Nondestructive Quantitative Determination of Docosahexaenoic Acid and n-3 Fatty Acids in Fish Oils by High-Resolution ^{1}H Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT: By using a 500 MHz proton nuclear magnetic resonance (^{1}H NMR) spectrometer we have developed a quantitative method for determining the contents of docosahexaenoic acid (DHA) in fish oils (mg/g), the molar proportions (mol%) of DHA to all other fatty acids composing the fish oils, and the molar proportions of total n-3 fatty acids to all other non-n-3 fatty acids in the fish oils. After examining the suitability of ethylene glycol dimethyl ether (EGDM), methanol, and 1,4-dioxane as internal standards, experimental conditions were optimized by mainly using EGDM as an internal standard. By setting the pulse repetition time at 30 s, five times longer than the longest T_1 of the ^{1}H NMR signals of fish oils, good reproducibility of data and analytical times less than 10 min were achieved. The use of the internal standard also allowed us to quantify DHA on a weight basis (mg/g). Verification of the method was carried out in an interlaboratory study between Japan and Norway on bonito, tuna, and salmon oils. The relative errors in the ^{1}H NMR data between Japan and Norway were 0.57–5.29% for quantification of DHA, 0.7–2.09% for the molar proportion of DHA, and 0.1–1.41% for the molar proportion of total n-3 fatty acids. Good agreement was observed between the ^{1}H NMR data and those obtained by gas chromatography (GC). The sample preparation before ^{1}H NMR measurements required only two steps: sample weighing and preparation of an internal standard solution. Based on the high reproducibility, simplicity of the procedure, and clarity of principle, the proposed ^{1}H NMR method was judged to be a promising alternative to the GC method in quantification of DHA and n-3 fatty acids in fish oils.

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KEY WORDS: DHA, n-3 fatty acids, fish oil, highly unsaturated fatty acids, internal standard, ^{1}H NMR spectroscopy.

Since the epidemiological reports on Inuit living in Greenland appeared in the 1970s (1–3), the beneficial effect of ω-3 (n-3) highly unsaturated fatty acids (HUFA) on human health has been emphasized (4–6). In recent years, the potential use of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has been further investigated for treatment of chronic diseases, e.g., diabetes, AIDS, and Alzheimer's (7–9). Consequently, there is worldwide interest in marine products such as fish and fish oils that contain high levels of DHA and EPA. The market for health foods enriched with the n-3 fatty acids including DHA and EPA has rapidly expanded in the European countries, the United States, and Japan. Thus, the quality of these important nutrients in marine products should be strictly controlled. In Japan, DHA contents (mg/g) are taken as an indispensable criterion for judging the quality of fish oils (10). For that purpose HUFA in fish and fish oils are customarily analyzed by gas chromatography (GC) (11–14). However, GC methods are time-consuming, involve considerable manipulation steps that may cause oxidation of lipids, may give variable results depending on the columns used, and pose difficulty in correctly identifying each fatty acid in the chromatograms (15). Additionally, as reported in an official collaborative study for GC (11), the interlaboratory precision index, RSD_R, of DHA in fish oils is relatively large (7.5–16.1%). It is easily expected that such deviation of each fatty acid is added up in totaling a group of fatty acids such as the n-3 fatty acids.

It has been recently shown that HUFA in fish and fish oils could be determined nondestructively and noninvasively by ^{1}H and ^{13}C NMR (proton and carbon nuclear magnetic resonance) spectroscopy (16–20). DHA is a unique fatty acid in having a C2 and C3 methylene (C2,3-CH_2) interposed between the carboxyl group at C1 and the double bond at C4. Because of this electronegative environment, DHA gives rise to unique signals for the C2,3 methylene protons (17,18,21) in an isolated downfield region. These signals are well separated from the signals of methylene protons of non-DHA fatty acids. In a non-DHA type fatty acid, a double bond, if there is one, is located at position 5 or greater, and the C2 methylene protons are distinguishable from other methylene protons in the molecule because of their proximity to the carboxyl moiety. The C2 methylene signals of non-DHA fatty acids are

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separated from the corresponding signals of DHA. It is thus possible to determine the molar proportions of DHA by comparing the signal areas for DHA C2,3-methylenes with those arising from C2-methylenes of other fatty acids. Furthermore, we deduced that the utility of isolated signals of C2,3 methylenes of DHA can be extended beyond the quantification of molar proportions. If a proper internal standard is used and ^{1}H NMR parameters are optimized, it should be possible to quantify the DHA content in oil on a weight basis (mg/g). Furthermore, the methyl protons of n-3 fatty acids show a unique signal. Because of their proximity to the double bond, the terminal methyl protons in all n-3 fatty acids resonate downfield compared with those in non-n-3 type fatty acids. Thus, by comparing the signal areas of methyl protons, the molar proportions of total n-3 fatty acids can be determined (17,19,22). Based on this principle, Sacchi *et al.* (19) measured the molar proportion of n-3 fatty acids (mol%) in fish lipids by both GC and ^{1}H NMR and observed good correlation between the two. However, in all previous ^{1}H NMR experiments the quantitative precision was not rigorously tested and no interlaboratory study was conducted. Nor was any attempt made to apply the ^{1}H NMR method to determine the weights of DHA in samples quantitatively. In this study, we report the use of an internal standard and optimized ^{1}H NMR parameters for determining the weights of DHA in fish oils (mg/g). Simultaneous determination of the molar proportions of DHA and those of total n-3 fatty acids by ^{1}H NMR is also reported. The reproducibility of the results was verified in an interlaboratory study between Japan and Norway.

EXPERIMENTAL PROCEDURES

Materials. Methanol (purity 99.8%; Wako Pure Chemicals Co., Tokyo, Japan), ethylene glycol dimethyl ether (EGDM, purity >99.0%; Wako Pure Chemicals Co.), and 1,4-dioxane (purity >99.0%, ACS Reagent; Sigma Chemical Co., St. Louis, MO) were tested as internal standards. $CDCl_3$ (99.5% purity in Norway, 99.8% purity with 0.03% tetramethylsilane (TMS) in Japan] was purchased from E. Merck (Darmstadt, Germany). Fatty acid methyl esters purchased from Sigma Chemical Co. (purity >99.0%) or from Supelco Inc. (FAME Mix, purity >99.0%; Bellefonte, PA) were used for GC standards. To confirm the linearity of the standard curve and to determine the optimal concentration of the internal standard, a series of samples with a known DHA content (0–400 mg/g) were prepared by mixing a salad oil (DHA free; Nisshin Oil Mills Ltd., Tokyo, Japan) with an aliquot of DHA triglyceride concentrate (911 mg/g; Harima Chemicals Inc., Tsukuba, Japan). A total of 11 refined fish oils from bonito or tuna were provided from the following Japanese companies: NOF Corp. (Tokyo), Harima Chemicals Inc., Maruha Corp. (Tsukuba), Croda Japan Co. (Shiga), and Ohno Chemical Machinery Co. (Tokyo). DHA concentrations were in the range from 20 to 48 mol%. These refined fish oils were used for testing the suitability of standards and to compare ^{1}H NMR data with those of GC. In the interlaboratory study, unrefined bonito and tuna oils produced in Japan (Yaizu Fish Meal Cooperative Association, Shizuoka, and Chuoh Feed Manufacturing Company Ltd., Chiba, respectively) and a salmon oil produced in Norway (Vikholmen Bioprocess AS, Vikholmen) were included.

