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High-Resolution ^{1}H and ^{2}H NMR Spectroscopy of Pure Essential Fatty Acids for Plants and Animals

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High-resolution ^{1}H and ^{2}H NMR spectroscopy were used to study the most important saturated (C14:0, C16:0, C18:0), monounsaturated (C16:1n-7, C18:1n-7, C18:1n-9, C20:1n-9, C22:1n-9) and polyunsaturated [C18:3n-3, C20:5n-3 (EPA), C22:6n-3 (DHA)] fatty acids which may be found in fish lipids, in order to prepare the tools for a subsequent study of the recognition of the origin of fish oils by isotopic analysis. The interpretation of the ^{1}H and ^{2}H NMR spectra of the different acids is reported. Additionally, the overall D/H and the $^{13}C/^{12}C$ isotope ratios are given. The overall D/H ratio ranges from 114.0 to 137.2 ppm/V.SMOW (V.SMOW = water standard) and the $^{13}C/^{12}C$ ratio from −23.5 to −33.2‰. From the ^{2}H NMR spectra, an investigation of the internal deuterium distribution in the saturated and mono- and polyunsaturated fatty acids was made. The deuterium distribution can be given for nearly all the chemical sites, except for the different double bonds, in EPA and DHA. The precision of the measurements was of the order of 0.5–4% depending on the nature of the cluster considered.

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INTRODUCTION

In recent years there has been growing interest in the nutritional benefits and eating quality of fish meat. Attention has been focused on the high levels of polyunsaturated fatty acids, especially ω-3 fatty acids, believed to play a preventive role in cardiovascular disease, inflammation and cancer.[1,2] The increasing production and consumption of fish oil as a health product and fish products manufactured from both farmed and wild fish has led to an increasing demand for methods useful in the authentication of these products. From this point of view, stable isotope analysis is considered 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[3] and hydrogen or oxygen isotope ratios are powerful indicators of environmental conditions.[4,5] 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, and NMR spectroscopy has the clear advantage over IRMS that natural abundance ^{2}H 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.

Isotopic fractionation studies of lipids have been mainly carried out by ^{13}C IRMS[6,7] and recently two SNIF-NMR studies have been devoted to plant lipids.[8,9] SNIF-NMR was developed in the early 1980s to detect adulteration of wine and has been adopted as an official method for the authentication of wine.[10] This technique has also been used in the authenticity testing of fruit juices[11] and aroma components.[12,13] However, detailed investigation of the deuterium distribution in lipids of marine origin has not been reported, as far as we know. On the other hand, ^{1}H NMR has been used to examine the ω-3 fatty acid content, 22:6n-3 fatty acid (DHA) and cholesterol content of lipids extracted from muscle of fish and fish oil.[14,15]

However, the study of lipids by NMR requires that preliminary treatment of the products must be applied beforehand. Indeed, even at a field of 11.4 T, ^{2}H NMR spectroscopy has a low resolution in terms of chemical shifts, and complex mixtures such as triglycerides lead to unusable spectra. It is then a requirement in such studies to hydrolyse the triglycerides into a mixture of fatty acids and to measure the ^{2}H NMR spectra of the mixtures after having carefully determined the acid composition by GC or HPLC, for example (Fig. 1). In the case of plant lipids, three or four molecular species, palmitic, stearic, oleic and linoleic acid, represent more than 95% of the whole pool of fatty acids and their NMR properties are relatively well documented. In the case of animal and fish lipids, however, the number of

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fatty acids involved in the metabolism is much higher and a treatment similar to that devoted to plant lipids would be unsuccessful if the NMR properties of the components of the mixture are not thoroughly studied. This paper presents a systematic ^1H and ^2H study of the most important saturated and mono- and polyunsaturated fatty acids which may be found in the hydrolysates of fish oils, in order to prepare the tools for a subsequent study of the recognition of the origin of fish oils by isotopic analysis.

EXPERIMENTAL

Materials

$CHCl_3$ ($\leqslant$0.0050% water content and stabilized with about 0.02% of amylene) was purchased from Prolabo (Paris, France) and $CDCl_3$ (99.8% purity) from Isotec (Miamisburg, OH, USA). Hexafluorobenzene (99% purity) was obtained from Acros Organics (Pittsburg, NJ, USA). C16:0, C16:1n-7, C18:0, C18:1n-9, C18:3n-3, C20:1n-9, C22:1n-9 and C22:6n-3 methyl esters (DHAme) was purchased from Sigma (St Louis, MO, USA). C22:6n-3 and C20:5n-3 ethyl esters were produced at Norsk Hydro. (Trondheim, Norway). EPAdel (health product, Japan) contained 94% C20:5n-3 ethyl ester. The different homologues of the fatty acids studied are characterized with their IUPAC and common names in Table 1.

