h (oil bath, 100 °C), with stirring. To the hot solution was added 75 mL of distilled water, and the solution was left to cool to room temperature. Then, 37% (v/v) HCl (~15 mL) was slowly added until the solution pH was ≈1, and 50 mL of distilled water was added to dissolve the KCl salts. The solution was decanted into a 500 mL separating funnel and washed with 4 × 75 mL of cyclohexane/diethyl ether (50/50 v/v). Fatty acids could be recovered from the organic phase. The aqueous phase was filtered through a sinter into a 500 mL round-bottomed flask and water removed by rotary evaporation (50 °C) to leave a white powdery residue. This was suspended in 100 mL of EtOH and filtered through a sinter into a preweighed 250 mL round-bottomed flask. The residue was washed with a further 100 mL of EtOH and the solvent removed from the combined phase by rotary evaporation. The viscous oil recovered was dried for 1 h at 70 °C under vacuum pump. Final traces of solvent were removed with a stream of N_2 gas during 1 h at 70 °C. The viscous oil containing principally glycerol and choline was stored under vacuum in a desiccator over P_2O_5.

Preparation of FAMEs by Transmethylation. To 2 g of fish oil in a 250 mL three-necked flask fitted with a condenser was added 30 mL of NaOH/MeOH (2% w/v). The mixture was refluxed for 30 min (oil bath, 90 °C) with stirring. After cooling, 20 mL methanol was added, followed by the slow addition of 6 mL of BF_3/MeOH (50% w/v) from a 50 mL dropping funnel. The solution was further refluxed for 30 min, after which 80 mL of cyclohexane was added, and it was left to cool to room temperature. After the addition of 20 mL of distilled water to dissolve the boron salts formed, 80 mL of saturated NaCl solution was added, and the whole mixture was transferred to a 500 mL separating funnel. The organic phase was recovered and the aqueous phase washed with 2 × 80 mL of cyclohexane. The combined organic phase was washed with 80 mL of distilled water, dried over anhydrous Na_2SO_4 sulfate, and filtered through a sinter. The residue was washed with 80 mL of cyclohexane. The solvent was removed by rotary evaporation from the combined organic phase in two stages: on a water bath at 30 °C maximum without vacuum, followed by continued evaporation for 1 h at 30 °C under low vacuum. The FAME sample was stored in a 5 mL vial at −30 °C, under N_2.

Gas Chromatography for FAME Composition Analysis. FAME composition was determined by capillary GC calibrated with authentic standards. In each sample vial was placed 6 μL of FAME sample, 1 mL of hexane, and 4 μL of internal standard solution [800 ppm 2,6-di-*tert*-butyl-*p*-cresol, also called BHT = bis(*tert*-butyl)hydroxytoluene]. Each sample was prepared in duplicate, and each vial was analyzed in duplicate within 24 h.

Samples (1 μL) were injected by autoinjection onto a SPB-PUFA fused silica column (30 m × 0.32 mm × 0.2 μm; www.supelco.com, product 24323) and mounted in a gas chromatograph (e.g., Agilent 6890N; www.home.agilent.com) with autosampler (e.g., Agilent 7683 injector), and the chromatogram was developed under the following conditions: carrier gas, He; flow, 1 mL/min in flow-control mode; injector temperature, 250 °C; detector temperature, 260 °C; thermal gradient, 140 °C for 2 min, then ramped at 8 °C/min to 210 °C, and then held at 210 °C for 42.25 min (total run time = 53 min). In these conditions, the retention times (minutes) for the 12 target FAMEs, representing the most important fatty acids found in salmon oil, were myristic acid C14:0 (9.36); palmitic acid C16:0 (11.87); palmitoleic acid C16:1n-7 (12.14); stearic acid C18:0 (15.34); vaccenic acid C18:1n-7 (15.78); oleic acid C18:1n-9 (15.63); linoleic acid C18:2n-6 (16.50); eicosenoic acid C20:1n-9 (21.66); eicosapentaenoic acid (EPA) C20:5n-3 (27.72); brassidic acid C22:1n-11 (32.10); docosapentaenoic acid (DPA) C22:5n-3 (43.57); docosahexaenoic acid (DHA) C22:6n-3 (45.84).

Quantification was carried out by reference to an internal standard (BHT, 5.00 g in 25 mL of pure hexane, stored at 5 °C) and multilevel calibration ($n = 3$) using a certified custom-designed standard solution (Supelco) of the 12 target FAMEs at equal concentrations (33.32 ± 0.04 mg/mL in 99.9% pure hexane, stored at −30 °C). The calibration

curves were established using the three following standard solutions: S1 (concentration of each FAME = 166 ppm), 5 μL of FAMEs standard solution + 1 mL of hexane + 4 μL of internal standard solution; S2 (concentration of each FAME = 330 ppm), 10 μL of FAMEs standard solution + 1 mL of hexane + 4 μL of internal standard solution; S3 (concentration of each FAME = 813 ppm), 25 μL of FAMEs standard solution + 1 mL of hexane + 4 μL of internal standard solution.

Integration of acquisition files and quantitative calculations were carried out automatically by the Agilent integration software. From these calibrations, a response factor for each FAME was calculated.

