Characterization of Farmed and Wild Salmon *(Salmo salar)* by a Combined Use of Compositional and Isotopic Analyses

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ABSTRACT: Gas chromatography, isotope ratio mass spectrometry, and high-resolution ^2H site-specific natural isotope fractionation/nuclear magnetic resonance spectroscopy have been used to study the different kinds of fish oils and lipids extracted from muscle of wild and farmed salmon (Norway, Scotland). A statistical analysis of the fatty acid compositions, overall ^2H and ^{13}C isotope ratios, and molar fractions of the isotopomeric deuterium clusters was carried out to select the most efficient variables for distinguishing the different groups of salmons and fishes studied. A classification analysis based on four fatty acid compositions, three deuterium molar fractions, and the overall $(D/H)_{tot}$ isotope ratio of fish oils completely assigns the oils to the right group.

Paper no. J9338 in *JAOCS 77,* 659–666 (June 2000).

KEY WORDS: Atlantic salmon, farmed fish, fish lipids, SNIF-NMR deuterium, wild fish.

In recent years there has been growing interest in the nutritional benefits and eating quality of fish meat. It has been focused on the high levels of polyunsaturated fatty acids, especially the ω-3 fatty acids, believed to play a preventive role in cardiovascular disease, inflammation, and cancer (1,2). World sales of farmed fish increase annually, and it is estimated that the annual demand will grow from 70 to 90 million metric tons in the course of the next decade (3). The increasing production and consumption of fish oil as a dietary supplement and of fish products produced both from farmed and wild fish have led to an increasing demand for methods efficient in the authentication of fish products. From this point of view, stable isotope analysis is reputed to be an excellent tool for origin assessment since $^{13}C/^{12}C$ fractionation gives straightforward responses concerning the primary photosynthetic metabolism of plant products (4), and hydrogen or oxygen isotope ratios are powerful indicators of environmental conditions (5). Isotope ratio mass spectrometry (IRMS) and site-specific natural isotope fractionation studied by nuclear magnetic resonance (SNIF-NMR) are the two main techniques used for the determination of isotope ratios of natural products. NMR spectroscopy has a definitive advantage over IRMS in the sense that natural-abundance ^2H isotopomers may be precisely and accurately quantified by SNIF-NMR, whereas IRMS gives only a mean value of the deuterium content of a given chemical species. The SNIF-NMR technique was developed in the early 1980s (6) and was applied to detect adulteration of wine (7,8). Today this method has been adopted as an official European method for authentication of wines (9) and as an AOAC approved technique for the control of sugar addition in fruit juices (10). Isotopic fractionation studies of lipids have been mainly carried out by ^{13}C IRMS (11,12), and several SNIF-NMR studies have been devoted to plant lipids (13) and to olive oils (14–16). The ^1H and ^{13}C NMR spectra of the most important saturated, mono- and polyunsaturated fatty acids found in fish oils have been interpreted (17,18), and the ^2H spectra of pure fatty acids, including eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) were recently published (19).

The aim of this work was to test the possibility to use the SNIF-NMR in origin recognition of farmed and wild salmon and fish oil by a combined use of compositional and isotopic analyses.

EXPERIMENTAL PROCEDURES

Materials. $CHCl_3$ (≤0.0050% water; stabilized with about 0.02% amylene) was purchased from Prolabo (Paris, France) and $CDCl_3$ (99.8% purity) was purchased from Isotec Inc. (Miamisburg, OH). Hexafluorobenzene (99% purity) was obtained from Acros (Fairlawn, NJ). The commercial salmon oil was a product from Vikholmen Bioprocess AS (Vikholmen, Norway); haddock liver oil, cod liver oil, tuna oil, anchovy oil, and tortoise oil were provided by Lipro AS (Aalesund, Norway); and lipids extracted from white muscle of wild (from the middle part of the Norwegian coast) and farmed Atlantic salmon (*Salmo salar*) from Scotland and from Norway. In addition, lipids were extracted from two feedstuffs used for farmed salmon from Norway and Scotland and were studied to determine the dependence of the deuterium composition of salmon oils on that of the feed.

Preparation of the samples. (i) Lipid extraction. Lipids were extracted from white muscle of farmed Atlantic salmon (*S. salar*) according to Bligh and Dyer (20).

(ii) Methylation of the lipid extracted from fish muscle and commercial marine oils. The fatty acid methyl esters (FAME)

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were formed by reaction with BF_3/methanol at 100°C (British Standard Methods of analysis of fats and fatty oils, BD 684: section 2.34: 1980). When the reaction with BF_3/methanol was complete, the esters were extracted into hexane and the solvent was evaporated under vacuum before ^2H NMR examination. The isotopic composition of methanol used in the derivation process is equal to 135 ppm for ^2H and –46.9‰ on the Vienna Pee Dee Belemnite (V.PDB) scale for ^{13}C.

Gas chromatography (GC). An internal standard, 21:0 methyl ester, was added to the extracted sample prior to methylation. FAME were prepared as described in References 21 and 22. The FAME were determined quantitatively by capillary GC [Carlo Erba HRGC 5160 series (Milano, Italy) equipped with a SP2330 glass capillary column, on-column injection, and flame-ionization detector] fitted with a Shimadzu-Cromatopac C-3rA computing integrator (Tokyo, Japan). The GC oven was programmed from an initial 60 to 150°C at a rate of 25°C/min and held for 3 min at the latter temperature. Thereafter, the temperature increase was 2°C/min to a final temperature of 190°C. Hydrogen was used as the carrier gas. Identification of FAME and quantitation were based on the comparison to standard reference samples (Nu-Chek-Prep, Elysian, MN) and up to 12 different fatty acids.

