methods

Multicomponent analysis of encapsulated marine oil supplements using high-resolution ^{1}H and ^{13}C NMR techniques

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Abstract Multicomponent high-resolution ^{1}H and ^{13}C NMR analysis has been employed for the purpose of detecting and quantifying a wide range of fatty acids (as triacylglycerols or otherwise) in encapsulated marine cod liver oil supplements. The ^{1}H NMR technique provided quantitative data regarding the docosahexaenoic acid content of these products, which serves as a valuable index of fish oil quality, and a combination of both ^{1}H and ^{13}C spectroscopies permitted the analysis of many further components therein, including *sn*-1 monoacylglycerols, *sn*-1,2 and -1,3 diacylglycerol adducts, together with a range of minor components, such as *trans*-fatty acids, free glycerol and cholesterol, and added vitamins A and E. The identities of each of the above agents were confirmed by the application of two-dimensional ^{1}H-^{1}H spectroscopies. The NMR techniques employed also uniquely permitted determinations of the content of non-acylglycerol forms of highly unsaturated (or other) fatty acids in these products (i.e., ethyl esters), and therefore served as a means of distinguishing "natural" sources of cod liver oils from those subjected to chemical modification to and/or supplementation with synthetic derivatives such as ethyl docosahexaenoate or eicosopentaenoate. The analytical significance and putative health effects of the results acquired are discussed.—Siddiqui, N., J. Sim, C. J. L. Silwood, H. Toms, R. A. Iles, and M. Grootveld. **Multicomponent analysis of encapsulated marine oil supplements using high-resolution ^{1}H and ^{13}C NMR techniques. *J. Lipid Res.* 2003.** 44: **2406–2427.**

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There are currently many reports available suggesting that the consumption of dietary n-3 (Omega-3) fatty acids are beneficial to human health, in particular their abilities to reduce cardiovascular mortality (1, 2) and putatively control the progression of a variety of inflammatory diseases (e.g., rheumatoid arthritis) (3). Marine oils rich in the highly unsaturated n-3 fatty acids, eicosopentaenoic acid [EPA, 20:5(n-3)], and docasahexaneoic acid [DHA, 22:6(n-3)] are an integral component of the traditional, predominantly carnivorous diet of Greenland Inuit (Eskimos) who display an unusually low incidence of ischemic heart disease and diabetes (4).

With respect to coronary heart disease, the protective actions of these highly unsaturated fatty acids (HUFAs) were originally believed to be ascribable to their roles in impairing platelet function (5), and two in vivo investigations have shown that the feeding of fish oils to animals markedly alleviated platelet accumulation at sites of arterial injury (6). Moreover, these n-3 fatty acids have been demonstrated to exert many striking effects on cellular function that indicate their anti-atherosclerotic, anti-inflammatory and antithrombotic properties (7–9), specifically the inhibition of leukotriene synthesis, protein kinase C activity, and cellular adhesion to surfaces, the stimulation of nitric oxide synthesis (a phenomenon causing vasodilation), and impairment of the production of inflammatory cytokines (e.g., interleukin-1 and tumor necrosis factor) by monocytes. Notwithstanding, further studies have shown that n-3 fatty acids also have a powerful antiarrhythmic property, which probably arises from the modulation of cellular calcium ion (Ca^{2+}) flux during episodes of arrhythymogenic stress (10, 11). Indeed, the presence of these fatty acids in the plasma membrane of monocytes readily influences Ca^{2+} channel structure and/or function (12).

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It should also be noted that the antithrombotic and vasodilatory actions exerted by n-3 fatty acids may enhance local myocardial oxygen supply, a process that would synchronously diminish the possibility of arrhythmia.

More recent investigations have indicated the potential therapeutic applications of EPA and DHA in various chronic conditions, e.g., AIDS, Alzheimer's disease, and diabetes (13–15).

In view of the above possible health benefits offered by marine oils and diets containing high EPA and DHA contents, the market for these products has substantially and rapidly expanded in North America, Europe, and Japan. Such products include encapsulated fish oil supplements, many of which are enriched with both EPA and DHA and fortified with essential nutrients such as vitamins A, D, and E. Consequently, there has been a corresponding demand for analytical methods with the capacity to routinely monitor the nature and levels of n-3 fatty acids (as glycerides or alternative forms) in these products in order to ensure their quality control and quality maintenance. Methods for monitoring the contents of added vitamins and for detecting and quantifying additional low-level components such as cholesterol are also essential requirements for quality assurance purposes.

Although gas chromatographic (GC) methods have previously been extensively employed for the purpose of determining these HUFAs in both fish and fish oils (16, 17), these methods are labor intensive and time consuming and also involve a complex series of chemical manipulation stages that, together with high GC injector and oven temperatures, may give rise to their artifactual oxidation; in view of their high degree of unsaturation, both EPA and DHA are particularly susceptible to oxidative deterioration, a process generating conjugated hydroperoxydiene and hydroxydiene and, subsequently, aldehydic products, among others (18). More recently, the analysis of these HUFA components in marine oils has been augmented by the employment of high-resolution ^{1}H and ^{13}C NMR spectroscopy (19).