Lipid extraction

The spectrum shown in Fig. 1 was obtained with the hydrolysate of lipids extracted from fish muscle of farmed Atlantic salmon (*Salmo salar*) according to the method of Bligh and Dyer.[16]

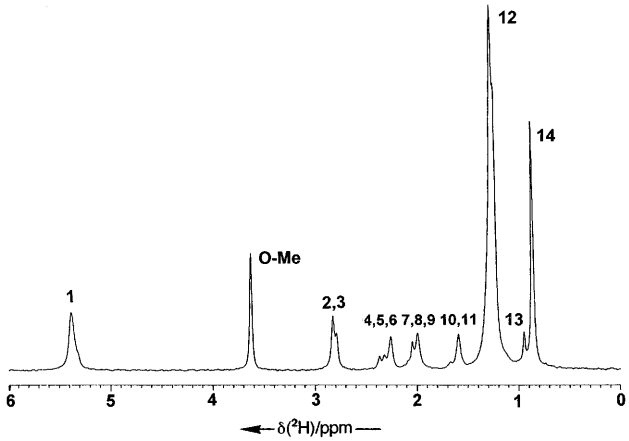


Figure 1. ^2H NMR spectrum of hydrolysate of lipids extracted from muscle of Atlantic salmon (*Salmo salar*). The signals are numbered according to Scheme 1 and Table 2.

NMR spectroscopy

The ^1H NMR spectra of the fatty acid methyl esters were examined in $CDCl_3$ using 5 mm tubes (maximum 20 mg per 0.6 ml $CDCl_3$), obtained at a frequency of 500.13 MHz using a Bruker DPX 500 spectrometer. The free induction decay (FID) was acquired with a pulse delay of 8.1 s, using a sweep width of 4 kHz and 65K data points. Scans (40) were collected at 300 K using a 45° excitation pulse. The chemical shifts were referenced indirectly to tetramethylsilane (TMS) by using the peak of $CDCl_3$ ($\delta = 7.26$ ppm/TMS). The interpretation of some of the clusters in the ^1H spectra of the different fatty acids is based on multi-irradiation experiments.

Determination of isotope ratios. The overall D/H isotope ratios of the methyl esters were obtained using a VG SIRA 9 spectrometer and the deuterium measurements were performed on the hydrogen gas resulting from reduction of water. The results were referenced to the

Table 1. Names, formulae, symbols and CAS Registry Numbers of the fatty acids studied

Name				CAS Registry
IUPAC	Common	Formula	Symbol	Number
Tetradecanoic	Myristic	C14H28O2	C14:0	544-63-8
Hexadecanoic	Palmitic	C16H32O2	C16:0	57-10-3
Octadecanoic	Stearic	C18H36O2	C18:0	57-11-4
9-Hexadecenoic (*Z*)	Palmitoleic	C16H30O2	C16:1n-7	373-49-9
9-Octadecenoic (*Z*)	Oleic	C18H34O2	C18:1n-9	112-80-1
11-Octadecenoic (*Z*)	Vaccenic	C18H34O2	C18:1n-7	693-72-1
11-Eicosenoic (*Z*)	Gandoic	C20H38O2	C20:1n-9	5561-99-9
13-Docosenoic (*Z*)	Erucic	C22H42O2	C22:1n-9	112-86-7
9,12,15-Octadecatrienoic (*Z,Z,Z*)	Linolenic	C18H30O2	C18:3n-3	60-33-3
5,8,11,14,17-Eicosapentaenoic (*Z,Z,Z,Z,Z*)	EPA	C20H30O2	C20:5n-3	10417-94-4
4,7,10,13,16,19-Docosahexaenoic (*Z,Z,Z,Z,Z,Z,*)	DHA	C22H32O2	C22:6n-3	6217-54-5

water standard V-SMOW.[17] The $^{13}C/^{12}C$ ratio examination was carried out using a Finnigan Delta E mass spectrometer coupled with a Carlo Erba Model 1500 microanalyser. The carbon isotopic parameters were expressed on the $\delta‰$ scale, which refers to the isotope ratio of the sample, S, to that of the international reference PDB:[18]

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

The site-specific isotope ratios of the methyl and ethyl ester were determined by SNIF-NMR in $CHCl_3$ (1.3–3.5 g $CHCl_3$) in 10 mm tubes. The ^{2}H NMR spectra of the methyl and ethyl esters dissolved in $CHCl_3$ (1.3 g per 3.5 g $CHCl_3$, 10 mm tubes) were obtained using a Bruker DPX 400 spectrometer operating at 61.4 MHz and a Bruker DRX 500 spectrometer operating at 76.7 MHz, respectively, fitted with a ^{19}F field-frequency locking device. Each sample was analysed three times. The spectra obtained at 61.4 MHz (DPX 400 spectrometer), were recorded at 303 K with a pulse delay of 6.9 s, including an acquisition time of 6.84 s, a sweep width of 1197.32 Hz and 16K data points, using a 90° excitation pulse, and 3200 scans were collected. The spectra of 20:5n-3 and 22:6n-3 ethyl esters, measured at 76.7 MHz (DRX 500 spectrometer), were obtained at 302 K with a pulse delay of 6.9 s including an acquisition time of 6.81 s, a sweep width of 1201.92 Hz and 16K data points, using a 90° excitation pulse, and 4000 scans were collected. The signal intensities were measured using a software program based on complex least-squares curve fitting.[19] The longitudinal relaxation times, T_1, of the DHA ethyl ester were measured on the Bruker DPX 400 spectrometer by using the inversion–recovery Fourier transform (IRFT) method at 303 K. The site-specific isotope ratios $(D/H)_i$ measured by ^{2}H NMR were reported on the V.SMOW-SLAP scale according to Eqn (1) and calculated according to