Isotopic determinations. The overall D/H isotope ratios of the methyl esters were obtained after combustion into CO_2 and water by IRMS using a VG SIRA 9 spectrometer (Micromass; formerly, VG Instrument, Manchester, United Kingdom), and the deuterium measurements were performed on the hydrogen gas resulting from reduction of water. The results were initially referred to the Vienna Standard Mean Ocean Water (V:SMOW) standard (23,24) and converted to D/H ratios expressed in ppm.

The ($^{13}C/^{12}C$) ratio examination was carried out by IRMS using a Finnigan Delta E mass spectrometer (Finnigan-MAT, Bremen, Germany) coupled with a Carlo Erba Model 1500 microanalyzer. The carbon isotopic parameters were expressed on the δ scale (‰) which refers the isotope ratio of the sample, *S*, to that of the international reference, V.PDB (25); see Equation 1.

$$\delta^{13}C = 1000\left[\frac{\left(^{13}C/^{12}C\right)_S - \left(^{13}C/^{12}C\right)_{PDB}}{\left(^{13}C/^{12}C\right)_{PDB}}\right] \quad [1]$$

The site-specific isotope ratios of the methyl esters of lipid extracts and fish oils were determined by SNIF-NMR using a DPX 400 Bruker spectrometer (Karlsruhe, Germany) operating at 61.4 MHz, fitted with a ^{19}F field-frequency locking device tuned to the frequency resonance of hexafluorobenzene. The samples were examined in $CHCl_3$ (1.3 g per 3.5 g $CHCl_3$) in 10-mm tubes. Each sample was analyzed three times. The spectra were recorded at 303 K with a pulse delay of 6.9 s, including an acquisition time of 6.84 s. Since the T_1 relaxation times of the different isotopomers observed were lower than 1 s, the pulse repetition time used enabled us to recover more than 99.9% of the magnetization. The sweep width was equal to 1197.32 Hz; and 16 K data points, using a 90° excitation pulse and 3,200 scans, were collected. The signal intensities were measured using a software program based on complex least-squares curve fitting (26). The site-specific isotope ratios $(D/H)_i$ measured by ^2H NMR were calculated according to Equation 2,

$$(D/H)_{ij} = \left(\frac{fm_{ij}}{FMP_{ij}}\right)(D/H)_{i_{tot}} \quad [2]$$

where fm_{ij} represents the effective molar ratio of the isotopomeric cluster *j* observed in the ^2H spectrum of product *i*, FMP_{ij} is the weighted statistical molar ratio of cluster *j* and the $(D/H)_{i_{tot}}$ the overall isotope ratio of product *i* determined by IRMS. The molar fractions were computed from the intensity of the clusters pertaining to the fatty acid fragment only and no correction due to the presence of the oxymethyl group of the ester moiety was needed .

Statistics. The experimental values of the different variables determined by GC (12 compositions of fatty acids in %w/w), IRMS [two overall isotope ratios (D/H) and ($^{13}C/^{12}C$)], and SNIF-NMR (14 molar fractions of isotopomeric clusters) for the fish oils studied were submitted to a variance analysis (ANOVA) in order to select the most discriminating parameters for the recognition of wild and farmed salmons (two groups). When a significant variable was sorted out, the Fisher Least Significant Difference (LSD) is computed at the 99% confidence level (27). To check the consistency of the computational data, ANOVA was also carried out on three groups of oils, including the cases of wild fishes other than salmon. A canonical discriminant analysis (CDA) (28) was performed using Unistat software (London), with the variables selected for GC ($\%m_{ij}$) and SNIF-NMR (fm_{ij}) to determine the classification power of the variables. In addition, CDA were also carried out with the deuterium isotope ratios of the observed clusters, whose computation involves the three kind of variables (Eq. 2), and with the whole set of the more discriminating variables measured ($\%m_{ij}$, fm_{ij}, and $(D/H)_{i_{tot}}$).

RESULTS AND DISCUSSION

Fatty acid composition of the fish oils studied. The values (%w/w) of the 12 fatty acids usually observed in the mixtures are given in Table 1. In some cases, the content in fatty acid is too low to be precisely determined and the corresponding entry is represented by n.q. (not quantified). However, for statistical analysis these missing data were replaced by the negligible value 0.1%w/w. According to the official method of fatty acid determination by GC method (22), a standard deviation of repeatability equal to 0.35%w/w (1% in relative value) may be expected for compositions higher than 5%w/w and the precision is lower for the less abundant acids. Then acids 16:0, 16:1n-9, 18:1n-9, 20:1n-9, 20:5n-3, 22:1n-9, and 22:6n-3 are conveniently determined, but acids 14:0, 18:0, 18:1n-7, 18:2n-6, and 22:5n-3 are more or less poorly quantified. An ANOVA was carried out in order to select the more discriminating compositional values in terms of the three

TABLE 1
Composition (%w/w) by Gas Chromatography of the Main Fatty Acids Extracted from Fish Oils