Stable Isotope Ratio Analysis (SIRA). Stable isotope ratio values were determined on several different isotope ratio mass spectrometers, each equipped with an elemental analyzer (EA) and/or pyrolysis oven, depending on the isotope to be measured.

Ratios $\delta^{13}C$ and $\delta^{15}N$ were determined by continuous helium flow EA-isotope ratio mass spectrometry (CF-EA-IRMS) using NA2100 Proteins (Carlo Erba Instruments, Milan, Italy) with oxidation temperature 1030 °C, reduction temperature 700 °C, and GC column temperature 25 °C, coupled to a Delta S mass spectrometer (ThermoElectron, Bremen, Germany).

Ratios $\delta^{18}O_{glycerol}$ and $\delta^{18}O_{oil}$ were determined by CF-EA-pyrolysis-IRMS (CF-EA-P-IRMS) using a NA1500 (Carlo Erba Instruments, Milan, Italy) with pyrolysis temperature 1060 °C and GC column temperature 55 °C, coupled to an Optima mass spectrometer (Micromass, Manchester, U.K.).

Ratios $\delta^{18}O_{water}$ were determined on the CO_2 gas resulting from off-line equilibration of water samples. Water (~4 mL) cryoextracted from salmon muscle was equilibrated with calibrated reference CO_2 gas as described in the EC regulation 822/97 (*15*). The CO_2 was introduced via a direct transfer dual-inlet interface into the Optima mass spectrometer (Micromass, Manchester, U.K.).

All IRMS results were expressed on the δ ‰ scale with respect to international standards according to the relationship

$$\delta\ (‰) = (R_{sample}/R_{standard} - 1) \times 1000$$

where R is the ratio of the heavy to the light isotope in the sample R_{sample} and in the standard $R_{standard}$.

International standards used were V-PDB (IAEA, Vienna) and V-SMOW (IAEA, Vienna) for $\delta^{13}C$ and $\delta^{18}O$, respectively (*16*), and atmospheric N_2 for $\delta^{15}N$. The working standards used for IRMS determinations were glutamic acid for $\delta^{13}C$ and $\delta^{15}N$, Nantes tap water for $\delta^{18}O_{water}$, and glucose for $\delta^{18}O_{glycerol}$ and $\delta^{18}O_{oil}$. These standards were calibrated against international standards. Uncertainties of the method used were estimated in our laboratory at 0.3‰ for $\delta^{13}C$, 0.5‰ for $\delta^{15}N$, 0.5 ‰ for $\delta^{18}O_{pyrolysis}$, and 0.35‰ for $\delta^{18}O_{water}$.

Secondary standards were run after every tenth sample, and data were drift corrected.

Data Treatment. Statistical analysis was carried out using the Statistica 6.0 software package (Statsoft, Inc.).

RESULTS AND DISCUSSION

Evaluation of Extraction and Transmethylation Methods for Salmon Muscle Lipids. The total analytical procedure required that ~30 g of lipid was prepared from each muscle sample.

Extraction. Three main lipid extraction methods, Bligh and Dyer (*14*), Folch (*17*), and petroleum ether (internal method) were evaluated for efficiency of extraction and ease of application. As shown in **Table 1**, although the Folch method (*17*) gave the highest yield of total oil, the Bligh and Dyer method (*14*) modified to include the addition of 0.88% KCl after filtration gave better recovery of phosphatidylcholine (with potential interest for $\delta^{15}N$), greatly sped up phase separation, and required significantly less solvent. This method was therefore adopted. Petroleum ether was abandoned because only triacylglycerides were extracted, which reduce the number of target molecules and number of parameters.

Table 1. Comparison of Methods for Extraction of Lipids from Salmon Muscle

method	total oil extracted (% wet wt)	PC	PE	CHOL	TAG
Bligh and Dyer, original	18.34	1.7	0.6	1.3	96.5
Bligh and Dyer, water added after filtering	16.84	2.1	0.8	1.2	95.5
Bligh and Dyer, 0.88% KCl added after filtering	16.48	3.0	0.8	1.5	94.6
Folch	20.36	2.4	0.6	1.3	95.0
petroleum ether	18.90	nd	nd	0.9	99.1

Transmethylation. Interlaboratory reproducibility for the transmethylation of oil to obtain FAMEs and the quantification of FAME composition was carried out by two laboratories (Eurofins and LAIEM), using the protocols described in Materials and Methods. For this purpose, a common sample of fish oil was used. The transmethylation was carried out at least three times by each laboratory. The mean values obtained for the GC quantification of FAMEs are given in **Table 2**. These results show that acceptable reproducibility was achieved.

Quantification of FAMEs. The quantification of FAMEs was assessed in an interlaboratory calibration study involving four laboratories (Eurofins, LAIEM, NAFC, SINTEF) and tested on five separate salmon muscle oil samples. Two examples are given in **Figure 2**. Good reproducibility and repeatability of the determination of fatty acid composition were obtained between and within laboratories. Cochran and Grubb's tests (*18*) for outliers showed that there were no statistically significant outliers in any of the analytical intercalibration data.