High-resolution NMR spectroscopy offers many advantages over alternative analytical methods, because it allows the rapid, simultaneous, noninvasive, and nondestructive study of a plethora of components present in complex, multicomponent samples such as foods, pharmaceutical formulations and biofluids (20–22). The principles of this spectroscopic technique involve the subjection of samples to an intense magnetic field. In view of their inherent magnetic moment, ^{1}H or other nuclei in organic (or other) molecules align themselves either with (lower energy state) or against the field (higher energy state), the former state being slightly predominant. However, in the presence of energy from the radiofrequency (RF) region of the electromagnetic spectrum, the nuclei may orient themselves in the higher energy state; because the absorption frequencies of such nuclei are critically dependent on their chemical, and therefore magnetic, microenvironment, varying levels of radiofrequency energy must be applied to excite the different classes of sample nuclei to the higher energy state. Data acquired are presented as a spectrum consisting of a plot of signal intensity versus resonance frequency in parts per million (ppm, a dimensionless unit). The majority of ^{1}H nuclei present in organic and biological molecules resonate (i.e., absorb RF energy) within the narrow spectral range of 0–10 ppm. The lower and higher frequency regions of the spectrum (smaller and larger ppm respectively) contain signals for ^{1}H nuclei that require lesser and higher amounts of RF energy, respectively, for resonance. The reference signal located at 0.00 ppm arises from an organic solvent- or water-soluble tetra- or trimethylsilane derivative, respectively (either internal or external). Samples examined are placed in a 5-mm outer diameter NMR tube which is then inserted into the magnetic field and irradiated with radiofrequency energy encompassing the entire 10 ppm spectral range. An appropriate deuterated [^{2}H] species [a deuterochloroform ($C^{2}HCl_3$) solvent system in the case of culinary oil sample analysis] is added to provide a field frequency lock, i.e., prevent any modifications or drift in the magnetic field during spectral acquisition. The appearance (multiplicity) of an ^{1}H signal is influenced by adjacent ^{1}H nuclei in a well-characterized manner, and the intensity of each resonance detectable is directly proportional to the product of the number of magnetically equivalent nuclei in the structural/functional group giving rise to it and the concentration of the molecule containing that group in the sample examined.

High-resolution NMR analysis has been previously successfully employed for determinations of the DHA and total n-3 fatty acid contents of fish oils and further fish products (23–28). However, with the exception of a ^{13}C NMR study focused on the positional distribution of DHA and EPA between the 1(3)- and 2-glycerol backbone positions of triacylglycerols (29), this technique has not been applied to the multicomponent analysis of encapsulated marine oil supplements fortified with essential vitamin nutrients. In the present study, we have employed high-field (9.4 and 14.1 T), high-resolution ^{1}H and ^{13}C NMR spectroscopy to detect and/or determine the concentrations of a wide range of fatty acid and alternative components present in such products (specifically those based on cod liver oil), the latter including cholesterol, added dl-α-tocopherol acetate (vitamin E derivative) and all-*trans*-retinol palmitate (vitamin A). Included are methods for determining the DHA content ($mg \cdot g^{-1}$ and $mol \cdot kg^{-1}$), its molar proportion expressed relative to all fatty acids therein (mol%), together with the molar proportion of the total n-3 fatty acids (as glycerides or alternative esters) in the samples investigated. These NMR techniques have also been employed, either individually or in tandem, to provide valuable molecular information regarding the substitutional status and positional distribution of individual fatty acids on the glycerol backbone (both involving the identification of mono- and diacylglycerol adducts), and also to monitor any free and *trans*-fatty acids detectable in the products investigated. Furthermore, many of the ^{1}H NMR assignments were confirmed by the application of two-dimensional (2D) ^{1}H-^{1}H NMR techniques. We also demonstrate, for the first time, a novel ^{1}H NMR method

for determining the relative proportions of naturally derived and synthetic (chemically modified) forms of these HUFAs in such marine oil supplements, the latter presumably generated via chemical manipulation in one of the products examined. The analytical significance and health aspects of the results obtained are discussed in detail.

MATERIALS AND METHODS

Materials

Encapsulated marine (cod liver) oil supplements were purchased from local retail outlets. The first of these (product I) purportedly contained cod liver oil enriched with fish oil n-3 fatty acids (1,050 mg), the n-3 fatty acid content being 400 mg, of which EPA and DHA accounted for 360 mg, dl-α-tocopherol acetate (10 mg), vitamin A as all-*trans*-retinol palmitate (800 μg), and vitamin D as a D_3 preparation (5 μg); the content of the second product (product II) was described as cod liver oil (1,000 mg) containing EPA (120 mg), DHA (110 mg), a 'natural' source of vitamin E antioxidant compound (unspecified), vitamin A (800 μg), and vitamin D (5 μg).

α-Tocopherol, α-tocopherol acetate, and 1,3,5-trichlorobenzene (TCB) were obtained from Sigma-Aldrich Chemical Co. (Poole, Dorset, UK), and deuterated chloroform (C^2HCl_3) was purchased from Goss Scientific Ltd. (Great Baddow, Essex, UK).