Case	Region	Type	Species	14:0	16:0	16:1n-9	18:0	18:1n-9	18:1n-7	18:2n-6	20:1n-9	20:5n-3	22:1n-9	22:5n-3	22:6n-3
1	Norway	Farm	Salmon	3.5	6.4	4.8	2.3	10.8	2.4	2.8	11.5	7.4	13.4	3.4	14.3
2		Farm	Salmon	3.7	6.6	4.9	2.5	10.8	2.7	2.9	13.8	6.6	1.4	2.6	11.5
3		Farm	Salmon	4.2	7.8	5.8	3.1	12.2	3.2	2.9	11.7	5.5	14.8	2.2	10.1
4		Farm	Salmon	5.0	10.2	6.8	2.6	14.4	3.3	2.7	10.0	5.7	12.0	1.8	10.1
5		Farm	Salmon	3.8	7.2	6.5	2.5	14.8	0.0	2.9	12.7	9.3	15.8	2.8	15.2
6		Farm	Salmon	3.8	7.5	6.4	2.6	14.8	0.0	2.7	13.8	7.8	16.2	2.8	14.4
7		Farm	Salmon	3.9	7.5	6.5	2.5	15.0	0.0	2.9	13.5	8.4	15.0	2.9	14.9
8		Farm	Salmon	3.6	6.9	6.4	2.6	14.1	0.0	2.8	12.9	8.7	15.2	2.9	17.0
9		Farm	Salmon	4.5	9.1	7.1	2.4	16.4	0.0	2.4	14.6	7.6	15.7	2.6	12.1
10		Farm	Salmon	3.7	7.1	6.4	2.6	12.9	0.0	2.8	13.5	9.5	16.1	3.0	14.8
11		Farm	Salmon	5.7	13.5	6.5	2.3	12.6	2.4	2.5	9.6	7.3	10.2	2.8	10.6
12		Farm	Salmon	5.8	14.5	6.6	2.9	12.4	2.9	1.9	6.9	9.5	0.0	3.8	11.8
13		Oil	Salmon	5.1	12.4	6.3	2.6	16.0	2.8	2.6	6.6	9.2	7.1	4.0	11.2
14		Feed	Salmon	4.2	8.0	7.1	1.4	13.1	0.0	2.5	14.6	11.5	20.0	0.8	9.5
15	Scotland	Farm	Salmon	4.8	9.1	7.1	2.6	16.7	0.0	3.6	13.1	7.4	13.9	2.7	12.5
16		Farm	Salmon	3.9	7.7	6.4	2.7	14.1	0.0	3.8	13.0	8.5	14.9	3.1	13.8
17		Farm	Salmon	4.3	8.5	7.0	2.7	15.6	0.0	3.7	13.4	8.1	13.9	2.7	12.9
18		Farm	Salmon	4.0	7.1	7.0	2.8	14.3	0.0	4.2	12.4	8.6	15.1	3.2	14.3
19		Farm	Salmon	4.6	8.7	6.9	2.7	15.9	0.0	3.6	13.5	7.8	14.0	2.7	12.6
20		Farm	Salmon	4.2	8.2	7.1	2.8	16.0	0.0	4.0	13.6	7.8	15.7	2.5	12.3
21		Feed	Salmon	3.6	7.0	6.6	2.1	13.4	0.0	4.4	12.5	13.4	17.3	1.0	10.2
22	Atlantic	Wild	Salmon	2.5	7.3	3.8	1.8	11.2	2.6	1.8	11.5	10.2	1.4	2.9	18.5
23		Wild	Salmon	1.5	11.1	3.0	2.1	14.8	3.3	1.3	7.6	11.9	1.2	3.9	21.7
24		Wild	Salmon	4.6	9.7	7.1	3.1	15.6	6.9	1.6	8.9	7.5	1.3	2.2	8.8
25		Wild	Salmon	5.6	8.3	5.5	2.5	12.3	2.9	1.3	11.7	5.4	1.4	2.0	10.0
26		Wild	Salmon	3.7	7.3	5.4	3.3	11.0	4.0	1.3	11.5	6.0	1.6	2.8	13.5
27		Wild	Salmon	3.4	8.7	5.5	5.4	13.1	5.2	1.9	8.2	6.8	1.4	3.5	15.4
28		Wild	Salmon	4.0	7.2	5.0	2.5	10.8	3.3	1.5	10.7	6.3	15.2	2.2	12.6
29		Wild	Salmon	6.0	13.3	6.1	2.3	16.2	3.3	1.2	9.8	5.4	0.0	1.6	9.5
30		Wild	Salmon	2.3	10.8	3.1	2.6	17.0	2.9	1.2	10.1	6.5	0.0	3.0	20.4
31		Wild	Salmon	2.4	10.6	3.7	2.2	14.0	2.7	1.1	10.9	8.8	0.0	3.6	19.5
32	Norway	Wild	Cod	4.5	10.4	5.4	2.0	16.3	2.9	1.6	13.2	7.9	9.0	1.2	12.3
33	Thailand	Wild	Tuna	3.3	19.7	5.1	5.5	12.3	2.4	1.2	0.8	5.4	0.5	1.1	25.4
34	Norway	Wild	Haddock	3.2	12.2	4.7	0.2	14.0	3.5	1.3	7.2	11.1	4.7	1.4	15.3
35	Chile	Wild	Anchovy	4.9	16.0	5.4	4.6	19.9	3.2	1.2	4.0	9.0	1.8	3.2	13.0
36	Ocean	Wild	Seal	4.7	8.1	11.4	0.9	19.2	4.1	1.8	10.9	6.8	4.3	3.7	10.0
37	Ocean	Wild	Whale	5.1	9.1	8.5	1.9	20.2	3.5	2.0	14.8	3.3	9.4	2.1	5.1
38	Korea	Wild	Tortoise	2.7	17.6	8.1	4.8	26.9	3.7	8.4	1.1	4.3	0.6	2.0	9.9

main groups of oils studied: farmed salmon, wild salmon, and oils of marine origin. When groups 1 and 2 only are considered the composition in the fatty acid 18:2n-6 is significant at the 99% confidence level and that of acid 16:1n-9 is only significant at 96.9%. The other compositions are statistically equivalent for the three groups considered. However, if attention is focused on the recognition of the three groups of oils, four fatty acid compositions are significant at the 99% confidence level: 22:1n-9, 18:1n-9, 16:0, and 16:1n-9, to distinguish farmed and wild salmons from the other marine oils. The fatty acids 22:1n-9 and 18:1n-9 enable a good differentiation to be made between group 1 or group 2 and group 3, whereas acid 16:0 or 16:1n-9 discriminates between 1 and 3 or 2 and 3, respectively. To conclude this section, it should be kept in mind that owing to its rather high coefficients of variation, GC-compositional analysis is not a very robust authentication method but can give useful trends.