Evaluation of Isotopic Analysis for Salmon Muscle Lipids. To test the performance of the IRMS methods, two laboratories (Eurofins and LAIEM) took part in an interlaboratory comparison. The method was applied on the same salmon oil sample extracted following the method verified above.

Saponification. The saponification protocol as described in Materials and Methods was evaluated on the basis of the isotopic values obtained for the components. Replicate saponifications were carried out on a commercial sample of salmon oil within each laboratory. Internal standard deviations were 0.1 ‰ for $\delta^{13}C_{glycerol}$, 0.2 ‰ for $\delta^{13}C_{fatty\ acids}$, and 1.0 ‰ for $\delta^{18}O_{glycerol}$. These values are within acceptable error ranges for each parameter. In order to assess the dependability of these values, the glycerol fraction was distilled to purify the glycerol. No significant difference was found between the $\delta^{13}C_{glycerol}$ of raw ($\delta^{13}C = -22.70 \pm 0.15$ ‰, $n = 7$) and distilled ($\delta^{13}C = -22.57 \pm 0.18$ ‰, $n = 7$) glycerol. The fatty acids were isolated from the saponification reaction, their FAMEs were prepared, and the composition was analyzed by GC. This was confirmed as identical to that obtained by direct transmethylation of the oil fraction (data not shown), confirming that the oil responded to the two protocols in the same way.

In addition, the $\delta^{13}C$ and $\delta^{18}O$ of the raw oil and the $\delta^{13}C$, $\delta^{15}N$, and total nitrogen content (N %) of the glycerol + choline fraction were measured on ten replicate samples (results not shown). Good comparability was obtained between the laboratories: $S_R = 0.4$ ‰ ($R = 1.1$ ‰) for $\delta^{15}N_{choline}$, and $S_R = 0.8$ ‰ ($R = 2.2$ ‰) for $\delta^{18}O_{oil}$. This repeatability and reproducibility are within the usually accepted limits for this technique.

Statistical Analysis of Samples. *Intergroup Variability.* The isotopic analyses and fatty acid compositional analyses for the 171 authentic salmon samples are summarized in **Table 3**. Means and standard deviations for the 18 parameters (6 isotopic,

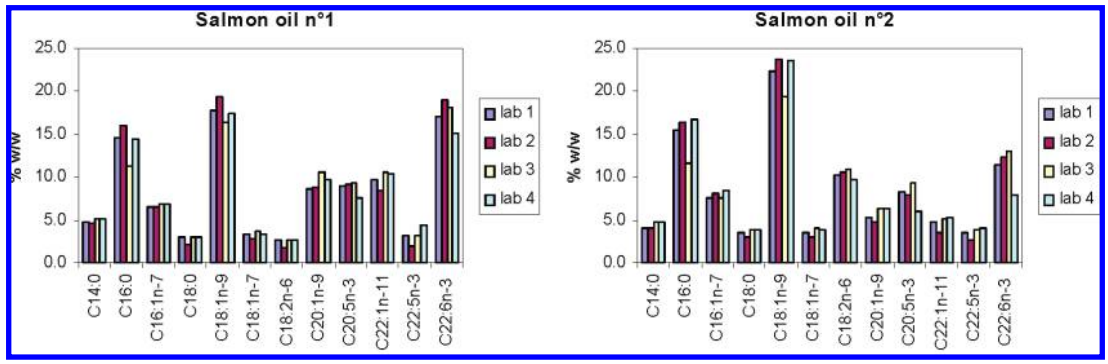

Figure 2. Interlaboratory calibration of FAME analysis for two salmon muscle oil samples.

Table 2. Interlaboratory Comparison for Quantification of FAMEs Obtained by Transmethylation of Fish Lipids

	FAME composition (% w/w)											
laboratory	C14:0	C16:0	C16:1n-7	C18:0	C18:1n-9	C18:1n-7	C18:2n-6	C20:1n-9	C20:5n-3	C22:1n-11	C22:5n-3	C22:6n-3
1	6.7	15.0	8.4	2.6	16.8	3.7	3.3	10.8	8.6	10.3	3.4	10.5
2	6.5	14.5	7.5	2.4	15.2	4.6	3.1	10.3	10.0	8.9	3.6	13.5

Table 3. Average and Standard Deviation of Isotopic and GC Results for Each Group of Authentic Salmon Samples

	farmed salmon (n = 130)		wild salmon (n = 41)	
	average	SD	average	SD
$\delta^{13}C_{oil}$	−25.3	0.8	−26.4	1.4
$\delta^{18}O_{oil}$	20.6	1.2	23.4	0.8
$\delta^{13}C_{gly}$	−24.1	0.7	−23.7	0.9
$\delta^{18}O_{gly}$	31.7	1.2	32.8	1.5
$\delta^{15}N_{chol}$	2.1	1.6	−0.8	1.0
$\delta^{13}C_{FA}$	−25.3	0.8	−26.7	1.6
C14:0	5.6	1.0	5.1	1.7
C16:0	18.1	4.3	19.2	6.6
C16:1n-7	7.4	1.2	5.6	1.2
C18:0	3.5	1.1	4.3	2.0
C18:1n-9	18.9	2.8	19.5	4.4
C18:1n-7	4.4	0.5	3.6	0.8
C18:2n-6	4.4	2.1	1.4	0.2
C20:1n-9	9.8	4.5	10.6	2.9
C20:5n-3	9.0	1.9	8.1	1.1
C22:1n-11	2.5	2.4	4.6	5.4
C22:5n-3	3.2	0.8	3.0	0.7
C22:6n-3	13.8	3.4	15.6	2.4

12 fatty acid composition) are presented for the total groups farmed and wild without differentiating geographical or temporal origin.