$$(D/H)_i = (f_i/F_i)(D/H)_{ms} \quad (2)$$

were f_i and F_i are the effective and statistical molar ratios of the different isotopomers i observed in the ^{2}H spectra and $(D/H)_{ms}$ are the overall isotope ratios of the products determined by IRMS.

Note: Since NMR chemical shifts and isotope ratios are expressed in the same relative units, ppm and 10^{-6}, these two parameters will be specifically expressed in the form: chemical shifts as ppm/TMS and isotope ratios as ppm/V.SMOW, where TMS and V.SMOW are internationally accepted references.

RESULTS AND DISCUSSION

The fatty acids studied are listed in Table 1. For the sake of convenience, the fatty acids will be represented by their symbols in the following. In order to optimize the acquisition parameters of the ^{2}H NMR spectra, the relaxation times of a typical fatty acid, C22:6n-3 ethyl ester (**11b**), were determined by the inversion–recovery method. The ethylenic (RCD=CHR′), allylic (RCHDCH=CHR′) and α- or β-carbonyl (RCHDCOR′ or $RCHDCH_2COR'$) isotopomers have relaxation times of the order of 0.25–0.35 s but the end-chain carbon atoms (CH_2DR and CH_3CHDR) relax significantly slower (T_1 = 1.05 and 0.8 s). For this reason, the acquisition time was set equal to 6.8 s in order to recover 99% of the spin magnetization. The overall isotope ratios of the different products considered were determined by IRMS and their values are given in Table 5.

Although the purpose of the present work was not to consider changes in the isotopic composition of fatty acids in the trophic chain of fishes, the $^{13}C/^{12}C$ isotope ratios were measured to check their consistency with the D/H ratios, since a rough relationship between the ^{2}H and ^{13}C isotopic composition has been observed for terrestrial C_3 and C_4 plants.[3]

All the products studied have the typical ^{13}C composition of marine sources[20–22] and $\delta\,^{13}C$ values are of the order of −28.3 to −33.2‰, but **10c** and **11c** have relatively higher values (−23.5 and −23.8‰). It should also be noted that the ^{2}H NMR spectra of the fatty acids were measured on the methyl esters and in two cases, **10a**, **b**, **c**, **d**, and **11a**, **b**, on the ethyl esters. In order to take into consideration the influence of the isotopic content of this extra methyl group on the overall isotope ratio, the isotope ratio of the starting material of the esterification was measured and found to be equal to 144.2 (0.2) ppm/V.SMOW. As a consequence, the isotope ratios of the fatty acid moiety are given in excess (⩽2 ppm or 1.5% in relative values), but this difference should only be considered when connecting the isotopic data of fatty acids with those of other compounds. In the present work, no correction was made.

Assignment of proton and deuterium chemical shifts

In general, the 500 MHz proton signals of a fatty acid methyl ester are not completely resolved in the aliphatic part of the spectrum and the situation is worst for the ^{2}H NMR spectra. The ^{1}H and ^{2}H NMR spectra of three fatty acids having an increasing number of double bonds are shown in Figs 2 and 3, respectively. Since the primary purpose of this research was to provide efficient tools for the recognition of the origin of fish oils from their D/H isotope ratios, the discussion of signal assignments will be focused on the ^{2}H NMR spectra. In such relatively complex ^{2}H NMR spectra, it is more appropriate to deal with signal clusters rather than individual signals. A cluster is defined as a number of signals which have the same apparent chemical shift and a Lorentzian shape. The second condition is not always fully satisfied and this point will be discussed later, since it leads to artefacts in the results of the curve fitting procedure. For the different fatty acids studied, 14 clusters were defined and are presented in Table 2 and Scheme 1.