Overall ^{2}H and ^{13}C contents of the fish oils. The introduction of a methyl group in the fatty acid molecule during the derivatization process induces a small but significant change of the isotopic abundance of FAME, and as a consequence, the measured ^{2}H and ^{13}C isotope ratios of the methyl esters must be corrected in order to obtain the true isotopic contents of the fatty acids. For example with methanol batches containing between 135 and 150 ppm of deuterium and –45 to –25‰ of ^{13}C, the theoretical correction is on the order of 1 to 2 ppm and –0.2 to +1‰, respectively, for most of the fatty acids studied (C_{14} to C_{22}). On the other hand, no significant isotopic fractionation has been observed during the derivatization process (15,28) and it has been shown that oils, hydrolyzed fatty acids, and FAME of a given product have practically the same values (29). However, since the experimental protocol of the derivatization procedure is quite well rationalized in the AOAC procedure (21,22), the relative consistency

TABLE 2
Site-Specific Isotope Ratios of the Different Clusters Observed in the ^2H Nuclear Magnetic Resonance Spectra of the Mixtures of Fatty Acids Extracted from Fish Oils

	Sample													
Case	$(D/H)_1$	$(D/H)_2$	$(D/H)_3$	$(D/H)_4$	$(D/H)_5$	$(D/H)_6$	$(D/H)_7$	$(D/H)_8$	$(D/H)_9$	$(D/H)_{10}$	$(D/H)_{11}$	$(D/H)_{12}$	$(D/H)_{13}$	$(D/H)_{14}$
1	99.3	102.1	112.4	92.8	103.1	127.9	120.0	192.2	121.0	120.0	146.1	129.5	107.5	125.8
2	88.0	80.7	99.5	85.8	96.8	118.1	122.0	168.0	114.8	122.0	135.3	140.7	88.2	138.6
3	102.4	101.1	127.2	118.6	135.1	125.8	188.2	227.8	115.4	188.2	143.6	128.1	115.8	113.1
4	109.6	119.6	127.2	108.4	108.4	116.5	176.5	229.7	112.4	176.5	133.4	123.6	125.4	117.0
5	85.2	90.7	75.2	84.4	124.7	123.9	156.2	172.7	112.6	156.2	138.1	135.5	78.3	161.3
6	86.3	88.7	84.9	88.3	109.8	122.9	142.3	178.8	113.0	142.3	138.5	132.8	78.3	164.4
7	80.6	78.4	79.3	77.4	106.4	113.0	156.4	159.2	101.0	156.4	125.2	135.6	73.5	216.3
8	75.7	73.5	70.9	63.7	86.9	114.4	127.9	152.6	105.5	127.9	126.7	143.5	69.3	232.7
9	97.8	103.9	96.6	85.5	111.8	116.0	192.5	203.4	112.8	192.5	135.1	130.8	97.7	154.8
10	81.8	76.8	81.9	74.4	83.7	121.6	131.4	157.0	112.8	131.4	139.9	137.7	74.8	179.0
11	114.4	109.2	176.9	103.2	123.1	127.0	168.1	250.4	118.2	168.1	144.7	123.2	122.6	117.0
12	100.6	96.6	134.2	102.6	98.5	120.8	105.0	183.3	123.9	105.0	139.2	132.4	90.1	145.1
13	104.5	106.6	150.8	125.3	169.6	121.0	160.5	211.7	109.4	160.5	140.4	124.7	95.8	144.2
14	91.1	92.4	104.0	98.9	128.3	117.7	142.1	208.4	117.9	142.1	132.0	128.3	95.3	164.9
15	100.7	104.8	105.3	108.9	114.0	131.2	221.2	222.7	114.5	221.2	142.8	131.3	95.8	116.7
16	93.1	89.3	101.0	102.2	124.7	131.7	190.4	195.0	114.2	190.4	146.2	136.4	96.5	115.5
17	95.9	100.8	104.4	103.4	118.2	126.4	180.4	213.2	112.8	180.4	138.6	131.1	100.5	127.2
18	90.6	90.3	90.2	96.5	125.2	133.0	155.1	189.5	116.0	155.1	146.0	135.5	87.5	121.1
19	97.3	95.3	102.2	100.9	150.5	127.8	191.3	222.7	112.4	191.3	141.8	132.1	97.9	114.2
20	100.5	105.8	108.5	100.0	135.3	129.2	186.0	222.6	112.7	186.0	140.9	129.8	99.2	115.3
21	89.3	85.4	107.4	132.2	160.0	124.7	105.9	191.3	124.1	105.9	145.7	133.1	80.6	130.0
22	88.7	93.7	80.1	105.4	111.0	133.0	126.9	146.4	141.7	126.9	147.1	141.1	94.9	147.0
23	93.4	103.6	85.1	110.1	116.5	139.5	143.1	169.9	136.7	143.1	139.8	139.8	100.1	126.7
24	95.9	102.6	93.1	103.4	139.3	110.7	176.4	191.5	115.5	176.4	134.2	132.2	92.5	143.3
25	81.1	91.2	63.0	82.8	94.1	101.8	130.4	164.4	117.0	130.4	115.1	136.0	59.2	216.1
26	70.4	71.5	57.7	63.3	76.5	98.2	114.0	122.3	105.7	114.0	116.6	146.0	51.8	255.8
27	72.1	74.0	54.8	72.8	81.2	104.8	103.3	118.1	110.0	103.3	128.2	147.0	50.3	222.7
28	83.6	90.9	73.3	81.4	96.3	108.5	142.1	158.5	103.0	142.1	126.4	136.6	68.1	206.3
29	99.4	108.3	82.4	96.5	108.6	98.6	119.2	176.0	107.3	119.2	108.1	134.0	78.9	159.1
30	84.0	83.5	98.1	85.1	85.1	123.5	148.1	143.5	121.9	148.1	136.6	145.8	77.9	220.0
31	69.5	72.7	63.2	77.6	89.1	102.4	90.3	117.6	101.8	90.3	113.8	156.2	62.3	302.5
32	116.4	131.5	140.4	111.8	124.9	129.8	173.0	240.4	117.1	173.0	150.7	123.3	115.1	118.5
33	102.0	106.0	109.8	123.7	123.7	110.9	217.1	133.7	101.9	217.1	134.2	143.9	102.3	137.1
34	106.6	116.0	125.9	123.4	141.9	140.8	169.2	212.8	115.0	169.2	164.9	131.6	98.0	122.9
35	111.8	123.7	123.1	142.2	150.5	127.0	179.3	206.0	112.7	179.3	151.3	126.0	102.1	116.5
36	113.5	117.5	152.6	121.5	142.0	122.3	189.3	245.5	114.3	189.3	143.1	124.4	122.5	132.5
37	118.9	127.2	166.2	141.7	190.1	115.9	225.6	316.5	121.6	225.6	133.1	123.4	119.6	129.9
38	113.5	122.8	121.0	122.7	142.9	110.8	302.1	297.8	154.0	302.1	142.2	124.6	113.0	124.6