Internal standard solutions. Four different concentrations [0.5, 1, 2, and 5 (wt/vol%)] of internal standard solutions were prepared by dissolving methanol, EGDM, and 1,4-dioxane, in $CDCl_3$, respectively. A 0.75-mL portion of the solution was accurately added to a sample oil (*ca.* 250 mg) in a 5-mm (outer diameter) NMR tube. The tubes were capped, and the contents were mixed by inverting the tubes several times. With these samples, quantification was done by directly comparing the signal areas of fatty acids with those of the standard.

Calibration curves for DHA. To 250, 225, 200, and 150 mg of DHA-free salad oil were added 0, 25, 50, and 100 mg of the DHA triglyceride concentrate, respectively, to make up 250-mg samples, respectively. Then the internal standard solutions were added. Therefore, a total of 48 sample tubes were prepared for the calibration curve linearity test. The calibration curves prepared with DHA-added solutions were designated the DHA calibration curves.

^{1}H NMR spectroscopy. For ^{1}H NMR measurements a Varian Inova 500 spectrometer (Varian, Palo Alto, CA) was used in Japan and a Bruker AM 500 (Karlsruhe, Germany) in Norway. ^{1}H T_1 measurements were performed in Japan by an inversion recovery method on refined tuna oils (250 mg) containing 1% of the respective standards. The parameters were as follows: acquisition time, 2.69 s; data points, 128 K; number of scan, 8; τ, 0.05, 0.5, 1.0, 2.0, 3.0, 5.0, 10, 20, 40 s; pulse delay, 160 s; temperature, 25°C; spectral width, 5,000 Hz. Quantitative high-resolution one-dimensional ^{1}H NMR spectra for the DHA triglyceride-salad oil mixtures and refined fish oils were obtained with the following parameters: data points 64 K; spectral width, 5,000 Hz; 16–32 scans; pulse angle, 45°; acquisition time, 7.0 s; pulse delay, 1.5 s. The pulse delay was changed from 1.5 to 3.0–53 s for measuring the unrefined fish oils. Chemical shifts were referenced indirectly to the methyl signal of EGDM (3.35 ppm), to the methylene signal of 1,4-dioxane (3.65 ppm), or to the methyl signal of methanol (3.30 ppm). Assignments of signals in the ^{1}H NMR spectra (Fig. 1) were based on the studies on n-3 fatty acids by Aursand *et al.* (17) and Sacchi *et al.* (19). For quantification of DHA, the signals arising from C2,3 methylenes in DHA (around 2.38 ppm, four protons) were compared with those of the C2 methylene signals from all other fatty acids in the sample (around 2.28 ppm, two protons) (16). The molar proportion of total n-3 fatty acids was also determined simultaneously by comparing the methyl signals at 0.81–0.89 ppm, which originated from n-3 fatty acids, with those at 0.90–0.98 ppm, from other fatty acids, as practiced by Sacchi *et al.* (19).

Signal intensity measurement. For quantitative ^{1}H NMR measurements, signal areas were measured for the following regions in fish oil spectra: C2,3 protons from DHA (2.325–2.425 ppm), C2 protons from other fatty acids (2.225–2.325

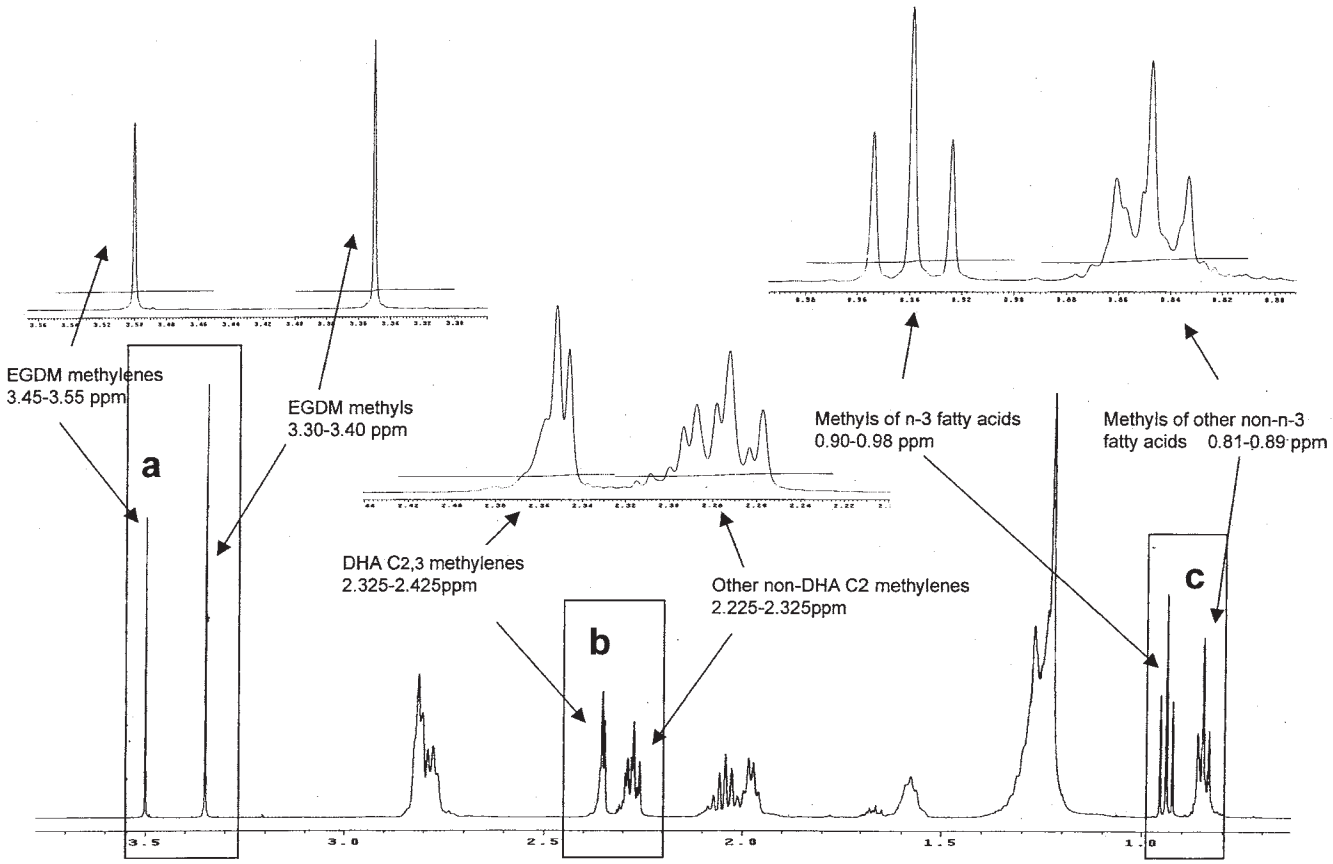

FIG. 1. An example of the manual peak separation for ethylene glycol dimethyl ether (EGDM) signals, docosahexaenoic acid (DHA) C2,3 and other fatty acids' C2 methylenes signals, and n-3 and other fatty acids methyl signals. Each framed region (**a**, **b**, **c**) is enlarged above.

ppm), methyl protons from n-3 fatty acids (0.90–0.98 ppm), and methyl protons from the other fatty acids (0.81–0.89 ppm). For measuring the singlet signals of the internal standards, the regions for the chemical shift value ±0.05 ppm were chosen. Signal areas were measured mainly by automatic integration after correcting baseline and drift. The process was called "manual peak separation" (Fig. 1). To compare the accuracy of integration, signal areas were also measured by a deconvolution program (Varian) on a workstation (Sun Sparc station 5). The process was called "deconvolution peak separation" (Fig. 2) (23). For this treatment, Gaussian and Lorenzian fractions were set to 2:8.