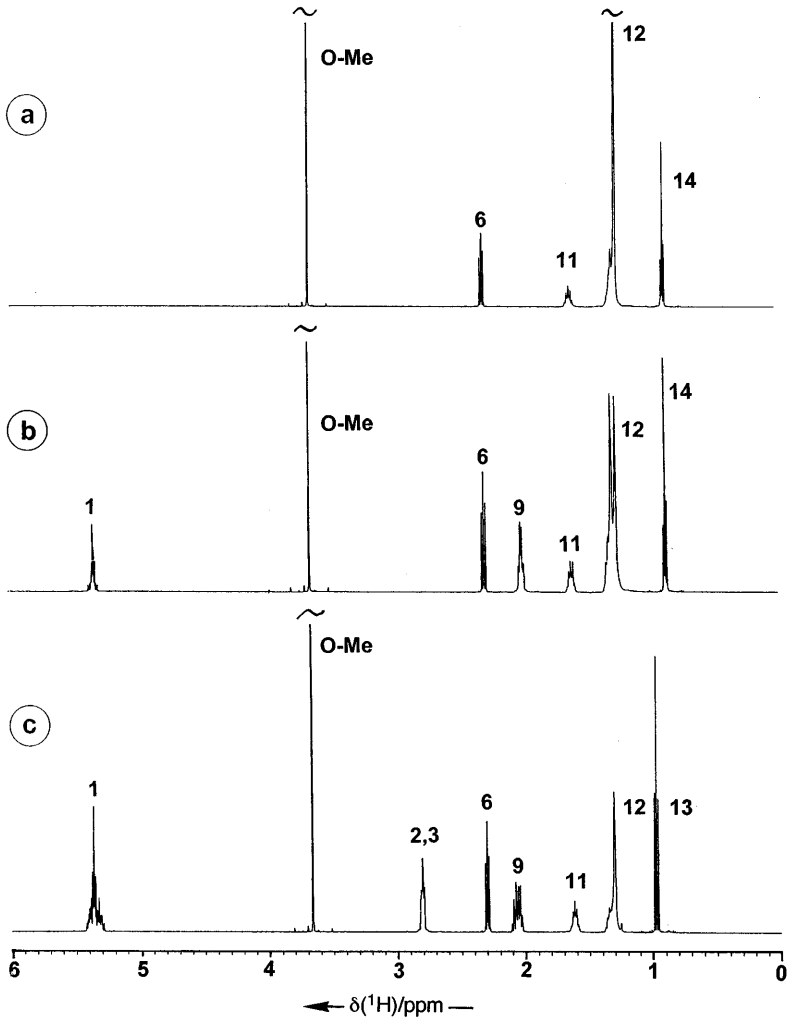


Figure 2. 500 MHz ^{1}H NMR spectra of C18 fatty acids having (a) 0, (b) 1 and (c) 3 double bonds. The signals are numbered according to Scheme 1 and Table 2.

Obviously, all the fatty acids considered here do not display the same number of clusters, and the populations of the clusters were calculated from the ^{1}H site populations of the corresponding signals. For example, myristic acid (**1**) has no ethylenic signals and the population of cluster 1 is zero; conversely, the polyunsaturated docosahexaenoic acid (**11**) displays no signal in the methylenic region of the spectrum and the population of cluster 10 is also zero. In Fig. 4(a) and (b) are given the ^{1}H and ^{2}H NMR spectra, respectively, of docosahexaenoic acid methyl ester.

The mean chemical shifts of the 14 clusters studied are given in the second row of Table 2 and those of the individual products investigated are given in Table 3.

Table 2. Contribution of the hydrogen atoms of the different fatty acid fragments studied to the NMR clusters observed at the field used (11.4 T)

		Cluster [δ(ppm/TMS)]:														
Fatty acid	Case	CLU1 5.35	CLU2 2.80	CLU3 2.75	CLU4 2.35	CLU5 2.30	CLU6 2.25	CLU7 2.05	CLU8 2.00	CLU9 1.95	CLU10 1.64	CLU11 1.55	CLU12 1.25	CLU13 0.95	CLU14 0.85	Total
C14:0	**1**	0	0	0	0	0	2	0	0	0	0	2	20	0	3	27
C16:0	**2**	0	0	0	0	0	2	0	0	0	0	2	24	0	3	31
C18:0	**3**	0	0	0	0	0	2	0	0	0	0	2	28	0	3	35
C16:1n-7	**4**	2	0	0	0	0	2	0	0	4	0	2	16	0	3	29
C18:1n-9	**5**	2	0	0	0	0	2	0	0	4	0	2	20	0	3	33
C18:1n-7	**6**	2	0	0	0	0	2	0	0	4	0	2	20	0	3	33
C20:1n-9	**7**	2	0	0	0	0	2	0	0	4	0	2	24	0	3	37
C22:1n-9	**8**	2	0	0	0	0	2	0	0	4	0	2	28	0	3	41
C18:3n-3	**9**	6	2	2	0	0	2	0	2	2	0	2	8	3	0	29
C20:5n-3	**10**	10	4	4	0	0	2	2	2	0	2	0	0	3	0	29
C22:6n-3	**11**	12	8	2	2	2	0	0	2	0	0	0	0	3	0	31