of the isotope ratios may be assumed and the data safely considered for analytical purposes. From this point of view, the $\delta^{13}C$ values are not efficient for distinguishing wild from farmed salmon, and the difference between the $(D/H)_{tot}$ isotope ratios of the two groups of salmon is only significant at the 98% confidence level (Table 2). When the third group (oils of marine origin) is included in the ANOVA computation, it appears that a significant difference exists between the $\delta^{13}C$ values of wild and farmed salmon.

Site-specific deuterium composition of fish oils. A typical ^2H NMR spectrum of fish oil is shown in Figure 1. The assignment of the ^2H signals from the proton spectra is straightforward (19,30,31), but it is apparent that even at relatively high magnetic fields (9.4 to 11.6 T) it is impossible to resolve all the signals of the different isotopomers of the fatty acids mixture shown on Figure 1. We have defined a group of signals having a pseudo-Lorentzian shape as an isotopic cluster. The identification of the different fatty acids contributing to a given cluster is given in Table 3 and the experimental molar fractions of the isotopomeric clusters obtained by a line shape analysis in the complex plane (25) from the SNIF-NMR spectra. These data correspond to the overall intensity of the cluster, and it is not possible to compute the individual isotope ratios of the fatty acids constituting the cluster. However, according to the mass and isotopic balance law which governs any mixture and transformation, it is possible to introduce a cluster isotopic abundance (Eq. 2) which is nearly equal to a cluster isotope ratio in the case of deuterium. As a consequence of Equation 2, where fm and $(D/H)_{tot}$ are experimental parameters, the weighted statistical molar fraction FMP

TABLE 3
Hydrogen Population of the Different Clusters Observed in the ^{2}H Nuclear Magnetic Resonance Spectra of Fish Oils

	Column #; δ (ppm)													
Fatty acid	1 5.35	2 2.80	3 2.75	4 2.50	5 2.30	6 2.25	7 2.05	8 2.00	9 1.95	10 1.64	11 1.55	12 1.25	13 0.90	14 0.85
14:0	0	0	0	0	0	2	0	0	0	0	2	20	0	3
16:0	0	0	0	0	0	2	0	0	0	0	2	24	0	3
16:1n-9	2	0	0	0	0	2	0	0	4	0	2	16	0	3
18:0	0	0	0	0	0	2	0	0	0	0	2	28	0	3
18:1n-9	2	0	0	0	0	2	0	0	4	0	2	20	0	3
18:1n-7	2	0	0	0	0	2	0	0	4	0	2	20	0	3
18:2n-6	4	2	0	0	0	2	0	0	4	0	2	14	0	3
20:1n-9	2	0	0	0	0	2	0	0	4	0	2	24	0	3
20:5n-3	10	4	4	0	0	2	2	2	0	2	0	0	3	0
22:1n-9	2	0	0	0	0	2	0	0	4	0	2	28	0	3
22:5n-3	10	6	2	0	0	2	0	2	2	0	2	4	3	0
22:6n-3	12	8	2	2	2	0	0	2	0	0	0	0	3	0

should be clarified. When a single chemical species is considered, the statistical molar fraction of a given isotopomer of the species is equal to the ratio of the site population, i.e., the number of hydrogen atoms in a given chemical position, to the overall number of hydrogen atoms of the chemical species. In the case of a mixture such a cluster, the fatty acid composition (%w/w) of the lipid extract *i* should be transformed into molar fractions that are weighted by the site populations given in Table 3. By using this procedure, the GC compositions (Table 1), the overall isotope (D/H) ratios of the fatty acids mixtures (Table 4), and the measured ^{2}H molar ratios (Table 5) are combined to produce the cluster isotope ratios of Table 2.