From an initial examination of these data, it can be seen that there is a large difference in the values of $\delta^{18}O_{oil}$, of $\delta^{15}N_{choline}$, and of C18:2n6 (%) between the two groups. Variability in these three parameters is presented in detail as Box and Whiskers plots in **Figure 3**.

A variance analysis (ANOVA) was also performed on the combined results from the isotopic results and the GC data in order to assess which parameters had the greatest discriminatory potential to differentiate between wild and farmed salmon. **Figure 4** represents the F value (intergroup variability/intragroup variability weighted ratios) used for ranking all the variables.

As **Figure 4** shows, this test confirms that the greatest variability is in the $\delta^{18}O_{oil}$ and the $\delta^{15}N_{choline}$, with the $\delta^{13}C_{fatty}$ acids also showing marked variability. In contrast, the other isotopic values, $\delta^{18}O_{glycerol}$ and $\delta^{13}C_{glycerol}$, show negligible contribution to discrimination. A bidimensional representation using the $\delta^{18}O_{oil}$ and $\delta^{15}N_{choline}$ parameters is given in **Figure 5**. This clearly shows that, on the basis of these two parameters alone, complete discrimination between authentic samples of farmed and wild salmon is achievable.

The C18:2n-6 percentage forms the next most important discriminatory parameter (**Figure 4**). To assess whether the inclusion of this parameter improved resolution between groups, a principal component analysis was carried out combining these three variables. The first two components discriminate all wild from all farmed salmon (**Figure 6**).

PC1 is mainly linked to $\delta^{18}O_{oil}$ (positively) and C18:2n-6 values (negatively), and PC2 is mainly linked to $\delta^{15}N_{choline}$ (positively). The inclusion of the compositional parameter has not improved the discrimination. This is in marked contrast to the recent report by Molkentin et al. (*12*), who found $\delta^{18}O$ of total muscle not to differ between wild and organically farmed salmon. However, these authors only used total muscle flesh, whereas in the present study the $\delta^{18}O_{oil}$ is used.

Thus it can be concluded that by measuring just the two parameters, $\delta^{18}O_{oil}$ and $\delta^{15}N_{choline}$, a distinction can be made between wild-caught and farmed (or farm-escape) fish. It was not possible to determine the $\delta^{15}N_{choline}$ values on whole oil without saponification in order to avoid the time-consuming extraction step, due to the low content of nitrogen in the oil (∼1%). On the other hand, $\delta^{15}N$ could be determined on the salmon flesh. However, although a clear correlation between $\delta^{15}N_{choline}$ and $\delta^{15}N_{muscle}$ was observed (**Figure 7A**), the bidimensional representation with $\delta^{18}O_{oil}$ and $\delta^{15}N_{choline}$ results in insufficient discrimination between the two groups (**Figure 7B**). Thus the use of the choline fraction is necessary.

Intragroup Variability. Within each group, wild and farmed, fish were obtained with a range of different parameters: different locations (see **Figure 1**), different seasons (spring/summer/autumn/winter), different years (2002/2003), different aquaculture practices of farming (slow growth/fast growth), and different diets (variable proportions of marine/vegetable oils). The variability within each group due to these factors was assessed in order to estimate the robustness of the data set. The dietary influences will be presented elsewhere.

The isotopic data were examined in relation to geographical origin for fish within each group. **Figure 8** presents a PCA based on isotopic data alone obtained from 41 authentic wild salmon samples. PC1 is mainly linked to $\delta^{18}O_{oil}$ (positively) and $\delta^{13}C_{glycerol}$ values (negatively), and PC2 is mainly linked to $\delta^{13}C_{oil}$ and $\delta^{15}N_{choline}$ (both negatively). The graph shows that