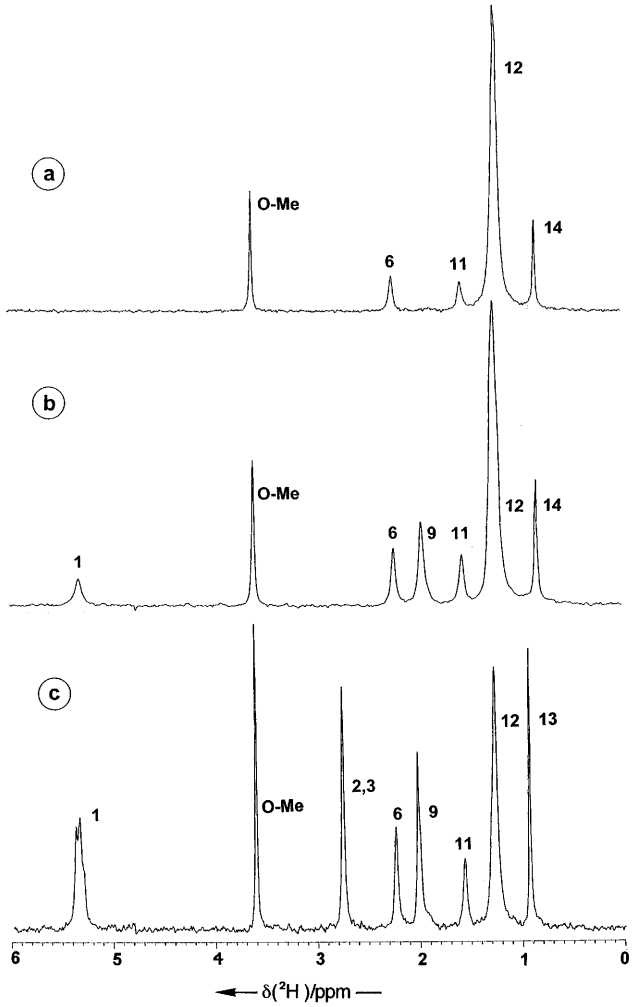


Figure 3. 76.8 MHz ^{2}H NMR spectra of C18 fatty acids (a) 0, (b) 1 and (c) 3 double bonds. The signals are numbered according to Scheme 1 Table 2.

The assignment is straightforward from the simple consideration of their chemical shifts for some cluster signals such as the end-chain methyl group, the ethylenic atoms in monounsaturated acids, the methylenic group in the α-position with respect to the carboxylic function and the methylenic chain. The α-methyl methylenic group is also easily identified from the other methylenic fragments by its relaxation time, which is substantially longer than those of the other groups. In the case of polyunsaturated acids, multiple irradiation experiments were successful for confirming the identification of the allylic methylene groups situated either in the α-position to the methyl group or near the carboxylic function. In the acids C18:3n-3, C20:5n-3 and C22:6n-3, it is possible to refine the assignment of the olefinic protons situated on the double bond close to the methyl group by homonuclear irradiation of the α-methyl allylic protons. Two-dimensional NMR experiments do not give more substantial information.

Quantitative ^{2}H NMR: determination of isotope ratios

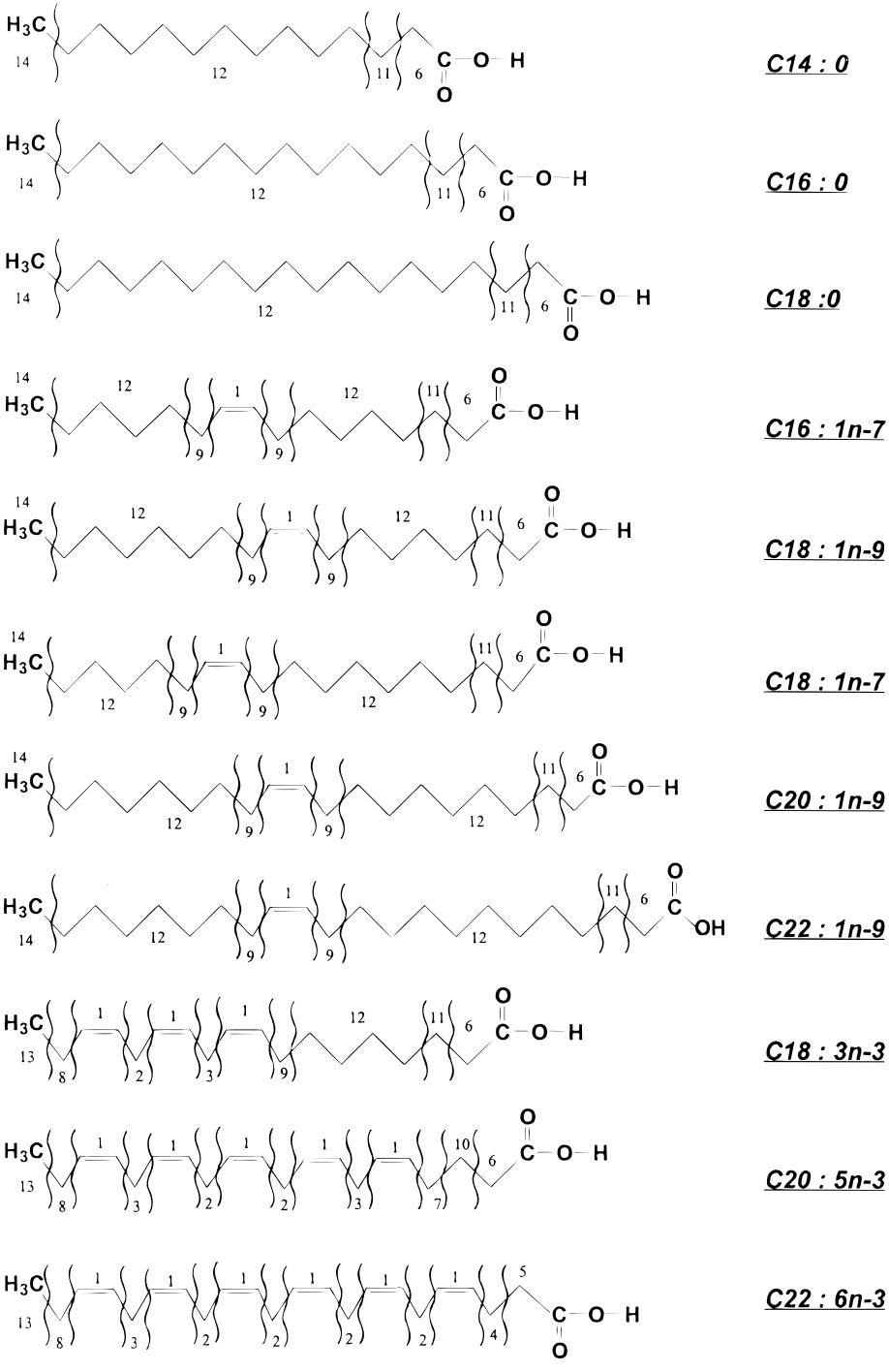
The signal intensities of the clusters were obtained using a modification of the curve fitting software Interliss,[19] which takes into account a Gaussian contribution to the cluster arising from a small degree of overlap of Lorentzian signals. In a first step, the effective molar fractions f_i of the different isotopomers observed for the fatty acid considered were calculated from the cluster intensities and are reported in Table 4. The molar fractions were normalized to unity, ignoring the contributions of the isotopomers of the ester methyl or ethyl groups to the overall spectrum intensity. In a second step, the isotope ratios were computed using Eqn (2), where the statistical molar fractions F_i were directly deduced from the data in Table 2 by dividing the site population of cluster i by the total number of hydrogen atoms considered in the fatty acid. The site-specific isotope ratios are given in Table 5 and the overall isotope ratios obtained by mass spectrometry, $(D/H)_{ms}$, are shown in the last column of this table.