The precision of the measurements of deuterium molar fractions is on the order of 0.001 in absolute value, as determined by the mean square deviation of the pooled variance of the individual deviation of the 14 clusters. In relative values, the standard deviation of repeatability varies between 1 and

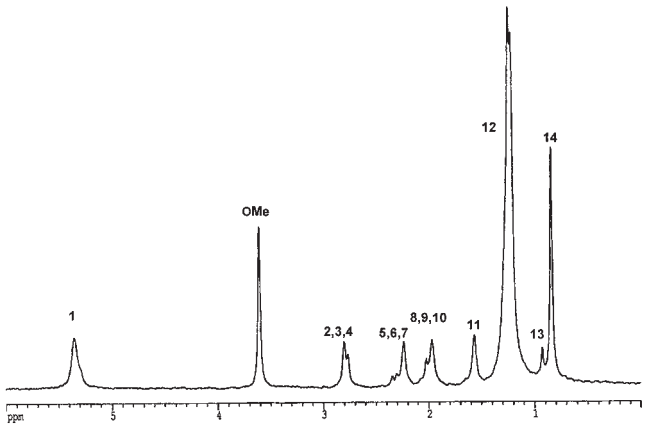

FIG. 1. ^{2}H Nuclear magnetic resonance spectrum (61.4 MHz) of the hydrolysate of lipid extracted from muscle of Atlantic salmon (*Salmo salar*). Hydrogen population of the different clusters is given in Table 3, and the site-specific isotope ratios of the different clusters are given in Table 2.

TABLE 4
Overall ^{2}H and ^{13}C Isotope Ratios of the Fish Oils Studied

Case	$(D/H)_{tot}$	$\delta^{13}C$
1	123.3	–26.5
2		–26.9
3		–27.9
4	122.4	–27.9
5	123.9	–27.8
6	123.5	–27.9
7	124.3	–27.9
8	126.5	–28.0
9	125.7	–28.1
10	123.9	–28.1
11	125.2	
12	125.4	
13		
14	124.1	–28.5
15	125.6	–27.2
16	125.4	–27.3
17	124.3	–27.2
18	124.1	–27.2
19	124.5	–27.3
20	124.3	–27.1
21	123.6	–27.6
22	124.3	–27.3
23	124.5	–27.6
24	125.2	–27.9
25	126.1	–28.1
26	125.9	–27.8
27	124.8	–27.6
28		
29	124.5	–28.0
30	128.6	
31	127.8	
32	126.1	–27.6
33	128.3	–25.0
34	128.6	–27.5
35	125.8	–25.4
36	126.3	–26.4
37	126.0	–25.9
38	128.3	–24.9

TABLE 5
Molar Fractions[a] of the 14 Isotopic Clusters Observed in the Deuterium Spectra of the Fish Oils Studied

Case	Sample type	Deuterium molar fractions ($\times 10^{-4}$)													
		fm1	fm2	fm3	fm4	fm5	fm6	fm7	fm8	fm9	fm10	fm11	fm12	fm13	fm14
1	Farm	1040.7	473.5	202.9	71.1	79.0	526.5	51.5	264.4	667.1	51.5	538.8	5149.6	221.8	661.8
2	Farm	901.3	364.2	178.4	62.3	70.4	502.5	55.1	225.3	615.4	55.1	514.6	5521.5	177.5	756.6
3	Farm	848.9	326.2	159.5	62.1	70.7	539.9	58.1	215.4	674.7	58.1	572.1	5593.6	164.2	656.7
4	Farm	901.2	381.3	160.4	57.0	57.0	518.1	56.7	215.9	647.7	56.7	550.3	5513.4	176.9	707.5
5	Farm	870.8	397.0	135.4	60.6	89.6	513.5	74.3	228.8	626.6	74.3	506.6	5411.1	155.5	855.8
6	Farm	837.3	362.1	137.3	60.4	75.1	512.3	57.1	217.7	645.1	57.1	522.1	5477.1	142.9	896.5
7	Farm	801.5	332.7	134.8	54.5	74.9	467.9	67.2	202.1	565.5	67.2	464.9	5465.8	140.0	1160.3
8	Farm	785.9	338.3	128.0	50.5	68.8	454.4	56.1	208.4	565.7	56.1	447.7	5512.2	142.0	1186.0
9	Farm	850.0	356.6	137.0	47.1	61.6	490.6	72.1	212.2	644.8	72.1	521.1	5513.8	152.9	868.2
10	Farm	838.8	337.7	150.1	52.6	59.2	503.7	64.6	210.5	621.9	64.6	510.9	5493.1	150.4	941.9
11	Farm	970.9	382.0	259.9	55.1	65.7	556.6	66.9	268.3	594.5	66.9	576.5	5264.9	197.0	674.8
12	Farm	1002.8	437.7	270.4	67.5	64.8	535.6	60.2	264.2	517.5	60.2	537.3	5192.6	194.8	794.2
13	Oil	996.8	434.1	270.3	71.7	97.1	536.3	81.5	272.0	545.4	81.5	550.9	5072.8	184.7	804.9
14	Feed	804.5	295.8	171.9	44.1	57.2	513.2	83.0	222.6	690.1	83.0	498.5	5458.4	152.7	924.8
15	Farm	895.2	378.9	151.7	62.9	65.8	556.6	81.8	238.6	647.3	81.8	552.9	5476.3	153.9	656.3
16	Farm	907.2	369.0	168.7	66.8	81.5	546.9	83.0	240.8	633.9	83.0	543.6	5478.5	178.8	618.5
17	Farm	895.1	387.5	163.3	62.9	71.9	537.9	74.9	245.1	640.5	74.9	532.7	5431.2	173.4	708.9
18	Farm	906.6	386.0	154.2	65.4	84.8	557.2	68.4	240.3	655.4	68.4	547.0	5446.3	166.5	653.5
19	Farm	888.0	355.3	154.1	59.6	88.9	545.2	75.7	247.8	638.5	75.7	548.7	5517.7	163.3	641.2
20	Farm	906.9	383.0	159.1	56.9	77.0	551.6	72.7	239.3	657.9	72.7	546.6	5465.2	159.9	651.1
21	Feed	873.2	320.5	208.2	65.0	78.7	546.4	74.0	237.0	712.1	74.0	536.2	5416.5	149.7	708.5
22	Wild	1167.5	601.3	208.9	117.1	123.3	520.3	84.1	285.1	643.5	84.1	477.8	4731.8	277.0	678.2
23	Wild	1278.9	710.3	238.4	130.4	137.9	538.7	100.6	356.5	557.6	100.6	441.6	4533.0	315.1	560.3
24	Wild	829.3	345.7	142.6	51.2	69.0	499.7	80.6	205.9	632.7	80.6	544.3	5523.7	149.2	845.6
25	Wild	714.5	338.7	93.6	52.0	59.0	440.5	47.8	184.0	599.0	47.8	455.4	5625.3	99.4	1242.9
26	Wild	727.2	337.1	103.5	52.4	63.3	403.2	45.4	170.8	529.2	45.4	432.5	5624.3	108.6	1357.2
27	Wild	769.5	371.8	104.0	63.2	70.6	433.2	42.9	174.9	523.1	42.9	476.7	5639.1	111.8	1176.5
28	Wild	767.7	349.2	110.6	54.5	64.4	444.4	51.5	181.9	564.0	51.5	472.0	5652.0	117.2	1119.2
29	Wild	797.9	352.8	109.4	53.5	60.1	445.5	40.6	173.8	536.2	40.6	451.3	5847.5	116.9	974.0
30	Wild	972.1	480.8	199.7	94.3	94.3	462.5	56.6	237.3	536.5	56.6	459.3	5100.2	193.1	1056.7
31	Wild	846.9	438.2	147.0	83.6	95.9	391.7	47.5	211.8	433.9	47.5	375.3	5305.9	168.2	1406.6
32	Wild	1029.6	469.1	213.2	68.5	76.5	549.0	73.1	263.3	630.7	73.1	573.7	5124.6	189.0	666.5
33	Wild	1155.5	659.7	221.2	165.8	165.8	404.0	66.7	228.2	287.4	66.7	447.7	5195.7	261.9	673.5
34	Wild	1171.5	578.7	281.0	103.7	119.2	568.0	111.4	334.8	513.2	111.4	556.8	4710.5	231.2	608.7
35	Wild	1047.6	517.1	222.1	93.8	99.3	554.6	88.1	270.5	484.7	88.1	586.2	5097.7	201.2	649.0
36	Wild	1006.7	401.2	217.7	61.2	71.5	542.0	69.8	259.7	700.7	69.8	581.6	5052.7	194.4	770.9
37	Wild	745.5	227.7	118.4	35.7	47.9	528.2	40.2	169.0	798.8	40.2	582.9	5737.0	95.8	832.8
38	Wild	850.4	372.6	120.8	56.9	66.3	494.9	66.4	231.8	839.7	66.4	603.9	5321.9	131.9	776.1