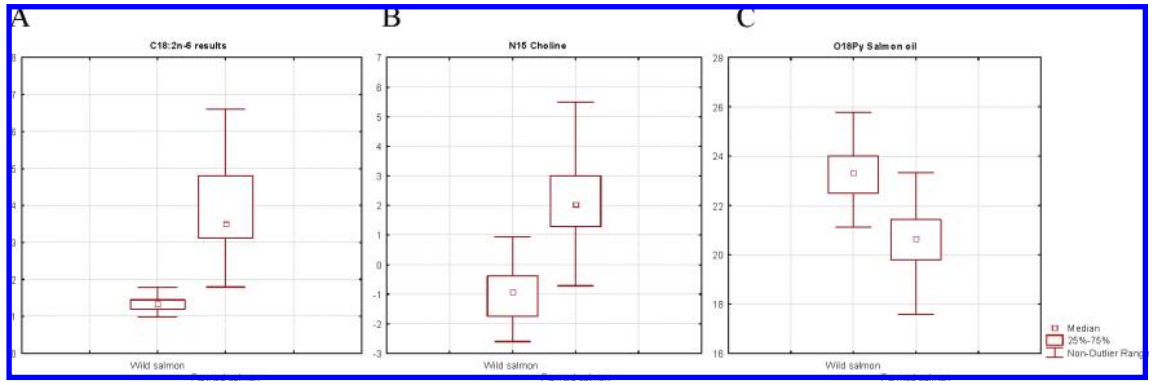

Figure 3. Box and whiskers plots for all wild and all farmed salmon: (**A**) linoleic acid (C18:2n-6) content as % of total fatty acid composition; (**B**) $\delta^{15}N_{choline}$ in total muscle oil extract; (**C**) $\delta^{18}O_{oil}$ for total muscle oil extract.

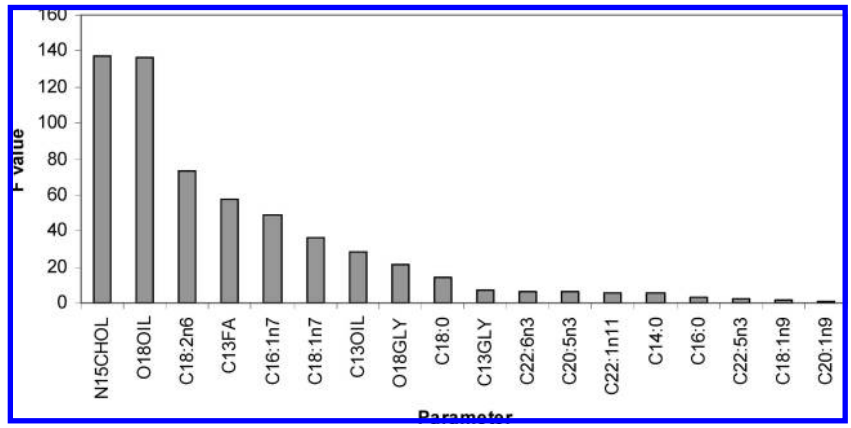

Figure 4. ANOVA on the IRMS and GC data obtained on the authentic salmon samples ($F_{critical} = 4$ at 5% confidence interval).

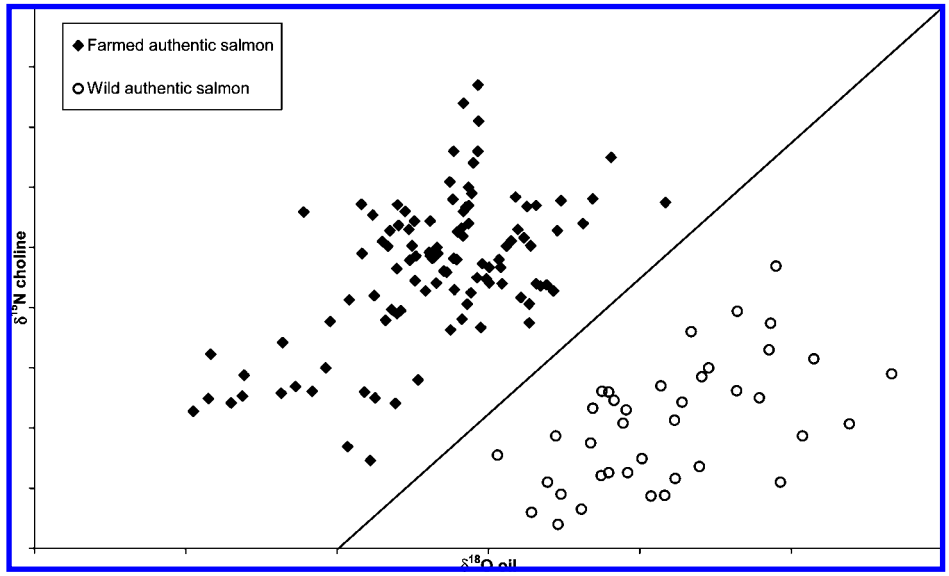

Figure 5. Results of $\delta^{18}O$ salmon oil and $\delta^{15}N$ choline measured on 171 authentic salmon samples.

it is possible to distinguish between wild salmon from different locations, and especially between Ireland and other origins. In contrast, distinguishing fish from Scotland and Norway is not possible. However, a larger sample set needs to be considered.

A similar treatment using the IRMS data obtained on the authentic farmed salmon samples shows that it is more difficult to distinguish origin in this case (**Figure 9**). PC1 is mainly linked to $\delta^{13}C_{oil}$ and $\delta^{13}C_{glycerol}$ values (both negatively), and PC2 is mainly linked to $\delta^{18}O_{oil}$ and $\delta^{15}N_{choline}$ (both negatively). It is possible to discriminate Canadian and Tasmanian salmon from the rest, probably due to the diet given to these fish, which is richer in vegetable oil and, in particular, in C4-derived oil (unpublished data). However, it is not possible to differentiate Norwegian from Scottish farmed salmon, most likely due to similar feed being used in most aquaculture regions. Further parameters need to be included in the analysis to separate these fish on the basis of their origin.