The data in Tables 4 and 5 elicit several comments. First, it is interesting to discuss the precision available in the determination of the molar fractions, which in turn governs substantially the precision of the isotope ratios, since the experimental error in $(D/H)_{ms}$ is significantly lower (0.35%) than those in f_i. The errors in the determination of the different clusters, which may be associated, at least in part, with the lineshape distortion of the cluster, are given in Table 6. A mean standard

Table 3. ^{2}H chemical shifts of the signals of the fatty acid methyl esters observed at a 11.4 T field (in ppm/TMS)

		Cluster													
Fatty acid[a]	Case	CLU1	CLU2	CLU3	CLU4	CLU5	CLU6	CLU7	CLU8	CLU9	CLU10	CLU11	CLU12	CLU13	CLU14
C14:0	**1**						2.21					1.54	1.19		0.81
C16:0	**2**						2.23					1.56	1.20		0.82
C18:0	**3**						2.24					1.57	1.22		0.84
C16:1n-7	**4**	5.32					2.23			1.96		1.56	1.24		0.83
C18:1n-9	**5**	5.32					2.24			1.97		1.57	1.25		0.84
C18:1n-7	**6**	5.32					2.24			1.97		1.57	1.25		0.84
C20:1n-9	**7**	5.34					2.25			1.95		1.58	1.25		0.85
C22:1n-9	**8**	5.34					2.25			1.98		1.58	1.25		0.85
C18:3n-3	**9**	5.36	2.76	2.76			2.25		2.03	2.017		1.57	1.27	0.93	
C20:5n-3 ee	**10**	5.38	2.80	2.77			2.24	2.07	2.02		1.64			0.94	
C22:6n-3	**11**	5.39	2.82	2.79	2.37	2.33			2.04					0.95	
C22:6n-3 ee	**11**	5.37	2.81	2.77	2.35	2.29			2.03					0.93	

[a] ee = Ethylester.



Scheme 1

deviation (MSD) was calculated for each cluster *i* from the standard deviations of cluster *i* measured in the different fatty acids. MSD is the square root of the mean pooled variance computed by column on the fatty acids and has roughly the same absolute value, i.e. 0.0022, whatever the cluster, except cluster 2, which is characterized by a larger value (0.0041). However, as a relative value, expressed by the relative standard deviation (RSD = MSD/mean in %), the error is obviously higher for small signals (4–4.5%) than for the more intense peaks (0.5–1.5%). Now, if we examine the distribution of the experimental error as a function of the nature of the fatty acid, the value of MSD computed by row is of the same order of magnitude: the values for the saturated, monounsaturated, pentaunsaturated and hexaunsaturated acids are 0.0020, 0.0017, 0.0026 and 0.0022, respectively. It may be concluded that the repeatability of SNIF-NMR does not depend significantly on the nature of the cluster or on the nature of the fatty acid. As a consequence of the imprecision of the molar fractions, the errors in the isotope ratios are of the order of 2.5%, ranging from 0.6% for the more intense and better resolved signals to 4.2% for the lower and badly shaped peaks.