Quantification of DHA (mg/g). The DHA content (mg/g) in samples (X) was calculated from Equation 1.

$$X = (\text{Adha} / \text{Astd}) \times (\text{Hstd} / \text{Hdha}) \times (\text{Mdha} / \text{Mstd}) \times Y/Z \qquad [1]$$

where Adha = signal area of DHA C2,3 methylene protons, Astd = signal area of the internal standard protons, Hstd = number of protons of the internal standard, Hdha = number of protons of DHA C2,3 methylene (4), Mdha = molecular weight of DHA (328.5), Mstd = molecular weight of the internal standard, Y = weight of the internal standard in the NMR tube (mg), Z = weight of the sample oils in the NMR tube (g).

Quantification of molar proportions (mol%). The molar proportions (mol%) of DHA (Rdha) and total n-3 fatty acids (Rn3) in samples were determined by using Equations 2 and 3, respectively;

$$\text{Rdha} = (\text{Adha}/2)/(\text{Adha}/2 + \text{Aotdha}) \times 100 \qquad [2]$$

$$\text{Rn3} = \text{An3}/(\text{An3} + \text{Aotn3}) \times 100 \qquad [3]$$

where Aotdha = signal area of the C2 methylene protons from fatty acids other than DHA, An3 = signal area of the methyl protons of n-3 fatty acids, and Aotn3 = signal area of the methyl protons of all fatty acids except n-3 fatty acids.

The data from ^{1}H NMR and GC were assessed on the basis of the relative error expressed by either Equation 4 or 5:

$$\text{relative error} = (^1\text{H NMR data} - \text{GC data})/[(^1\text{H NMR data} + \text{GC data})/2] \times 100 \qquad [4]$$

$$\text{relative error} = |(^1\text{H NMR data} - \text{GC data})|/[(^1\text{H NMR data} + \text{GC data})/2] \times 100 \qquad [5]$$

(The numerator is expressed as absolute value.)

GC. The fatty acid composition and the DHA content (mg/g) of fish oils were measured after methylation by GC

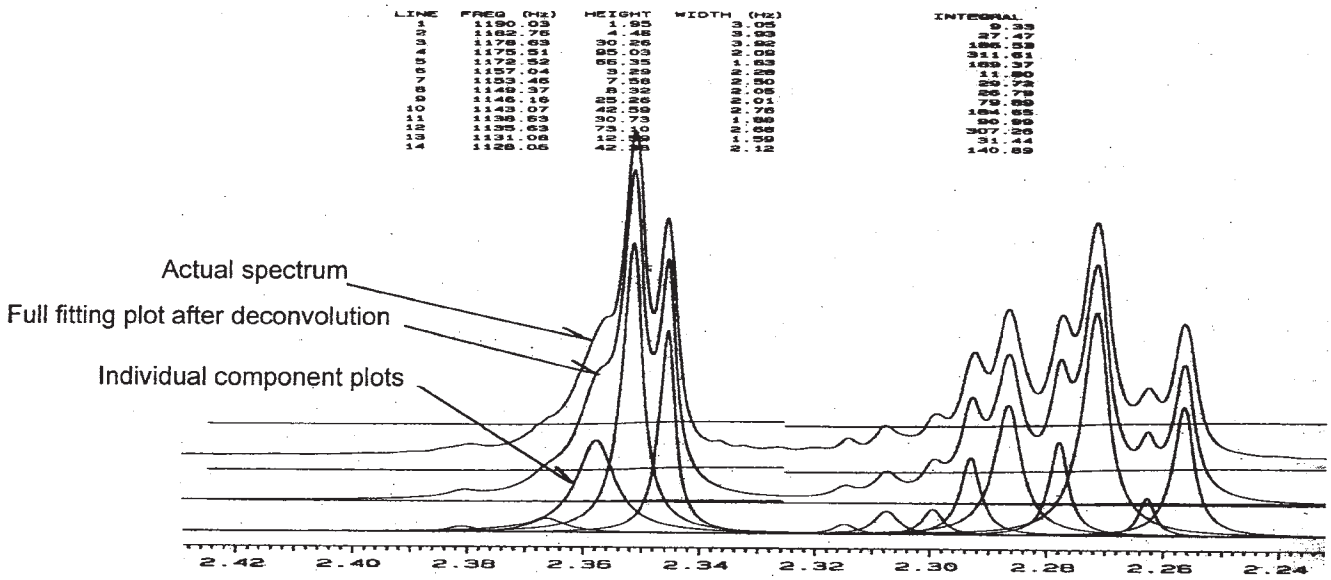

FIG. 2. An example of the deconvolution peak separation for DHA C2,3 and other fatty acids' C2 methylene regions. Inserted data (top) show the individual peak frequency, height, width, and integral. For abbreviation see Figure 1.

under the following conditions: apparatus, Shimadzu GC-17A (Shimadzu, Tokyo, Japan); column, Supelco PAG, i.d. 0.32 mm × 30 m, depth of liquid film (df) 0.25 μm (Supelco, Inc., Bellefonte, PA); detector, flame-ionization, at 250°C; oven temperature, 80°C, hold 1 min, 8°C/min to 190°C, 1.1°C/min to 215°C; injector temperature, 250°C; carrier gas, He, 3 mL/min; gas pressure, air–0.5 kg/cm^2, H_2–0.5 kg/cm^2; injection mode, splitless; internal standard, tricosanoic acid (Sigma Chemical Co., 99%). Methylation of fatty acids of refined fish oils was carried out with boron trifluoride (11). Fatty acids of the unrefined fish oils were methylated by the alkaline methanol method (14). By calculating from the molecular weight and the weight-based proportion (wt%) of each fatty acid obtained by GC, the proportion of each fatty acid was converted into a molar fraction (mol%).

Other analyses. Peroxide values (PV) and acid values (AV) of the sample oils were measured according to the AOAC method (24) and a modified AOCS method (25), respectively.

RESULTS AND DISCUSSION

Linearity of the DHA calibration curves. In order to secure a good separation between the internal standard signal(s) and those of lipids, the spectral region from δ3.3 to δ3.7 was selected as suitable for the internal standard. Methanol, EGDM, and 1,4-dioxane were chosen as internal standard candidates based on the following criteria: stability, solubility in both chloroform and fish oils, commercial availability, simplicity of the signal shape, and short relaxation time. As shown in Table 1, T_1 values of methyl/methylene protons of the internal standards added to a refined tuna oil were all less than 6.0 s and thus longer than those of the lipid signals (DHA C2,3 methylene, 1.0 s; other C2 methylene, 0.9 s; n-3 methyl, 4.1 s; other methyl, 2.4 s). According to Becker *et al.* (26), the pulse repetition time should be five times longer than the longest T_1 to obtain quantitative data when a flip angle of 90º is used. However, to shorten the analysis time and still achieve highly sensitive measurements, we started experi-

TABLE 1
Chemical Properties of Internal Standard Candidates

Substance	Molecular weight	Structural formula	Proton signal				Relaxation time[a] (s)
			Group	Proton quantity	Position (δ), ppm	Shape	
Methanol	32.04	MeOH	Methyl	3	3.30	Singlet	5.60
1,4-Dioxane	88.11		Methylene	8	3.65	Singlet	4.19
Ethylene glycol dimethyl ether (EGDM)	90.12		Methyl	6	3.35	Singlet	4.01
			Methylene	4	3.50	Singlet	3.69

[a]Measured in a refined tuna oil.