[a]fm, effective molar ratio.

2%, according to the intensity of the cluster. Considering that the repeatability of the GC determination is on the same order of magnitude and that of ^{2}H IRMS covers a 0.5 to 1% range, it is possible to estimate a higher limit for the repeatability of site-specific isotope ratio determinations in the range of 2 to 4%. The computation of several sets of $(D/H)_{ij}$ values including randomly the different sources of uncertainty gives a mean square standard deviation equal to 2.5%. It is interesting to compare the discriminating powers of pure isotopic parameters, i.e., the molar ratios of Table 5, and those of combined variables, i.e., the site-specific isotope ratios of Table 2. Whatever the number of groups considered, the molar fractions fm_j and the isotope ratios $(D/H)_j$ of clusters 6, 11 and 14 are efficient at the 99% confidence level, and the differences between the means of the two groups of salmon are also significant at the same degree of certainty. Moreover, the differentiation of wild and farmed salmons may be emphasized by using the molar fractions of cluster 9.

Origin recognition of farmed and wild salmon. CDA were systematically performed on the different lipid extracts studied with selected sets of variables. Indeed, using the whole set of parameters measured on the different samples will increase the information noise and give a deceptive feeling of discrimination efficiency. Then, CDA were computed successively with those of the GC compositions, IRMS isotope ratios, ^{2}H molar fractions, and site-specific isotope ratios which were found to be significant at the 99% confidence level in the ANOVA. By using these four kinds of variables, the samples were gathered either in two (farmed and wild salmon) or three (farmed, wild salmon, and marine oils) groups, and according to the properties of CDA (27), one or two discriminant functions were obtained, respectively. In the first situa-