Proton ^{1}H NMR measurements and sample preparation

Proton ^{1}H NMR measurements on the the above cod liver oil samples (encapsulated) were conducted on Bruker AMX-400 and AMX-600 spectrometers (Queen Mary University of London facilities, London, UK) operating at frequencies of 400.13 and 600.13 MHz, respectively, and a probe temperature of 298 K. Typically, 200–250 mg quantities of the marine oil preparations were removed from their capsules, accurately weighed, directly dissolved in 0.50 ml of C^2HCl_3 containing 5.00×10^{-3} mol·dm^{-3} TCB, thoroughly rotamixed, and then transferred to 5 mm-diameter NMR tubes. The TCB served as a chemical shift reference and an internal quantitative NMR standard (singlet resonance located at 7.227 ppm in C^2HCl_3) solution. This TCB solution was standardized spectrophotometrically against independently prepared solutions in the same medium ($\varepsilon = 1.71 \times 10^2$ M^{-1}·cm^{-1} at a wavelength of 273 nm).

Deuterated methanol (d_4-MeOH) extracts of these marine oil products were obtained by adding 0.75 ml of this NMR solvent (containing 1.153×10^{-2} mol·dm^{-3} ethanol added as an internal chemical shift reference and quantitative NMR standard) to accurately weighed samples of the oils (~250 mg), thoroughly rotamixing the mixture, centrifugation (30 min, 500 *g*), and removal of the upper, d_4-MeOH phase for ^{1}H NMR analysis.

Typical pulsing conditions for ^{1}H NMR experiments were: 128 or 256 free induction decays (FIDs) using 32,768 or 65,536 data points, 72° pulses, a relaxation delay of 2.00 s, and an acquisition time of 1.28 s. The spectral width was 4,831 or 7,246 Hz. Exponential line-broadening functions of 0.20 Hz were routinely employed for purposes of processing. 2D shift-correlated (COSY) spectra of marine oil formulations were acquired on the above 400 or 600 MHz spectrometers using the standard sequence of Au, Bartholdi, and Ernst (30), with 2,048 data points in the t_2 dimension, 512 increments of t_1, a relaxation delay of 2.00 s, and 48 transients. ^{1}H-^{1}H total correlation (TOCSY) spectra were recorded using the RD-(90°-t_1-spin lock)-ACQ pulse sequence (31), 90° on the AMX-600 spectrometer being equivalent to 8.4 μs for these experiments. The spin lock employed the MLEV-17 sequence (32), with a typical mixing time of 70 ms. Acquisition parameters were: 128 t_1 increments, each of magnitude 2,048 data points; spectral width 12,019 Hz in each dimension; 64 transients in each case; four dummy scans; relaxation delay 3.0 s; acquisition time 0.17 s. Sine-bell-squared window functions shifted by $\pi/2$ (i.e., a cosine^2 function) were employed in each dimension, with zero-filling twice in f_1, prior to transformation of the 2,048 × 1,024 data point-sized matrix. The standard phase-sensitive time-proportional phase incrementation method (33) for optimizing detection along the second dimension was utilized.

Chemical shifts were referenced to TCB (7.227 ppm) and/or residual chloroform (7.262 ppm). Resonances present in the ^{1}H NMR spectra of marine oil products were assigned by a consideration of chemical shift values, coupling patterns, and coupling constants, with special reference to previously conducted ^{1}H NMR studies of n-3 fatty acids present in fish oils (24, 25, 28). The relative intensities of signals were determined by electronic integration, and the concentrations of selected components were computed by comparing their resonance areas with that of the added TCB. The DHA content in mg·g^{-1} (glycerol-bound or otherwise) was determined by measurement of the combined intensities of its C2,3 (F_α,F_β) -CH_2- group multiplet signals at ~2.38 ppm and comparison of these with that of the TCB internal standard [Equation 1, where A_{DHA} and A_{TCB} represent the signal areas of DHA's F_α,F_β methylene group protons and TCB, respectively, H_{DHA} and H_{TCB} the number of protons giving rise to the combined DHA C2,C3 methylene resonances (4) and that of TCB (3), respectively, M_{DHA} and M_{TCB} the molecular masses of DHA (328.50) and TCB (181.45), respectively, and x and y the weights of internal standard (in mg) and oil sample (in g), respectively, present in the NMR tube]. Comparisons of the intensities of these isolated DHA resonances (4 ^{1}H nuclei) with those of the C2 (F_α) methylene group signals arising from all other fatty acid species in the samples examined (~2.28 ppm, 2 ^{1}H nuclei) (23) yielded the DHA content as a percentage of all fatty acid components present (Equation 2, where A_{FA} represents the signal area of the F_α methylene group protons of all fatty acids other than DHA). The total n-3 fatty acid molar percentages of the samples examined were concomitantly determined via direct comparisons of the intensities of their terminal-CH_3 (F_ω) group resonance(s) in the 0.90–0.98 ppm region (triplet) with those of the combined n-6, n-9, and saturated fatty acid triplet signal(s) located in the 0.81–0.89 ppm region (Equation 3, where $A_{n\text{-}3}$ denotes the signal area of the F_ω-CH_3 group protons of n-3 fatty acids, and $A_{non\text{-}n\text{-}3}$ that of the F_ω-CH_3 group of all non-n-3 fatty acids).