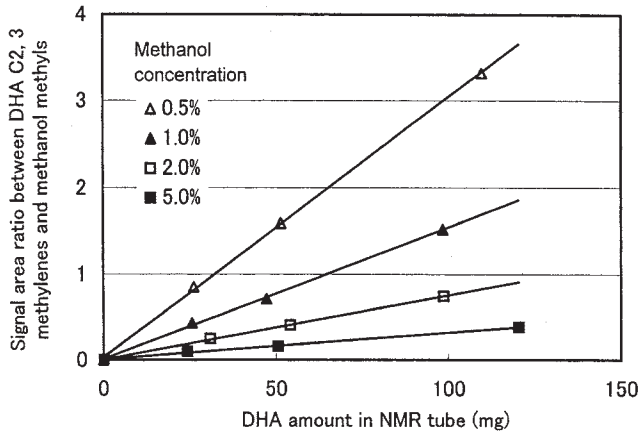

FIG. 3. DHA standard curves using methanol signal at 3.30 ppm as an internal standard. NMR, nuclear magnetic resonance; for other abbreviation see Figure 1.

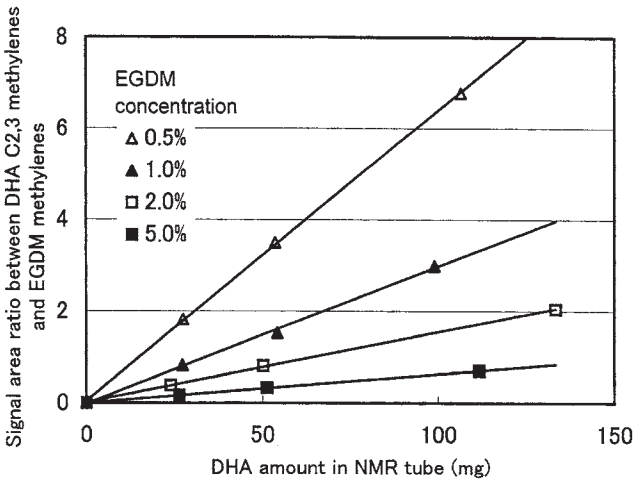

FIG. 5. DHA standard curves using methylene signal of EGDM at 3.50 ppm as an internal standard. For abbreviations see Figures 1 and 3.

ments by tentatively setting the repetition time at 8.5 s and the flip angle at 45°. Therefore, in the interlaboratory experiments, we further explored the optimal repetition time by using unrefined fish oils.

As shown in Figures 3 to 6, calibration curves obtained on the three internal standards showed good linearity at four different concentrations tested. EGDM provides two calibration curves: one from the methylene signal (Fig. 5) and the other from the methyl signal (Fig. 6). The ordinate axis of the calibration curves indicates the signal area ratio of the resonance of DHA C2,3 methylenes to that of either methyl or methylene of the internal standards. Signals of the hydroxyl proton in methanol appeared in the spectra as a broad singlet, and its chemical shift was concentration-dependent, that is, around 2.8 ppm at 5%, 2.1 ppm at 2%, and 1.65 ppm at 1% (data not shown). Unlike those of DHA, C2 and C3 methylene protons of EPA resonated at around 2.28 and 1.7 ppm, respectively, under a similar analytical condition. Therefore, these signals from methanol and/or EPA should not interfere with quantification of DHA and other fatty acids.

For all the calibration curves, good correlation was observed between the signal ratio and the amount of DHA in the NMR tube ($r^2 > 0.995$). As the actual concentrations of DHA in fish oils used in this study were in a range of 25–115 mg per NMR tube (100–450 mg/g in the sample oils), the good linearity in this range observed for all three internal standard candidates implied that any of them should be satisfactory for quantification of DHA (Figs. 3–6). Therefore, we tentatively chose the 1.0% solution of respective standards during the following experiments.

Comparative study between ^{1}H NMR and GC measurements of refined fish oil. Eleven samples of refined fish oil having DHA concentrations in the range of 20 to 48 mol% and PV less than 10 meq/kg (data not shown) were used for

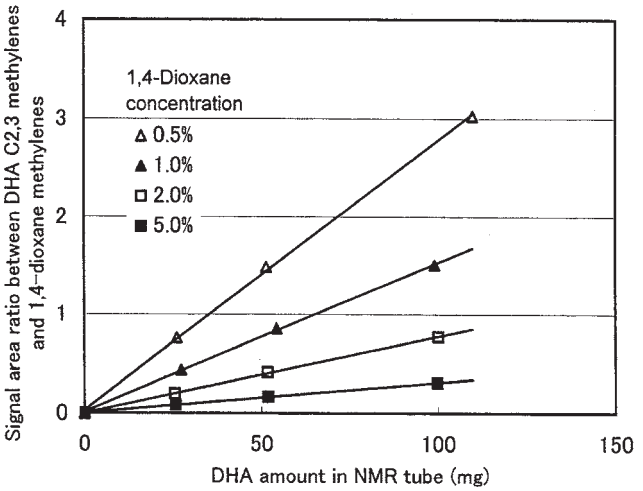

FIG. 4. DHA standard curves using 1,4-dioxane signal at 3.65 ppm as an internal standard. For abbreviations see Figures 1 and 3.

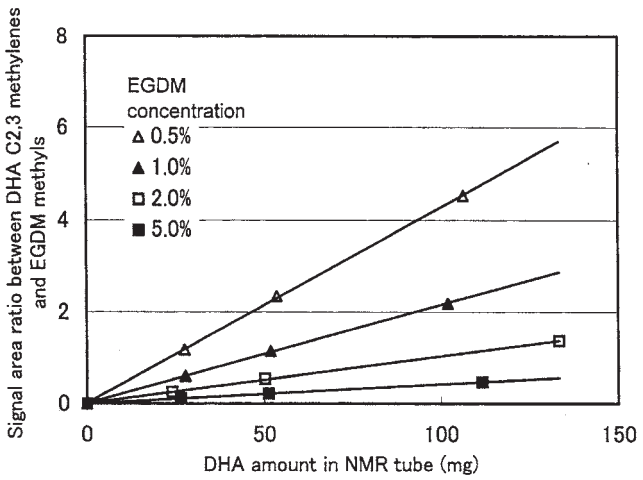

FIG. 6. DHA standard curves using methyl signal of EGDM at 3.35 ppm as an internal standard. For abbreviations see Figure 1.

TABLE 2

Fatty Acid Composition of Refined Fish Oils in Weight Percentage and Mole Fraction Percentage Determined by Gas Chromatography[a] (GC)