Market Sample Assessment. Fifty-four individual salmon were purchased from supermarkets and local markets in France (10 samples), Italy (4 samples), Norway (35 samples), and the United Kingdom (5 samples). Among the 54 salmon from the market, 43 were labeled "farmed" and 11 samples were labeled

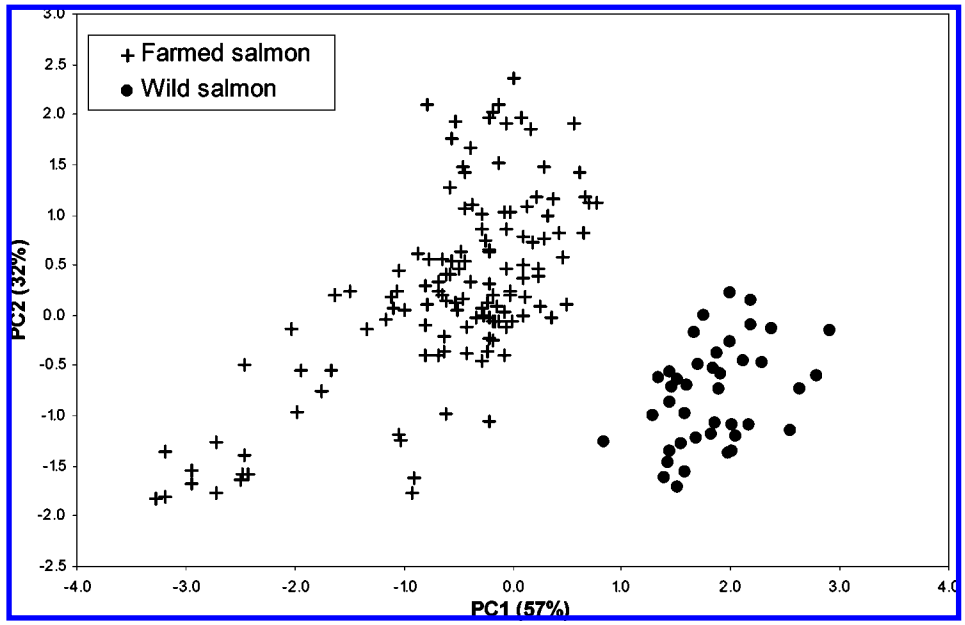

Figure 6. PCA diagram (combination of $\delta^{18}O_{oil}$, $\delta^{15}N_{choline}$, and the C18:2n-6 %) on salmon oil. F_1 and F_2 represent the first and second principal components, with percentage explained variance indicated in parentheses.

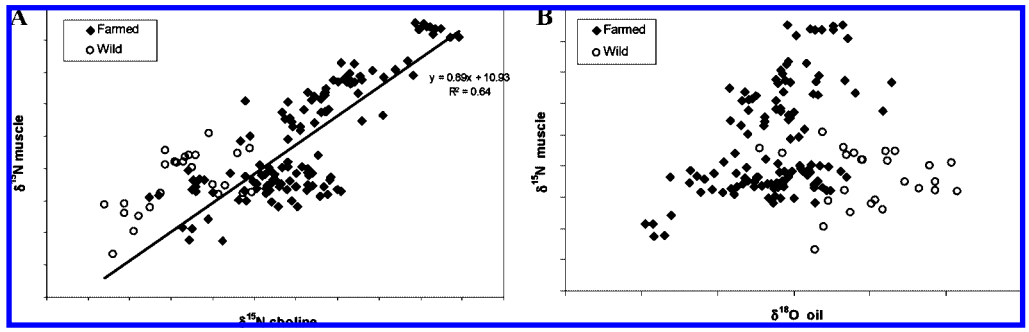

Figure 7. (**A**) Correlation between $\delta^{15}N$ (‰) for choline and $\delta^{15}N$ for whole muscle. (**B**) Bidimensional representation of data from $\delta^{18}O$ (‰) for salmon oil and $\delta^{15}N$ (‰) for whole muscle.

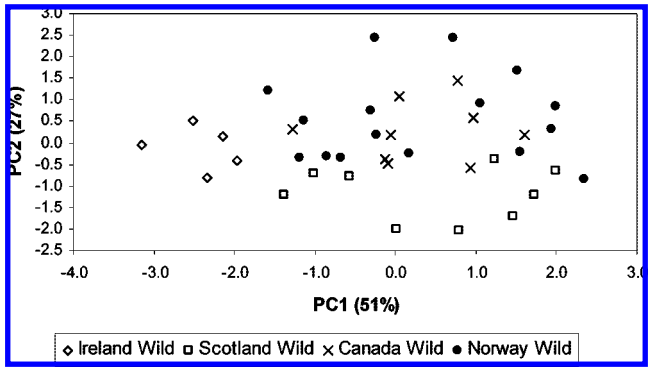

Figure 8. PCA diagram using all IRMS data obtained on 41 authentic wild salmon samples. PC1 and PC2 represent the first and second principal components, with percentage explained variance indicated in parentheses.