$$[\text{DHA}]\ (\text{mg.g}^{-1}) = (A_{DHA}/A_{TCB}) \cdot (H_{TCB}/H_{DHA}) \cdot (M_{DHA}/M_{TCB}) \cdot (x/y) \quad \textit{(Eq. 1)}$$

$$[\text{DHA}]\ (\text{mol\%}) = (A_{DHA}/2) \cdot [(A_{DHA}/2) + A_{FA}]^{-1} \cdot 100 \quad \textit{(Eq. 2)}$$

$$[\text{n-3 fatty acids}]\ (\text{mol\%}) = (A_{n\text{-}3} / A_{n\text{-}3} + A_{non\text{-}n\text{-}3}) \cdot 100 \quad \textit{(Eq. 3)}$$

The α-tocopherol acetate content of fish oil supplements (in mg·g^{-1} units) was determined by the method of standard additions. Briefly, ~1.00 g quantities of oil were accurately weighed and then treated with accurately weighed amounts of this antioxidant (~10 mg, approximately equivalent to the content specified by the manufacturers of product I). Subsequently, accurately weighed quantities (~0.25 g) of these α-tocopherol acetate-"spiked" oil samples were added to 0.50 ml aliquots of C^2HCl_3 containing 5.00×10^{-3} mol·dm^{-3} TCB and then transferred to 5 mm-diameter NMR tubes after thorough rotamixing as described above. The increase in the intensity of its heterocyclic ring 4 position -CH_2- group signal (*t*, δ = 2.555 ppm) expressed relative to that of the TCB internal standard was monitored via electronic integration. Each determination was conducted in duplicate.

The free (unesterified) cholesterol content of the fish oil formulations was estimated by integration of its C18-position -CH_3 group resonance (*s*, δ = 0.655 ppm) and comparison of its intensity with that of the TCB internal standard.

^{13}C NMR measurements

^{13}C NMR spectra were obtained on the Bruker AMX-600 MHz facility (at an operating frequency of 150.93 MHz). The 10,240 FIDs were acquired, each of magnitude 65,536 data points, a sweep width of 26,316 Hz, 8.00 μs pulses, a 10.0 s spin relaxation delay between pulses, and an acquisition time of 1.245 s. Sensitivity enhancement was achieved via the employment of a 5.00 Hz line-broadening function, followed by the zero-filling of FIDs to the above number of data points.

Application of computational methods to confirm ^{1}H NMR assignments

^{1}H NMR assignments and appropriate chemical shift values and coupling constants of resonances of selected marine oil components with complex spin systems (e.g., all-*trans*-retinol palmitate) were confirmed with the HNMR prediction software (version 3.0) from the Advanced Chemistry Development (ACD Inc., Toronto, Canada) software suite. This software was utilized to generate a theoretical spectrum that did not incorporate an allowance for solvent-mediated shielding effects, and the ^{1}H chemical shift and ^{1}H-^{1}H spin-spin coupling constant values obtained therefrom were then adjusted to reflect those derived from the experimental spectrum via a first-order analysis. The theoretical spectrum was then recalculated; a correct first-order analysis gave rise to an exact match between theoretically and experimentally derived spectra. The calculations conducted also made an allowance for the concentrations of each spectrally simulated component in fish oil samples.

Typically, the ^{1}H simulations were based on an internal database containing NMR data for >81K experimental ^{1}H spectra, the associated algorithms employing intramolecular interaction parameters for >300 structural fragments, and the associated subalgorithms estimating initial values for unique structural fragments. The compilation of fragment data were handled with a modified Hierarchical Organization of Shells Expert code, which allowed for explicit substituent charge and stereo bond conventions, optimizing to the maximal number of spheres. Subsequent quantum mechanical shielding calculations allowed for the number of these codes found in the internal database search, in addition to the number of those sought. Calculational errors were determined as the standard deviations of the experimental values found within the database, and typically ^{1}H chemical shift and ^{1}H-^{1}H coupling constant values were accurate to within 0.05 ppm and 0.2 Hz, respectively.

Statistical analysis

All data (concentrations in mg·g^{-1}, mmol·kg^{-1} or mol%) are expressed as mean value ± standard error (SE). Suspected extreme values ("outliers") in each group of repeated determinations made were tested for by computation of Grubbs' (extreme value − overall mean)/overall standard deviation ratio (34) (for each data set) and the value discarded if the probability of observing this value was <0.05.

Nomenclature

Fatty acid chains (F) are represented by the conventional notation, e.g., 22:6(n-3), which denotes a 22-carbon chain with six carbon-carbon double bonds, the first of these located three atoms distant from the the terminal-CH_3 group (i.e., at carbon number 20). The subsequent double bonds (abbreviated as Δ) are each separated from those preceding by one -CH_2- group (*bis*-allylic-CH_2's, abbreviated as Δ-CH_2-Δ). F_α and F_β correspond to the C2 and C3 methylene group protons, while F_ω and $F_{\omega-1}$ represent those of the terminal-CH_3 and directly adjacent -CH_2- groups, respectively (35).