Table 4. Molar fractions of the ^{2}H isotopomers of the fatty acid fragments studied

		Molar ratio													
Fatty acid	Case	f_1	f_2	f_3	f_4	f_5	f_6	f_7	f_8	f_9	f_{10}	f_{11}	f_{12}	f_{13}	f_{14}	Total
C14:0	**1**						0.0779					0.0741	0.7415		0.1065	1.0000
C16:0	**2**						0.0744					0.0713	0.7614		0.0929	1.0000
C18:0	**3**						0.0569					0.0476	0.8184		0.0772	1.0000
C16:1n-7	**4**	0.0647					0.0604			0.1276		0.0590	0.5819		0.1064	1.0000
C18:1n-9	**5**	0.0543					0.0712			0.1224		0.0672	0.5986		0.0864	1.0000
C18:1n-7	**6**	0.0526					0.0563			0.1252		0.0547	0.6105		0.1006	1.0000
C20:1n-9	**7**	0.0502					0.0453			0.1042		0.0436	0.6802		0.0764	1.0000
C22:1n-9	**8**	0.0444					0.0483			0.0922		0.0467	0.6978		0.0706	1.0000
C18:3n-3	**9**	0.1785		0.1384			0.0854		0.0641	0.0725		0.0611	0.3002	0.0998		1.0000
C20:5n-3	**10a**	0.3162	0.1708	0.1450			0.0656	0.0818	0.0526		0.0826			0.0855		1.0000
	10b	0.3178	0.1771	0.1388			0.0640	0.0806	0.0532		0.0836			0.0850		1.0000
	10c	0.3069	0.1741	0.1479			0.0667	0.0843	0.0566		0.0813			0.0823		1.0000
	10d	0.3189	0.1670	0.1512			0.0641	0.0762	0.0582		0.0802			0.0842		1.0000
C22:6n-3	**11a**	0.3843	0.2766	0.0626	0.0791	0.0549			0.0602					0.0824		1.0000
	11b	0.3839	0.2723	0.0660	0.0801	0.0535			0.0607					0.0836		1.0000
	11c	0.3932	0.2908	0.0460	0.0824	0.0552			0.0545					0.0779		1.0000

Table 5. Site-specific isotope ratios (in ppm/V.SMOW) of the fatty acid fragments studied[a]

Fatty acid	Case	15Molar ratios $(D/H)_1$	$(D/H)_2$	$(D/H)_3$	$(D/H)_4$	$(D/H)_5$	$(D/H)_6$	$(D/H)_7$	$(D/H)_8$	$(D/H)_9$	$(D/H)_{10}$	$(D/H)_{11}$	$(D/H)_{12}$	$(D/H)_{13}$	$(D/H)_{14}$	$(D/H)_{ms}$
C14:0	**1**						129.2					123.0	123.0		117.9	122.9
C16:0	**2**						139.8					134.0	119.2		116.4	121.0
C18:0	**3**						121.6					101.7	125.0		110.0	122.2
C16:1n-7	**4**	128.7					120.0			126.9		117.4	144.7		141.1	137.2
C18:1n-9	**5**	113.7					149.0			128.1		140.7	125.3		120.6	126.9
C18:1n-7	**6**	99.0					105.9			117.8		102.8	114.8		126.1	114.0
C20:1n-9	**7**	122.0					110.0			126.6		105.9	137.7		123.8	131.3
C22:1n-9	**8**	106.3					115.7			110.5		111.8	139.4		112.8	129.3
C18:3n-3	**9**	102.9		119.7			147.7		110.9	125.4		105.7	129.8	115.1		119.3
C20:5n-3	**10a**	107.5	145.1	123.2			111.5	139.0	89.3		140.3			96.9		117.2
	10b	116.6	150.4	117.9			108.8	136.9	90.4		142.1			96.3		117.2
	10c	110.6	156.8	133.2			120.1	151.8	101.9		146.4			98.8		124.2
	10d	107.4	140.7	127.4			108.0	128.4	98.0		135.2			94.5		116.2
C22:6n-3	**11a**	121.4	131.1	118.6	149.9	104.0			114.0					104.2		122.3
	11b	122.0	129.8	125.8	152.7	102.0			115.8					106.3		123.0
	11c	128.2	142.2	90.0	161.3	107.9			106.6					101.6		126.2

[a] The fragments correspond to the fatty acid moiety of the fatty acid methyl or ethyl esters.