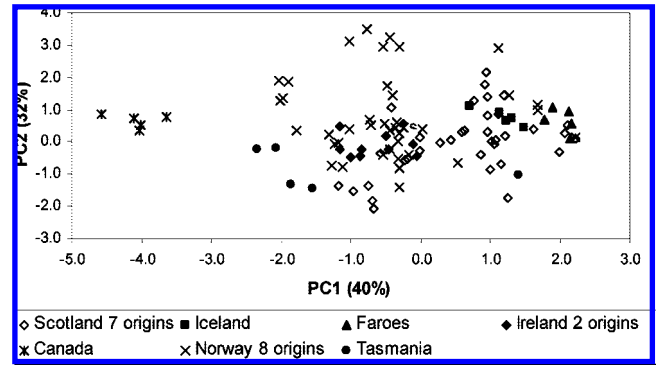

Figure 9. PCA diagram using all IRMS data obtained on 130 authentic farmed salmon samples. PC1 and PC2 represent the first and second principal components, with percentage explained variance indicated in parentheses.

"wild". These were subjected to blind analysis of both compositional and isotopic parameters, in the same conditions as used for the authentic samples. In view of the conclusions given above, of the isotopic parameters only the $\delta^{15}N_{choline}$, $\delta^{18}O_{oil}$, $\delta^{13}C_{oil}$, and $\delta^{13}C_{glycerol}$ were determined, together with the GC FAME profile. As with the data set samples, the $\delta^{18}O_{oil}$, the $\delta^{15}N_{choline}$, and the percent of linoleic acid (C18:2n-6) proved good indicators as to whether the fish was farmed or wild. **Figure 10** shows the bidimensional plot for $\delta^{18}O_{oil}$ versus $\delta^{15}N_{choline}$. As can be seen, in contrast to the situation with the authentic sample set, considerable overlap occurs, indicating that mislabeling of some individuals may have occurred. With only three exceptions, the market samples labeled farmed fell in the farmed group for the IRMS methods, while nine market samples (from Norwegian supermarkets) labeled wild showed isotopic parameters similar to farmed values. Additionally, when the C18:2n-6 content is also taken into account, five of these nine samples clearly have too high a C18:2n-6 content to be

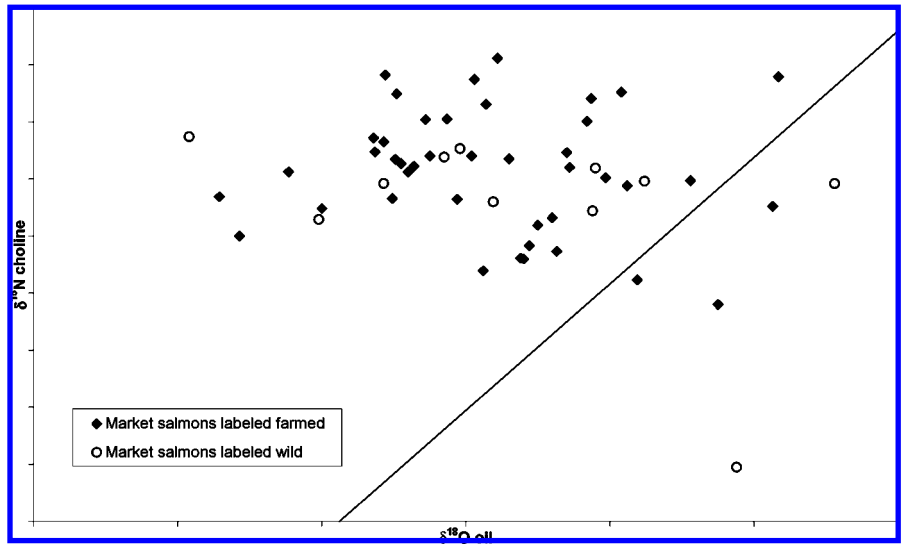

Figure 10. Results of δ^{18}O oil and δ^{15}N choline measured on market samples. Each point represents an individual fish. The separator line has been placed in the same position as in Figure 5.