	Sample oil code[b]																					
	A-22		A-30		A-50		B-25		B-45		C-25		C-40		D-23		D-27		E-A1		E-A2	
	Wt%[c]	Mol%[d]	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%
14:0[e]	3.5	4.2	2.9	3.6	1.9	2.4	2.6	3.2	1.4	1.8	2.5	3.1	2.1	2.7	3.0	3.7	2.5	3.1	3.3	4.0	3.2	3.9
15:1	1.1	1.3	0.7	0.8	0.6	0.7	0.7	0.8	0.4	0.5	0.7	0.8	0.5	0.6	0.7	0.8	0.6	0.7	1.3	1.5	1.2	1.4
16:0	23.3	25.6	13.9	15.6	11.2	13.0	17.8	19.7	9.3	10.8	15.1	16.8	9.2	10.6	17.9	19.8	12.1	13.6	19.9	22.0	20.4	22.5
16:1	4.3	4.8	5.2	5.9	2.8	3.3	4.8	5.4	2.5	2.9	4.9	5.5	3.5	4.0	4.8	5.4	5.5	6.2	4.7	5.2	5.0	5.6
16:2	1.1	1.2	1.2	1.4	0.8	0.9	1.1	1.2	0.6	0.7	1.0	1.1	0.8	0.9	0.7	0.8	0.8	0.9	1.3	1.5	1.3	1.5
17:0	1.5	1.6	0.8	0.9	0.8	0.9	0.9	1.0	0.5	0.6	0.8	0.9	0.7	0.8	0.8	0.8	0.5	0.5	1.4	1.5	1.3	1.4
18:0	6.6	6.6	3.1	3.2	3.4	3.6	4.4	4.5	2.5	2.7	3.8	3.9	3.6	3.8	4.4	4.5	2.4	2.5	5.2	5.3	5.1	5.2
18:1	14.2	14.3	15.1	15.5	8.3	8.8	21.0	21.4	10.7	11.5	21.4	21.9	10.1	10.7	20.1	20.5	19.1	19.7	13.1	13.3	14.0	14.2
18:2n-6[f]	1.1	1.1	2.2	2.3	0.7	0.7	1.1	1.1	0.6	0.6	1.3	1.3	0.9	1.0	1.2	1.2	1.4	1.5	1.1	1.1	1.1	1.1
18:3n-3[g]	0.8	0.8	0.8	0.8	0.5	0.5	0.6	0.6	0.4	0.4	0.6	0.6	0.6	0.6	0.7	0.7	0.7	0.7	0.9	0.9	0.7	0.7
18:4n-3	0.8	0.8	1.2	1.3	0.8	0.9	0.4	0.4	0.5	0.5	0.5	0.5	1.3	1.4	0.8	0.8	1.1	1.2	0.9	0.9	0.8	0.8
20:1	1.6	1.5	1.5	1.4	0.8	0.8	2.6	2.4	1.7	1.7	3.1	2.9	2.3	2.2	3.2	3.0	2.2	2.1	0.7	0.7	0.5	0.5
20:3n-3	0.3	0.3	0.2	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2
20:4n-6	1.5	1.4	2.2	2.1	2.1	2.1	2.1	2.0	2.7	2.7	2.2	2.1	2.6	2.6	1.9	1.8	2.4	2.3	1.4	1.3	1.7	1.6
20:4n-3	0.4	0.4	0.5	0.5	0.3	0.3	0.4	0.4	0.3	0.3	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.3	0.3	0.3	0.3
20:5n-3	5.1	4.9	8.5	8.2	4.2	4.2	5.3	5.1	3.9	3.9	6.7	6.5	7.5	7.5	6.4	6.1	9.1	8.8	4.6	4.4	5.2	5.0
21:5n-3	0.2	0.2	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.2
22:1	1.0	0.9	0.8	0.7	0.6	0.5	1.0	0.9	0.9	0.8	1.3	1.1	1.6	1.4	2.0	1.7	0.9	0.8	0.3	0.3	0.0	0.0
22:4n-3	1.8	1.6	1.8	1.6	4.0	3.7	1.5	1.3	3.1	2.9	1.3	1.2	3.0	2.7	1.3	1.1	1.5	1.3	2.3	2.0	2.4	2.1
22:5n-3	1.1	1.0	1.7	1.5	1.4	1.3	1.6	1.4	2.1	2.0	1.8	1.6	2.0	1.8	1.5	1.3	2.0	1.8	0.8	0.7	0.8	0.7
22:6n-3	23.7	21.0	30.9	27.9	49.6	46.2	23.5	21.0	51.4	48.2	25.8	23.1	40.8	37.8	22.5	20.1	29.8	26.9	29.8	26.6	28.7	25.5
Other[h]	2.3	2.1	1.7	1.7	2.0	1.9	2.7	2.5	1.9	1.8	2.0	1.9	2.5	2.5	2.6	2.4	1.7	1.7	2.2	2.0	2.0	1.9
Unknown[i]	2.4	2.3	2.8	2.8	2.4	2.5	3.2	3.1	1.9	2.0	2.2	2.2	3.0	3.1	2.5	2.5	2.5	2.5	3.7	3.6	3.6	3.5
n-3 f.a.[j]	34.2	30.8	45.9	42.3	61.3	57.6	33.8	30.7	62.2	58.8	37.7	34.5	56.3	52.9	34.2	31.2	45.4	41.9	40.1	36.3	39.3	35.5

[a]GC data were from single analysis.

[b]Sample oils coded E-A1 and E-A2 were refined from bonito; others were from tuna.

[c]Wt% denotes weight percentage.

[d]Mol% indicates molar fraction percentage.

[e]14:0 shows tetradecanoic acid; and so forth.

[f]n-6 denotes fatty acids assignable to n-6.

[g]n-3 denotes fatty acids assignable to n-3.

[h]Other minor fatty acids of which concentrations (wt%) were less than 1% except for n-3 fatty acids.

[i]Unassignable fatty acids.

[j]Total amount of n-3 fatty acids.

TABLE 3
Comparison Between GC and ^{1}H NMR Data on Refined Fish Oils[a]

Sample oil code[b]	Internal standard	DHA content (mg/g)					DHA proportion (mol%)			n-3 fatty acids (mol%)		
		GC	NMR-1[c]	RE[d]	NMR-2[e]	RE	GC[f]	NMR	RE	GC[f]	NMR	RE
A-22	Methyl	206	206	–0.1	212	2.9	21.0	20.2	3.7	30.8	27.1	–12.8
A-30	in EGDM	270	277	2.7	287	6.1	27.9	27.5	1.4	42.3	37.1	–13.1
A-50		424	419	–1.2	434	2.3	46.2	44.6	–3.5	57.6	47.1	–20.1
B-25		208	191	–8.5	204	–1.9	21.0	20.5	–2.3	30.7	25.9	–17.0
B-45		442	439	–0.8	455	2.9	48.2	46.6	–3.3	58.8	49.9	–16.4
C-25		229	224	–2.3	231	1.0	23.1	22.1	–4.5	34.5	33.3	–3.5
C-40		365	376	2.8	389	6.4	37.8	37.9	0.3	52.9	43.9	–18.6
D-23		208	198	–5.2	204	–2.0	20.1	19.8	–1.3	31.2	27.6	–12.2
D-27		276	267	–3.2	276	0.2	26.9	26.7	–0.7	41.9	36.7	–13.2
E-A1		277	267	–3.8	276	–0.3	26.6	26.8	0.7	36.3	29.9	–19.3
E-A2		270	278	3.0	288	6.4	25.5	26.8	4.9	35.5	29.5	–18.5
A-22	Methylene	206	210	2.0	222	7.7	—	—	—	—	—	—
A-30	in EGDM	270	271	0.4	287	6.0	—	—	—	—	—	—
A-50		424	419	–1.1	443	4.4	—	—	—	—	—	—
B-25		208	198	–4.8	210	1.0	—	—	—	—	—	—
B-45		442	435	–1.5	460	3.9	—	—	—	—	—	—
C-25		229	224	–2.1	234	2.3	—	—	—	—	—	—
C-40		365	372	2.0	389	6.2	—	—	—	—	—	—
D-23		208	198	–4.9	207	–0.4	—	—	—	—	—	—
D-27		276	277	0.3	289	4.6	—	—	—	—	—	—
E-A1		277	275	–0.8	287	3.5	—	—	—	—	—	—
E-A2		270	274	1.6	287	5.9	—	—	—	—	—	—
A-22	Methanol	206	227	9.7	222	7.5	21.0	21.0	0.2	30.8	27.3	–12.0
A-30		270	302	11.3	296	9.1	27.9	27.9	–0.1	42.3	37.6	–11.8
A-50		424	454	6.9	446	5.0	46.2	45.4	–1.7	57.6	45.5	–23.5
B-25		208	225	7.7	219	5.3	21.0	20.9	–0.4	30.7	28.2	–8.5
B-45		442	469	6.0	460	4.1	48.2	46.3	–4.1	58.8	48.9	–18.4
C-25		229	211	–8.2	206	–10.5	23.1	20.7	–11.1	34.5	31.2	–10.0
C-40		365	389	6.4	382	4.4	37.8	37.1	–1.8	52.9	43.1	–20.4
D-23		208	229	9.4	223	7.1	20.1	21.0	4.4	31.2	29.2	–6.6
D-27		276	301	8.5	294	6.4	26.9	27.6	2.7	41.9	37.6	–10.8
E-A1		277	284	2.5	278	0.4	26.6	26.6	–0.1	36.3	31.3	–14.8
E-A2		270	289	6.7	283	4.6	25.5	26.6	4.3	35.5	30.7	–14.5
A-22	1,4–Dioxane	206	224	8.2	201	2.6	21.0	21.3	1.4	30.8	26.6	–14.6
A-30		270	287	6.1	258	–4.4	27.9	27.7	–0.6	42.3	37.8	–11.2
A-50		424	444	4.5	401	–5.5	46.2	45.2	–2.1	57.6	46.1	–22.2
B-25		208	232	10.9	208	0.2	21.0	21.3	1.3	30.7	27	–12.8
B-45		442	454	2.7	411	–7.3	48.2	46.5	–3.5	58.8	49.5	–17.2
C-25		229	255	10.6	229	0.0	23.1	22.9	–0.8	34.5	30.4	–12.6
C-40		365	356	–2.5	322	–12.6	37.8	36.9	–2.4	52.9	43.7	–19.0
D-23		208	220	5.6	197	–5.3	20.1	20.7	2.8	31.2	28.4	–9.4
D-27		276	279	1.1	251	–9.4	26.9	26.8	–0.3	41.9	37.5	–11.1
E-A1		277	267	–3.8	240	–14.2	26.6	26.8	0.6	36.3	30.7	–16.7
E-A2		270	277	2.7	250	–7.7	25.5	26.2	2.6	35.5	30.1	–16.5