RESULTS

1D ^{1}H NMR and 2D ^{1}H-^{1}H NMR analysis of fatty acid components present in marine oil supplements

A typical 600 MHz ^{1}H NMR spectrum of a commercially available cod liver oil supplement (product I), accompanied by expansions of its 0.80–1.00 and 2.20–2.50 ppm regions, is shown in **Fig. 1**. The F_α and F_β methylene (-CH_2-) groups (C2 and C3 positions, respectively) located between the deshielding carboxylic acid function {esterified to glycerol [1(3)- or 2-positions] or otherwise} and the C4–C5 position double bond of DHA exclusively give rise to ^{1}H NMR signals downfield of and well resolved from those arising from the C2 (F_α) -CH_2- groups of all the other (non-DHA) fatty acids (Fig. 1), and this phenomenon readily permits the quantification of DHA in the fish oil supplements examined here, both as a molar proportion (mol%) of all fatty acids present and as mol·kg^{-1} or mg·g^{-1} units as outlined in the Materials and Methods section.

All n-3 fatty acids have a terminal-CH_3 (F_ω) group resonance that is significantly downfield of that of n-6, n-9, and saturated fatty acids (Fig. 1), a consequence of their closer proximity to the nearest carbon-carbon double bond and, as noted previously by our laboratory (36) and others (24, 25), the ratio of the intensities of these two clearly resolved signals (i.e., 0.940:0.846 ppm) yields the n-3:n-6 fatty acid ratio for fish oil supplements. Moreover, expression of the 0.940 ppm resonance intensity to that corresponding to the sum of both the terminal-CH_3 group signals provides a useful index regarding the ratio of total n-3 to non-n-3 fatty acids, and hence the molar percentage of the former present in the samples examined. The mean ± between-assay standard error values for each of these ^{1}H NMR determinations are given in **Table 1**.

The DHA content is similar for both products (∼9.5 mol%), and these values are in good agreement with those of Gunstone and Seth (29), who employed ^{13}C NMR spectroscopy for the determination of this HUFA in cod liver oil. However, product I clearly offers a higher total n-3 fatty acid content than product II.

Visual inspection of further fatty acid chain resonances in the spectra acquired permitted the complete or partial distinction between different classes of these species. **Figure 2** displays the expanded 1.50–1.85 (F_β), 1.90–2.20 (Δ-1), and 2.70–3.00 ppm (Δ-CH_2-Δ) regions of a typical ^{1}H marine oil supplement sample spectrum, and each of these contained two or more classes of resonances. The F_β -CH_2- group region has clearly resolved multiplet resonances centered at 1.59 and 1.69 ppm, the former predominantly arising from 16:0 and 18:1(n-9) and the latter from 20:5(n-3), because only this fatty acid and 20:4 (n-6) (arachidonate) have a signal at this chemical shift value and there are only low levels of the latter in marine oils [i.e., 1.3–2.7