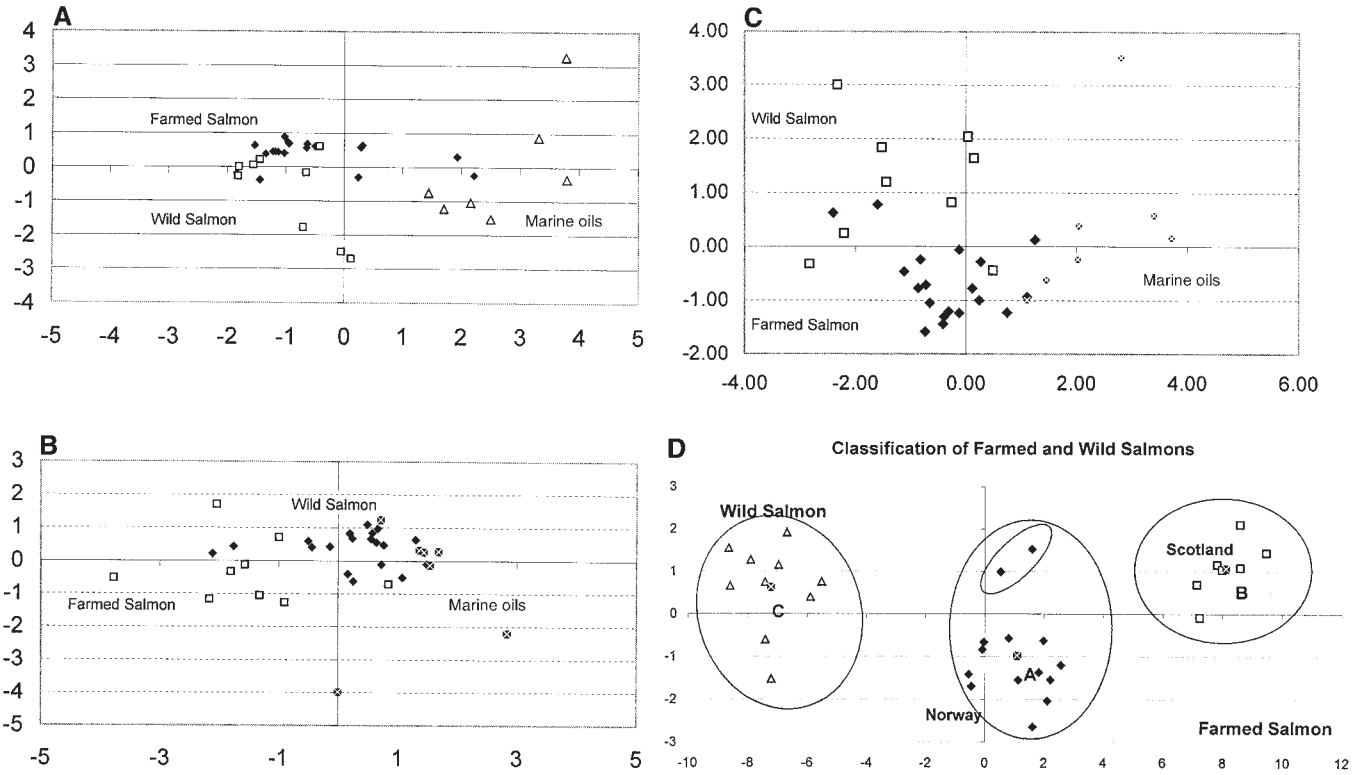

FIG. 2. Canonical representation of the two groups of farmed and wild salmons. (A) The four gas chromatographic compositions (18:2n-6, 20:5n-3, 18:1n-7, 16:1n-9) having the largest *F* values (>99.9%) in the analysis of variance procedure were used in this analysis. Classification achieved was 80%. (B) Three molar fractions (fm11, fm6, fm9) with $F > 99.9\%$ were used in this analysis. Classification achieved was 83%. (C) Five site-specific isotope ratios [$(D/H)_8$, $(D/H)_{12}$, $(D/H)_{11}$, $(D/H)_3$, $(D/H)_{14}$, $F > 99.9\%$] were used in this analysis. Classification achieved was 88%. (D) The discriminant scores of the samples are computed from the values of the eight original variables weighted by the discriminant loadings: four fatty acid compositions [16:0 (–0.47), 16:1n-9 (0.44), 18:1n-9 (0.19), 22:1n-9 (–0.17)], three molar ratios [fm6 (374.0), fm11 (167.9), fm14 (67.9)], the overall isotope ratio $(D/H)_{tot}$ (–0.23) and an added constant (–3.22). Classification achieved was 100% where A, B and C are the gravity centers of the groups.

tion (two groups), the deuterium molar fractions fm6, fm11, and fm14 lead to the highest degree of classification (89.3%), samples numbers 4, 10, and 28 being wrongly classified; and in the second situation (three groups), 85.7% of the oils were conveniently classified using the site-specific isotope ratios $(D/H)_4$, $(D/H)_6$, $(D/H)_{11}$, and $(D/H)_{14}$, samples 9, 10, 13, 28, and 38 being wrongly classified. These results are encouraging, but it should be kept in mind that an analytical procedure must minimize the second-order risk, i.e., considering false an honest product. From this respect, the previous results are not completely satisfying since a wild salmon (no. 28) is classified in the group of the farmed salmon. To obviate this problem tentatively, CDA was carried out with four compositional variables, 16:0, 16:1n-9, 18:1n-9, and 22:1n-9; three deuterium molar fractions fm6, fm11, and fm14; and $(D/H)_{tot}$ measured by IRMS. These eight mixed variables give a 100% classification (Fig. 2). Moreover, the Norwegian and Scottish origins of the farmed salmon are clearly differentiated. It is interesting to note that there is no significant difference between the $(D/H)_{tot}$ isotope ratios of feed no. 14 and 21, and that the feed given to the farmed salmon either in Norway or in Scotland is assigned the corresponding group of fishes. The differentiation between farmed salmon from Norway and Scotland seems not to be directly related to the composition of feed stuffs, rather it mainly depends on the deuterium distribution in the fatty acids. On the other hand, the differences observed between farmed and wild salmon can hardly be imputed to the isotopic composition of the wild feed, which is unknown. In turn, a possible difference of biological activities between farmed or wild salmon could be invoked as a discriminant factor.

A joint use of the compositional data and of the natural deuterium distribution of most of the fatty acids found in salmon and wild fishes, respectively, determined by GC and NMR spectroscopy lead to a straightforward differentiation of farmed and wild fishes. The classification was fully effective when four GC compositions, three deuterium molar fractions, and the overall $(D/H)_{tot}$ isotope ratio of the fatty acid mixtures were used in the calculation. The degree of classification was on the order of 80 to 88% when a single set of variables was considered. Moreover, it was possible to discriminate between salmon farmed in Norway and in Scotland. Work is in progress to investigate the classification efficiency of ^{13}C NMR fingerprinting methods in similar situations.

ACKNOWLEDGMENTS

This work was carried out as a part of two different projects supported by the Norwegian Research Council (NFR-project 107904/120 and 115147/112). We thank A. Sæbø, Lipro AS, Aalesund, Norway, for providing the commercial fish oils. Additionally, a research grant from the Norwegian Research Council is gratefully acknowledged. We thank also Brüker France for their support.

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[Received July 28, 1999; accepted January 31, 2000]