[a]All ^{2}H nuclear magnetic resonance (NMR) and GC data are from single analyses.
[b]Samples coded E-A1 and E-A2 are refined from bonito; others are from tuna.
[c]^{1}H NMR data determined by the internal standard method.
[d]The relative error (%) calculated by (^{1}H NMR data – GC data)/[(^{1}H NMR data + GC data)/2] × 100.
[e]^{1}H NMR data determined by the DHA calibration curve.
[f]Molar fractions were calculated from the fatty acid composition in wt% as indicated in Table 2. DHA, docosahexaenoic acid; for other abbreviations see Tables 1 and 2.

comparing ^{1}H NMR and GC-derived data. As shown in Table 2, n-3 fatty acids in these samples consisted mainly of docosapentaenoic acid (22:5), docosatetraenoic acid (22:4), octadecatetraenoic acid (18:4), and octadecatrienoic acid (18:3), as well as DHA and EPA. Since the ^{1}H NMR determines only the molar-based proportion (mol%), the wt% data of GC were converted to the molar proportion for comparison (Table 3).

Between the two peak separation procedures for area measurement in ^{1}H NMR spectra, the manual method was preferred to the other for measuring peak areas. The deconvolution technique occasionally gave incorrect-fitting results for the DHA C2,3 methylene region as seen in those of A-30, B-25, and E-A1, probably due to the complexity of the ^{1}H NMR spectra of fish oils (data not shown). Therefore, the manual

TABLE 4
General Properties of Unrefined Fish Oils Used in Interlaboratory Study

Unrefined fish oils		Peroxide value (meq/kg)		Acid value (mg/g)	
No.	Source	Norway	Japan	Norway	Japan
1	Bonito	14.2	10.6	0.96	1.76
2	Tuna	9.2	7.6	0.18	0.20
3	Tuna	10.6	9.4	0.21	0.20
4	Tuna	10.9	10.1	0.82	0.31
5	Salmon	0.0	0.6	1.08	2.61

peak separation was used in the following experiments. Table 3 shows the content of DHA, its molar proportion, and the molar proportion of the total n-3 fatty acids in refined fish oils, as determined by GC and ^{1}H NMR, respectively. The ^{1}H NMR data on the DHA content in Table 3 were obtained by the use of the three internal standards. In addition, DHA calibration curves (1% concentration), prepared as described in the previous section, were used. Since there is no other method than GC to evaluate the accuracy of the ^{1}H NMR data, we compared the GC data with the ^{1}H NMR data. When 1,4-dioxane was used as the standard, the DHA content as estimated by the internal standard method varied from that obtained by the DHA calibration curve. The DHA contents obtained by ^{1}H NMR with the internal standard method were slightly greater than those by GC, while the opposite was true when the DHA calibration curve was used. The ^{1}H NMR method gave slightly higher values than the GC method when methanol was used as a standard, regardless of its use as the internal standard or by the DHA calibration curve. In the case of EGDM, the relative errors for the DHA contents obtained by calibrating with either methyl signals or methylene signals were comparable with each other and were not affected by the use of either the internal standard or the DHA calibration curve. Among the three internal standards tested, EGDM gave the smallest relative errors (less than 7%). From these data we inferred that the DHA contents obtained by ^{1}H NMR using EGDM as the standard were more precise than those obtained by GC.

The data in Table 3 also show that the variations for the molar proportions of DHA and those for n-3 fatty acids determined by the ^{1}H NMR method are small regardless of the standard substance used. Furthermore, good agreement ($R^2 = 0.994$, relative error <5%) was observed between the ^{1}H NMR and the GC data for molar proportions of DHA and DHA quantification with EGDM. A good correlation ($R^2 = 0.976$) was also

TABLE 5
Fatty Acid Composition of Unrefined Fish Oils Used for Interlaboratory Study in wt% and mol% Determined by GC[a]