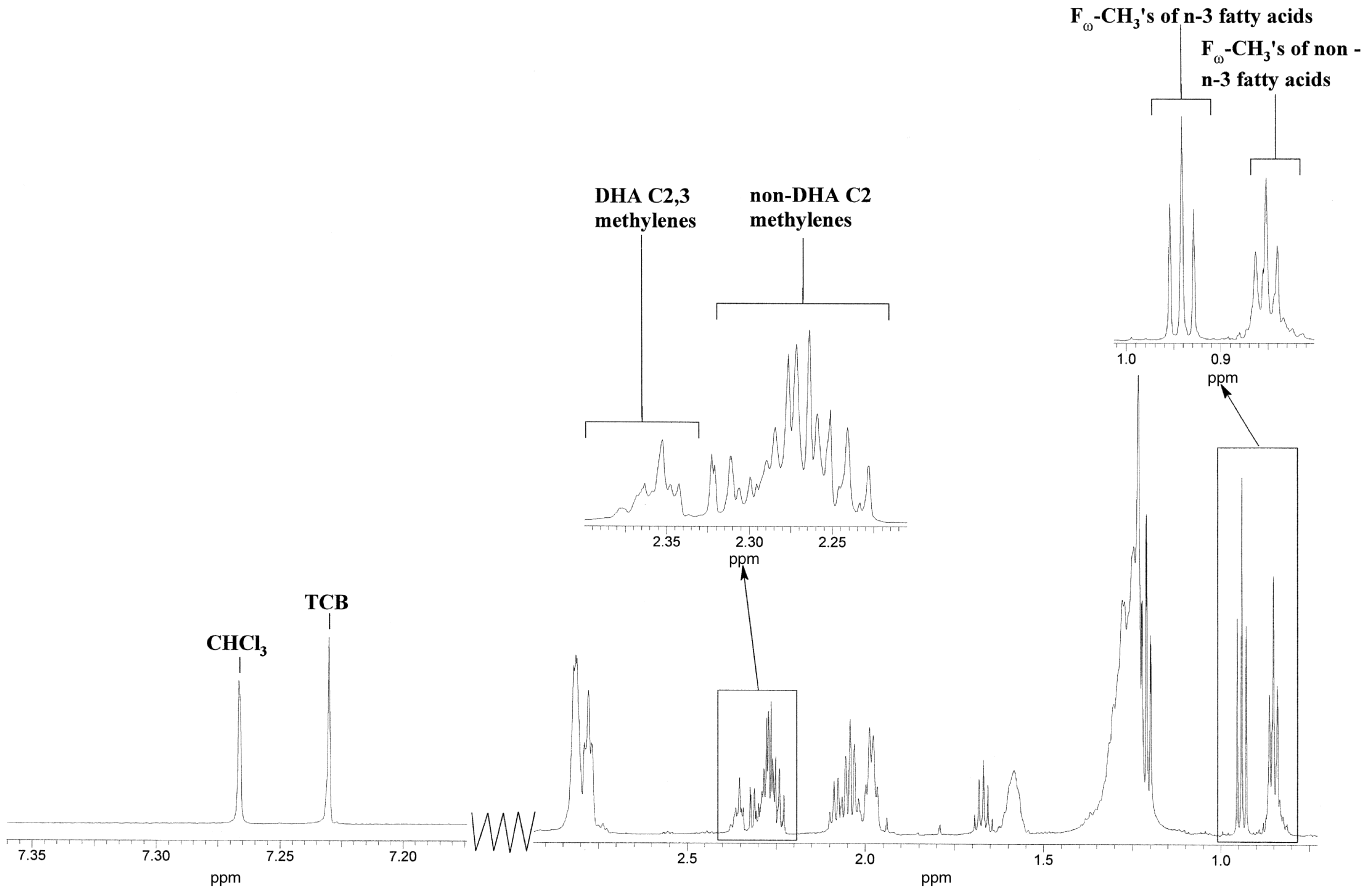


Fig. 1. Partial 600 MHz ^{1}H NMR spectrum of an encapsulated cod liver oil supplement (product I, $CDCl_3$ solution). The spectra exhibit clear resolution between *1*) C2 methylene group resonances (expanded 2.20–2.45 ppm region) of docasahexaenoic acid (DHA), C2,C3, and all other fatty acids; and *2*) those of the terminal-CH_3 groups (expanded 0.80–1.05 ppm region) of n-3 and all other fatty acids. A typical spectrum is shown. TCB, 1,3,5-trichlorobenzene.

mol% (28)]. For the pattern of resonances in the Δ-1 region, that at 2.00 ppm corresponds to 18:1(n-9) and that at 2.08 ppm to all n-3 fatty acids, predominantly 20:5(n-3), 22:6(n-3), and, to a much lesser extent, 20:4(n-3); contributions from 18:2(n-6) (2.06 ppm), 18:3(n-3) (2.07 ppm), and the C16 (Δ-1) -CH_2- group signal of 20:4(n-6) (2.06 ppm) (37) are much less discernable in view of their markedly lower concentrations [0.6–2.3, 0.4–0.9, and 1.3–2.7 mol%, respectively, in a range of marine oils (28)]. The C4 (Δ-1) resonance of 20:4(n-6) (δ = 2.13 ppm), which distinguishes this species from all other unsaturated fatty acids, was not detectable in the spectra acquired, an observation also consistent with its low content in the fish oil supplements examined here. As expected, all linoleate [18:2(n-6)] signals were of a very low intensity when expressed relative to those arising from 16:0, 18:1(n-9), 20:5(n-3), and 22:6(n-3) fatty acid components that are reported to be present at levels of 11–26, 9–22, 3–9, and 20–48 mol%, respectively, in various fish oils (28). Indeed, particularly notable is the very low intensity of its Δ-CH_2-Δ group resonance at 2.738 ppm.

2D ^{1}H-^{1}H COSY spectra of the fish oil supplements revealed clear connectivities between (1) the non-n-3-fatty acid F_ω group signal and those of two classes of magnetically distinct bulk chain (-CH_2-)$_n$ groups, these linkages being largely ascribable to 16:0 and 18:1(n-9), together with smaller quantities of 22:1 and all n-6 polyunsaturated fatty acid (PUFA) components (data not shown). Because the C3 (F_β) position ^{1}H resonance located at 1.69 ppm is predominantly attributable to EPA, there was no connec-

TABLE 1. Mean ± standard error (SE) DHA and n-3 fatty acid contents of encapsulated marine fish oil products

Product	DHA		n-3 Fatty Acids	
	mg·g^{-1}	*mmol·kg^{-1}*	*mol%*	
1	115.95 ± 4.40 (n = 8)	353 ± 13 (n = 8)	9.16 ± 0.19 (n = 10)	40.03 ± 0.36 (n = 10)
2	119.80 ± 2.49 (n = 8)	365 ± 8 (n = 8)	9.47 ± 0.13 (n = 14)	27.00 ± 0.36 (n = 13)

DHA, docosahexaenoic acid.