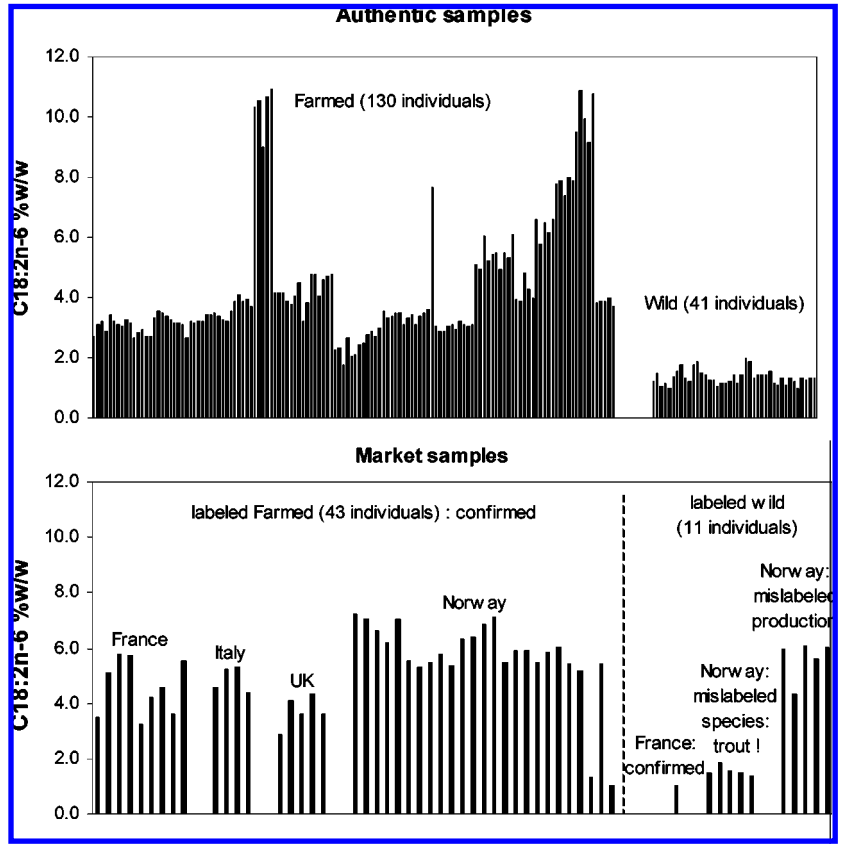

Figure 11. Distribution of C18:2n-6 (%) in the authentic and wild samples.

considered as wild (**Figure 11**) and can be putatively identified as mislabeled. This was confirmed on the basis of further compositional and ^{13}C NMR data (not shown). After further investigation by GC profiling and ^{13}C NMR (data not shown), the four samples labeled wild, but with isotopic parameters and low C18:2n-6 content similar to farmed values, were shown to be trout that had been mislabeled.

This additional market sampling indicates that the technique is appropriate for assessing whether a sample is wild or farmed. Relatively simple multivariate statistical treatment has shown that, of nine measured isotopic parameters ($\delta^{13}C_{oil}$, $\delta^{18}O_{oil}$, $\delta^{13}C_{glycerol}$, $\delta^{15}N_{choline}$, $\delta^{18}O_{glycerol}$, $\delta^{13}C_{fatty\ acids}$, $\delta^{13}C_{muscle}$, $\delta^{15}N_{muscle}$, $\delta^{18}O_{water}$), good discrimination between wild and farmed salmon can be achieved using just two of these ($\delta^{18}O_{oil}$, $\delta^{15}N_{choline}$). However, to assess labeling in the market place, it is necessary to combine these with the percent of linoleic acid C18:2n-6 in the fatty acids present. With these three parameters, it proved possible to identify 9 mislabeled fish out of 54 market-purchased samples. Not surprisingly, these were largely labeled as "wild salmon", even though 5 appeared to be trout.

Conclusion. This study adds to the growing body of evidence that stable isotope analysis, often combined with fatty acid composition, is a reliable and robust means to identify the biological and geographical origin of fish and fish products.

However, variability within the predominant parameters makes it difficult to propose a general solution. Thus, Molkentin et al. (*12*) found that $\delta^{13}C_{muscle}$ and $\delta^{15}N_{muscle}$ gave good discrimination between populations of wild salmon, even distinguishing fish from the south and north of Norway collected in the same year. When combined with the complete fatty acid data, conventional and organic farming practices could also be distinguished. These differences presumably reflect differences in diet, which have a marked influence on the isotopic profiles measured (unpublished data). This approach is also being extended to other species, such as the European sea bass (*Dicentrarchus labrax*) (*19*).

However, at present the methodology needs to be treated with caution. As shown in the present study, considerable variability in the parameters can be introduced by environmental factors, and further studies are required to define more precisely these influences. Thus, while it is now possible to have some degree of confidence in defining whether a fish is of wild or farmed origin, it is less facile to determine its geographical origin. Even the distinction made between fish farmed in the north Atlantic or Tasmania (**Figure 8**) undoubtedly reflects dietary differences, which can fluctuate depending on the material sources for the fatty acid and meal used. Nevertheless, the relatively straightforward determination of, at the most, two to four parameters appears to be sufficient to designate the wild/farmed nature of a fish of unknown origin. Most authors interpret these differences as due to dietary composition (*9, 10, 12*). Hence, any method to be applied to regulate the fraudulent labeling of fish as wild/farmed will be heavily dependent on the relationship between isotopic and compositional parameters in the feed and in the fish tissue. This relationship is the subject of a following publication.

ABBREVIATIONS USED

C14:0, myristic acid; C16:0, palmitic acid; C16:1n-7, palmitoleic acid; C18:0, stearic acid; C18:1n-7, vaccenic acid; C18:1n-9, oleic acid; C18:2n-6, linoleic acid; C20:1n-9, eicosenoic acid; C20:5n-3, eicosapentaenoic acid (EPA); C22:1n-11, brassidic acid; C22:5n-3, docosapentaenoic acid (DPA); C22:6n-3, docosahexaenoic acid (DHA); PCA, principal component analysis.

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