	Sample oil no. and source									
	No. 1 Bonito		No. 2 Tuna		No. 3 Tuna		No. 4 Tuna		No. 5 Tuna	
	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%	Wt%	Mol%
14:0[b]	3.6	4.4	4.2	5.1	2.4	3.0	3.1	3.8	5.8	7.1
15:0	1.4	1.6	0.4	0.5	0.6	0.7	0.7	0.8	0.4	0.5
16:0	20.2	22.3	14.9	16.5	16.2	17.9	16.7	18.5	13.4	14.6
16:1	5.5	6.1	5.5	6.2	5.7	6.3	5.0	5.6	7.8	8.6
17:0	1.5	1.5	0.4	0.4	0.7	0.7	0.8	0.8	0.4	0.4
18:0	5.3	5.3	4.0	4.0	3.7	3.8	4.6	4.6	2.8	2.8
18:1	13.6	13.8	22.8	23.2	25.6	26.1	20.3	20.6	18.0	18.1
18:2n-6	1.2	1.2	1.2	1.2	1.0	1.0	1.3	1.3	3.1	3.1
18:3n-3	0.6	0.6	0.7	0.7	0.4	0.4	0.7	0.7	1.1	1.1
18:4n-3	0.9	0.9	1.6	1.7	0.5	0.5	1.1	1.1	1.8	1.9
20:1	0.8	0.7	8.7	8.1	3.3	3.1	3.3	3.1	5.9	5.5
20:3n-3	0.1	0.1	0.1	0.1	0.3	0.3	0.2	0.2	0.1	0.1
20:4n-6	1.5	1.5	0.6	0.6	1.8	1.7	1.5	1.5	0.8	0.7
20:4n-3	0.4	0.4	0.7	0.6	0.6	0.6	0.7	0.7	1.7	1.6
20:5n-3	4.7	4.5	6.2	5.9	6.0	5.7	7.5	7.2	9.1	8.6
21:5n-3	0.2	0.2	0.3	0.3	0.2	0.2	0.3	0.2	0.5	0.5
22:1	0.3	0.3	9.1	7.9	1.0	0.9	1.8	1.6	5.3	4.6
22:4n-3	2.1	1.8	0.2	0.2	0.8	0.7	0.8	0.7	0.3	0.2
22:5n-3	0.8	0.7	1.3	1.2	1.6	1.4	1.9	1.7	3.9	3.5
22:6n-3	27.4	24.3	12.3	11.0	21.9	19.6	22.0	19.6	10.9	9.6
Other	3.2	3.1	2.5	2.4	2.8	2.7	3.0	2.9	4.6	4.6
Unknown	5.0	4.9	2.4	2.3	3.0	2.9	2.8	2.7	2.5	2.4
n-3 f.a.	37.0	33.4	23.4	21.7	32.2	29.3	35.2	32.1	29.4	27.0

[a]Data are expressed as the average of those from three parallel experiments.
[b]14:0 denotes tetradecanoic acid; and so forth. For other abbreviations and conventions see Table 2.

TABLE 6
Comparison of DHA Content from the Interlaboratory ^{1}H NMR Analyses[a] Between Japan and Norway Along with GC Data[b]

Sample oil	Internal standard	Data	DHA content (mg/g) ^{1}H NMR 10 s[c] Norway	10 s[c] Japan	30 s[c] Norway	30 s[c] Japan	60 s[c] Japan	GC
		Average	293.83	276.97	284.17	276.07	273.40	267.58
	EGDM	CV[d]	0.61	0.73	0.64	1.33	2.70	1.67
No. 1		Relative error[e]		5.90[f]		2.89[f]	—	4.58[g]
Bonito		Average	291.93	289.67	—	—	—	—
	1,4-Dioxane	CV	0.12	0.85	—	—	—	—
		Relative error		0.78	—	—	—	—
		Average	127.04	120.93	123.79	122.57	122.43	123.40
	EGDM	CV	3.64	2.39	4.01	1.38	1.07	0.73
No. 2		Relative error		4.92		0.99	—	0.18
Tuna		Average	128.30	127.07	—	—	—	—
	1,4-Dioxane	CV	0.47	0.88	—	—	—	—
		Relative error		0.97	—	—	—	—
		Average	221.63	208.17	214.51	208.27	212.40	215.70
	EGDM	CV	1.94	3.47	2.72	0.91	1.41	0.20
No. 3		Relative error		6.26		2.95	—	2.02
Tuna		Average	228.13	218.67	—	—	—-	—
	1,4-Dioxane	CV	0.26	1.51	—	—	—	—
		Relative error		4.24	—	—	—	—
		Average	229.14	213.27	217.97	206.73	208.77	215.42
	EGDM	CV	0.60	2.31	3.84	0.07	2.18	0.72
No. 4		Relative error		7.17		5.29	—	1.44
Tuna		Average	231.07	219.93	—	—	—	—
	1,4-Dioxane	CV	0.35	1.95	—	—	—	—
		Relative error		4.94	—	—	—	—
		Average	112.91	111.60	111.70	111.07	110.43	105.99
	EGDM	CV	3.07	2.41	3.27	0.95	2.41	1.47
No. 5		Relative error		1.16		0.57	—	4.96
Salmon		Average	120.63	111.53	—	—	—	—
	1,4-Dioxane	CV	0.37	1.93	—	—	—	—
		Relative error		7.84	—	—	—	—

[a]The ^{1}H NMR experiments in Japan were carried out on triplicated samples. In Norway, the sample preparation was not triplicated but the ^{1}H NMR measurement was triplicated.
[b]The GC data are from three parallel analyses.
[c]Pulse repetition time: the 60 s repetition time was tested only in Japan.
[d]Intralaboratory coefficient of variation (CV) (%).
[e]The relative error is expressed by an equation: |(data A – data B)|/(average of the two data) × 100.
[f]The relative error between Norway and Japan.
[g]The relative error between the ^{1}H NMR data (EGDM, 30 s of repetition time) and the GC data. For abbreviations see Tables 1–3.

observed between the ^{1}H NMR data and GC data for n-3 fatty acids. However, the GC method always gave higher values than those of the ^{1}H NMR. The observed relative errors (9–22%) were in accordance with those reported by Sacchi *et al.* (19). Such a large deviation between the ^{1}H NMR and GC data might have been caused at least partly by the inadequate pulse repetition time of 8.5 s (acquiring time 7 s + pulse delay 1.5 s), which might be too short to quantify n-3 fatty acids. We therefore examined the effect of the pulse repetition time on the quantification of n-3 fatty acids and DHA in subsequent experiments.

Interlaboratory ^{1}H NMR experiments on unrefined fish oils. In the interlaboratory study between Norway and Japan, five samples of unrefined fish oils extracted from bonito, tuna, and salmon were used. As shown in Table 4, all the PV and AV of the sample oils determined in each country prior to the ^{1}H NMR experiments were below 15 and 3.0 mg/g, respectively. As described in the preceding section, the optimal proton relaxation time for quantitative precision was further examined. For this purpose, the EGDM methyl signal was used, for the most part, as the internal standard for DHA quantification. As shown in Table 5, the fatty acid composition in the unrefined fish oils coded No.1 (bonito) and No. 4 (tuna), respectively, were comparable with those of the refined fish oils coded E-A2 (bonito) and D-23 (tuna), respectively. Additionally, no significant signals except for those of triglycerides and the standard were observed in the ^{1}H NMR spectra. Therefore, the quantitative conditions examined below were considered to be similarly applicable to the refined oils.

The results from the interlaboratory ^{1}H NMR experiments

TABLE 7
Comparison of the DHA Content from the Interlaboratory ^{1}H NMR Analyses[a] Between Japan and Norway Together with the GC Data[b]

Sample oil	Internal standard	Data	DHA proportion (mol%) ^{1}H NMR 10 s[c] Norway	10 s[c] Japan	30 s[c] Norway	30 s[c] Japan	60 s[c] Japan	GC
		Average	27.16	27.29	27.03	27.45	27.36	24.50
	EGDM	CV[d]	0.67	0.88	0.78	0.46	0.20	2.49
No. 1		Relative error[e]	5.50[f]		1.53[f]		—	10.59[g]
Bonito		Average	26.83	27.08	—	—	—	—
	1,4-Dioxane	CV	0.15	0.13	—	—	—	—
		Relative error	0.92		—	—	—	—
		Average	11.85	11.80	11.93	12.01	12.01	11.00
	EGDM	CV	2.45	2.67	2.98	0.43	0.79	1.71
No. 2		Relative error	0.42		0.70		—	8.43
Tuna		Average	11.80	11.89	—	—	—	—
	1,4-Dioxane	CV	0.48	0.38	—	—	—	—
		Relative error	0.76		—	—	—	—
		Average	20.70	20.45	20.65	20.71	20.96	20.16
	EGDM	CV	0.56	1.95	0.66	2.02	0.46	1.43
No. 3		Relative error	1.21		0.29		—	2.56
Tuna		Average	20.64	20.21	—	—	—-	—
	1,4-Dioxane	CV	0.22	2.19	—	—	—	—
		Relative error	2.11		—	—	—	—
		Average	21.22	21.12	21.18	20.75	20.76	19.69
	EGDM	CV	1.69	1.54	1.59	0.47	1.56	1.35
No. 4		Relative error	0.48		2.09		—	6.27
Tuna		Average	20.92	20.49	—	—	—	—
	1,4-Dioxane	CV	0.35	0.75	—	—	—	—
		Relative error	2.11		—	—	—	—
		Average	10.56	10.66	10.65	10.76	10.71	10.18
	EGDM	CV	1.01	1.46	0.88	0.32	2.56	1.15
No. 5		Relative error	0.93		1.00		—	5.04
Salmon		Average	10.78	10.57	—	—	—	—
	1,4-Dioxane	CV	0.38	0.75	—	—	—	—
		Relative error	1.94		—	—	—	—