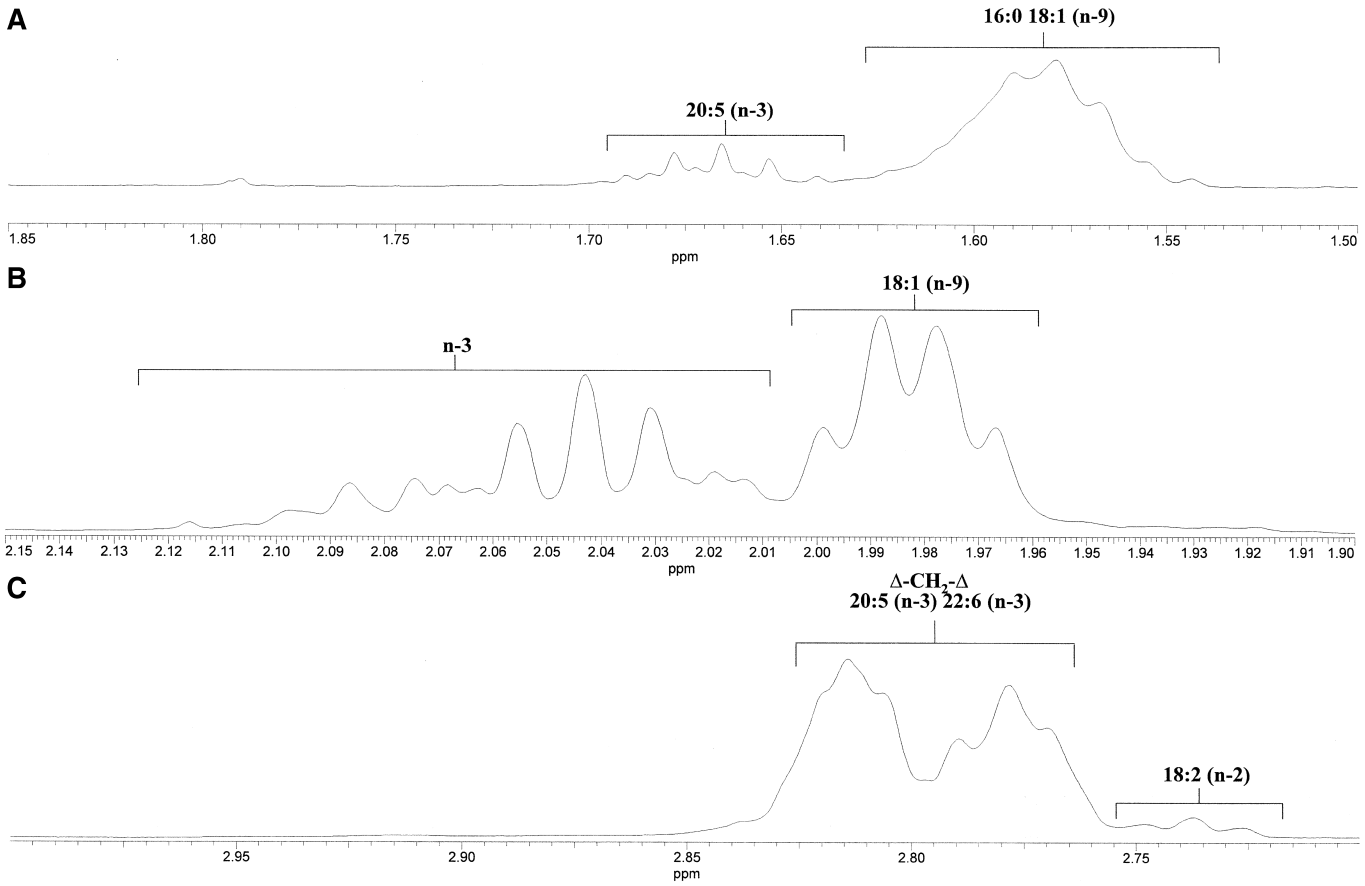


Fig. 2. Expanded F_β, Δ-1, and Δ-CH_2-Δ regions of a 600 MHz ^{1}H NMR spectrum of a cod liver oil supplement (product I). Expanded F_β (1.50–1.85 ppm) (A), Δ-1 (1.90–2.20 ppm) (B), and Δ-CH_2-Δ (2.70–3.00 ppm) (C) regions. A typical spectrum is shown.

tivity observed between this signal and that of bulk chain methylene groups; however, as expected, it was strongly linked to the F_α and C4(Δ-1) resonances at 2.32 and 2.13 ppm, respectively. Further connectivities revealed by the ^{1}H-^{1}H COSY spectra included: *1*) the 1.59 ppm F_β multiplet signal and that of the bulk chain $(-CH_2-)_n$ resonances centered at ~1.24 ppm, consistent with the assignment of the former resonance to 16:0 and 18:1(n-9) as noted above; *2*) the 2.00 ppm Δ-1 resonance to those at 1.59 and ~1.20 ppm, representing the relatively high levels of 18:1(n-1) present; *3*) the 2.08 ppm Δ-1 signal to that of the n-3 fatty acid terminal-CH_3 group, confirming its assignment to a combination of EPA, DHA, and further n-3 fatty acids; *4*) the multiple vinylic proton resonances centered at ~5.3 ppm, the Δ-CH_2-Δ group signals at 2.82 and 2.86 ppm, and those ascribable to Δ-1 protons at 2.00 and 2.08 ppm; and *5*) the two triacylglycerol glycerol backbone 1(3)-position -CH_2OCOR group resonances (δ = 4.12 and 4.25 ppm) and that arising from the 2-position -CHOCOR group (δ = 5.26 ppm). Of particular interest to this study is the observation of a linkage between these vinylic resonances and the unique, well-resolved F_β methylene group protons of DHA (overlapped with its F_α-position -CH_2- group signal in 1D spectra, δ = 2.38 ppm). Hence, this 2D ^{1}H-^{1}H connectivity also serves as a highly specific marker of DHA present in fish oil products.