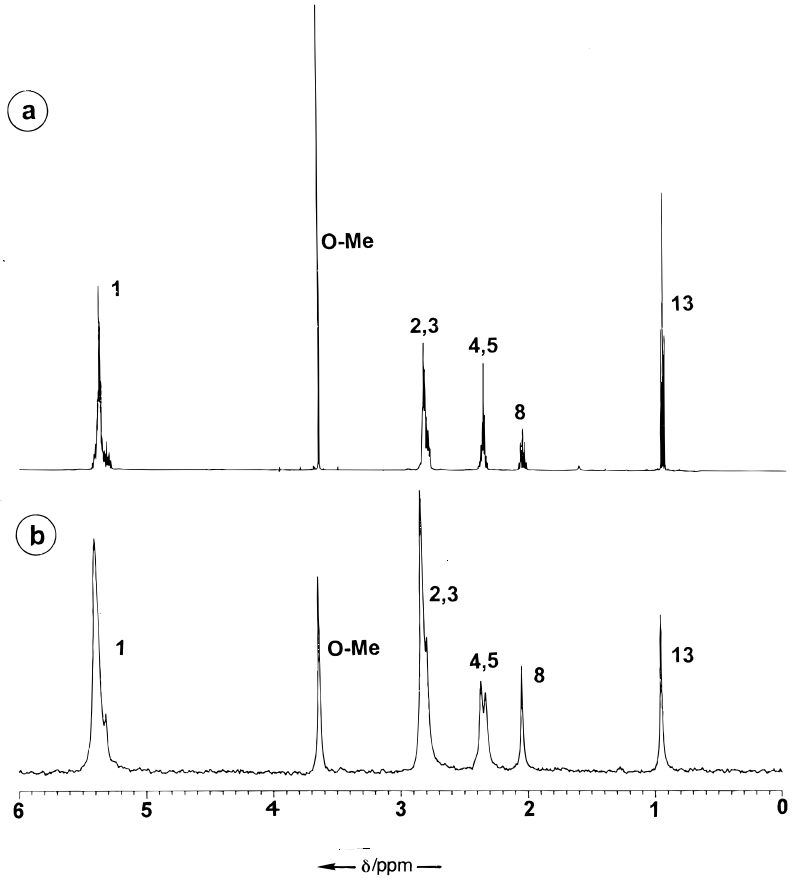


Figure 4. (a) 500 MHz ^{1}H and (b) 76.8 MHz ^{2}H NMR spectra of hexaunsaturated fatty acid C22:6n-3 methyl esters. The signals are numbered according to Scheme 1 and Table 2.

Variation of molar fractions and isotope ratios as a function of structure

The purpose of this work was to develop tools for the authentication of plant, animal and fish oils in terms of the nature of the living organism and environmental conditions, and the work was limited with most of the fatty acids studied to only one compound of each kind. Although it is not possible to define isotopic authentication criteria, some remarks can be made concerning the deuterium distribution in these products. Excepted for acid **4**, all the fatty acids studied have a mean $(D/H)_{ms}$ value of the order of 122 ppm/V.SMOW. Lipids of marine origin contain a relatively high proportion of the n-3 long-chain fatty acids such as EPA and DHA. In the ^{2}H NMR spectra of these fatty acids it was possible to distinguish between signals from nearly all the chemical sites except for the signals from the double bonds. We observed a deuterium depletion at the methyl end, the ω-2 site and site C-2 close to the car-

Table 6. Precision of the determination of the molar fractions of fatty acid ^{2}H clusters by ^{2}H NMR

	f_1 [a]	f_1 [b]	f_2 [c]	f_2 [d]	f_3 [c]	f_3 [d]	f_4	f_5	f_6
Mean	0.0532	0.3459	0.1722	0.2799	0.1443	0.0582	0.0805	0.0545	0.0643
MSD	0.0025	0.0025	0.0041	0.0041	0.0027	0.0027	0.0022	0.0023	0.0022
RSD(%)	4.64	0.71	2.36	1.45	1.84	4.56	2.76	4.22	3.39
	f_7	f_8	f_9	f_{10}	f_{11}	f_{12}	f_{13}	f_{14}	
Mean	0.0807	0.0575	0.1074	0.0819	0.0584	0.6434	0.0851	0.0896	
MSD	0.0013	0.0009	0.0030	0.0005	0.0025	0.0034	0.0012	0.0014	
RSD(%)	1.64	1.65	2.83	0.63	4.33	0.53	1.42	1.61	

[a] Monounsaturated.
[b] Polyunsaturated.
[c] Pentaunsaturated.
[d] Hexaunsaturated.

bonyl end and a strong enrichment in site C-3 in both EPA and DHA and in site C-4 (*cis*-allylic) in EPA. This is not in accordance with results reported for saturated and monounsaturated acids that are depleted in deuterium in the C-3 site.[8] In general, the highly unsaturated fatty acids such as EPA and DHA are converted from C18:3n-3 by a pathway combining the sequential action of Δ^6- and Δ^5-desaturases, elongases and retroconversion enzymes.[23–25] The enrichment of the C3 site in EPA and DHA can be related to the different origin of the hydrogen in the fatty acid elongation reaction and/or effects of the enzymic oxidation at positions 4 and 5 for DHA and EPA, respectively.

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