[a]For footnotes *a–g* see Table 6.

are shown in Tables 6–8 and can be summarized as follows: (i) The DHA contents calibrated with EGDM were lower than those with 1,4-dioxane; (ii) the DHA contents were lower when the repetition time was set at 30 s than at 10 s; (iii) conversely, molar proportions of n-3 fatty acids were higher with a repetition time of 30 s than with 10 s; (iv) the differences between the two laboratories in molar proportion data for n-3 fatty acids and DHA, respectively, were very small (less than 2.11% of relative error); (v) the precision of interlaboratory measurements on DHA quantification was improved by prolongation of the repetition time. The relative error of 1.16–7.17% at 10 s of repetition time was improved to 0.57–5.29% at 30 s.

As the above results suggest that the repetition time of 10 s was too short to quantify these fatty acids precisely, part of the marked differences (10–20% of the relative error) observed between GC and ^{1}H NMR data on n-3 fatty acids in the refined fish oils seemed to be explained by the inadequate setting of the repetition time. However, the longer T_1 (1.7 s) of the terminal methyl protons in n-3 fatty acids compared to that of other fatty acids cannot alone account for the large difference of 20%, as the difference in T_1 values is less than 2 s.

The insufficient accuracy and precision in GC analysis of fish lipids in the interlaboratory experiments (11) can be explained by the following reasons: (i) Strictly controlled time and temperature are required for methylation and GC measurements; (ii) owing to the wide variation of the fatty acids, many standard fatty acids have to be used; (iii) because of the high content of highly unsaturated fatty acids, oxidative deterioration occurs easily upon heating. Errors with each fatty acid are added up in quantifying a group of fatty acids, e.g., the n-3 fatty acids. These drawbacks of GC might have contributed to the errors of the GC results. As to the ^{1}H NMR method, attention should be paid to the following points to achieve good accuracy and precision: (i) The lipid content of the sample oil should be higher than 95%; (ii) contaminants having methyl proton signals at around 0.8–1 ppm and methylene proton signals at around 2.2–2.4 ppm should be avoided; (iii) the oxidative deterioration of sample oil should be monitored by PV, AV, and ^{1}H NMR, because oxidized oils, regardless of the position of the oxidation, may give signals that interfere with measurements leading to overestimates of n-3 fatty acids and/or DHA.

TABLE 8
Comparison of DHA Content from Interlaboratory ^{1}H NMR Analyses[a] Between Japan and Norway Together with GC Data[b]

			n-3 fatty acids proportion (mol%)					
			^{1}H NMR					
			10 s[c]		30 s[c]		60 s[c]	
Sample oil	Internal standard	Data	Norway	Japan	Norway	Japan	Japan	GC
		Average	31.34	31.55	32.05	32.08	32.10	33.60
	EGDM	CV[d]	0.23	0.49	0.08	0.04	0.60	2.03
No. 1		Relative error[e]	0.67[f]		0.10[f]		—	4.68[g]
Bonito		Average	31.55	31.57	—	—	—	—
	1,4-Dioxane	CV	0.05	0.32	—	—	—	—
		Relative error	0.07		—	—	—	—
		Average	22.03	22.12	22.56	22.24	22.18	21.62
	EGDM	CV	1.21	1.46	0.85	0.40	0.50	1.35
No. 2		Relative error	0.39		1.41		—	3.54
Tuna		Average	22.16	21.85	—	—	—	—
	1,4-Dioxane	CV	0.20	0.03	—	—	—	—
		Relative error	1.39		—	—	—	—
		Average	28.35	28.49	29.08	28.79	29.08	29.93
	EGDM	CV	0.90	0.89	0.05	0.73	0.60	0.89
No. 3		Relative error	0.49		1.02		—	2.56
Tuna		Average	28.60	28.38	—	—	—-	—
	1,4-Dioxane	CV	0.02	0.48	—	—	—	—
		Relative error	0.80		—	—	—	—
		Average	31.04	31.40	32.22	31.79	31.94	32.11
	EGDM	CV	1.38	0.42	0.23	0.34	0.60	1.07
No. 4		Relative error	1.17		1.35		—	0.32
Tuna		Average	31.69	31.45	—	—	—	—
	1,4-Dioxane	CV	0.05	0.30	—	—	—	—
		Relative error	0.77		—	—	—	—
		Average	27.63	27.76	28.03	28.13	28.20	28.60
	EGDM	CV	0.80	0.40	0.32	0.33	0.07	0.74
No. 5		Relative error	0.47		0.35		—	1.82
Salmon		Average	27.59	27.76	—	—	—	—
	1,4-Dioxane	CV	0.04	0.16	—	—	—	—
		Relative error	0.60		—	—	—	—

[a]For footnotes *a–g* see Table 6.

Since the relaxation time is affected by viscosity of the sample solution in ^{1}H NMR measurements, the sample concentration used in this study (250 mg/0.75 mL $CDCl_3$) can be higher than the optimum for quantitative purposes. Nevertheless, we selected this concentration in order to facilitate simultaneous measurements of ^{13}C NMR spectra, which were expected to provide useful information in future studies. It is very important therefore to control precisely the temperature and concentration of samples as well as the ^{1}H NMR parameters. In this study the pulse repetition time of 30 s was judged to be practical and yet to give reproducible results. With this setting, analysis of an ordinary sample solution takes 8 to 16 min. However, the high concentration of the sample solution used in this study allowed us to shorten the analysis time to 2 min (4 scan times) or less. Sample preparation before ^{1}H NMR measurement requires only two steps: sample weighing and preparation of an internal standard solution. The simplicity of the ^{1}H NMR method offers a great advantage over GC methods, since fish lipids deteriorate easily due to their high content of highly unsaturated fatty acids. Additionally, ^{1}H NMR is a nondestructive method and therefore allows us to re-use small samples, if further experiments are needed. Although this was the first study and involved only two laboratories, the high precision of the ^{1}H NMR method was demonstrated. Encouraged by the results presented here, we are planning an expanded collaborative study in the near future for both GC and ^{1}H NMR.

The results here show that the ^{1}H NMR method is a simple, rapid, and precise alternative to the GC method for the quantification of DHA, molar proportion of DHA, and molar proportion of total n-3 fatty acids in fish oils. Optimization of the experimental conditions for reducing analysis time will be an important task in future studies. Use of the ^{1}H NMR technique should be accelerated in view of the rapid improvement in cost performance of ^{1}H NMR. Spectrometers with lower magnetic fields (300–400 MHz) will be included in further studies.

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