For one of the products investigated (product I), a further, intense connectivity between resonances located at 1.21 (apparent triplet) and 4.09 ppm (apparent quartet) were visible. These linked signals are clearly attributable to an ethyl group in an ester derivative (38), i.e., the manufacturers of this particular product have either chemically modified the cod liver oil supplement or added DHA and EPA in the forms of ethyl docosahexaenoate and ethyl eicosopentenoate, respectively. Closer examination of the 1D ^{1}H NMR spectra of this product in the 1.15–1.25 ppm region (**Fig. 3**) revealed that the signal at 1.21 ppm consisted of two separate triplets of very similar but nevertheless distinguishable chemical shift values (δ = 1.211 and 1.215 ppm, j = 7.36 Hz in each case). In view of the molecular structures of the ethyl ester derivatives of DHA and EPA [i.e., a closer proximity of nearest >C=C< double bond in DHA (C4 position) to the -CH_3 moiety of the ethyl group, together with a larger number of double bonds in this fatty acid when considered as a whole], the separate 1.211 and 1.215 ppm -CH_3 group resonances are tentatively assigned to the ethyl esters of EPA and DHA, respectively, although, as outlined in detail below, it is important to note that ^{1}H NMR analysis of d_4-MeOH extracts of this product revealed that ~30% of these ethyl ester-CH_3 group resonances are accountable for by non-n-3 fatty acid species. The intensities of these two signals are very

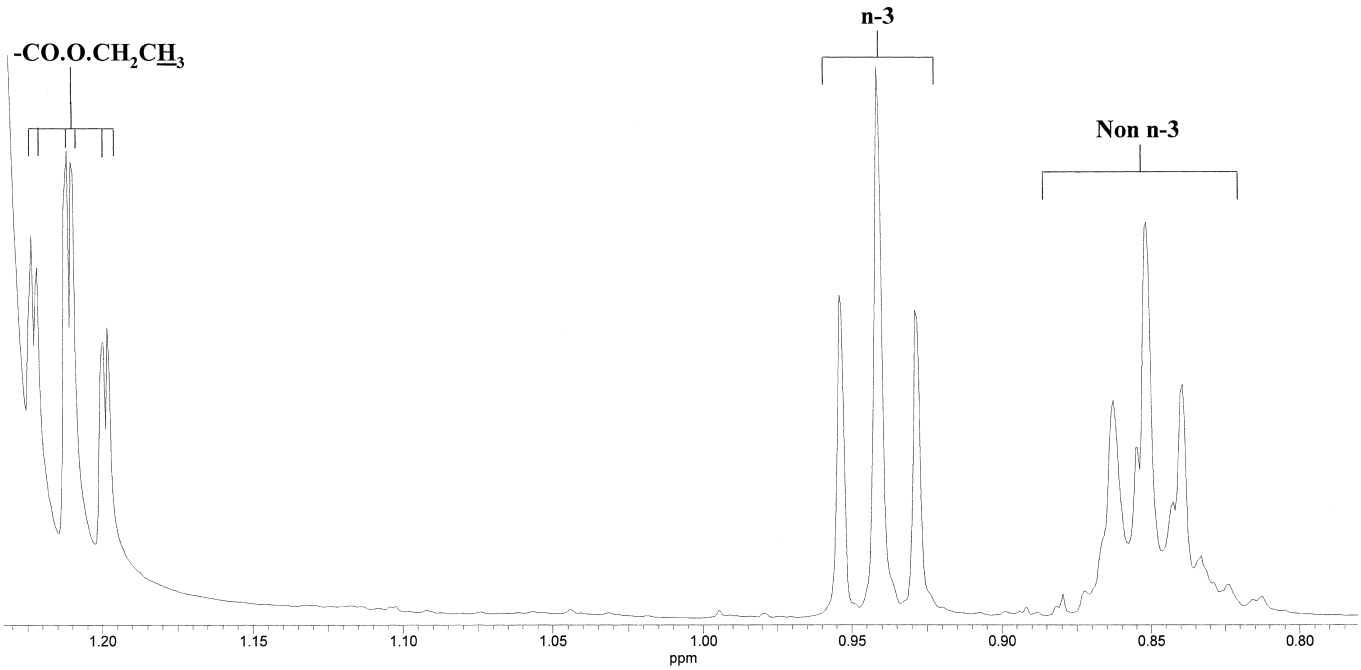


Fig. 3. The 0.89–1.27 ppm region of the 600 MHz ^{1}H NMR spectrum of product I ($CDCl_3$ solution). The spectrum contains resonances arising from the -CH_3 moiety of the ethyl group of fatty acid ethyl esters (predominantly n-3 HUFAs). A typical spectrum is shown. -CO.O.CH_2C$\underline{H}_3$, fatty acid ethyl esters' -CH_3 groups.

similar, suggesting that similar molar levels of each HUFA ester have been generated from or added to the product, although the line-width of that assigned to DHA ethyl ester appeared to be somewhat broader than that assigned to the corresponding EPA ester. The DHA and EPA ethyl ester resonances were not detectable in the 600 MHz ^{1}H NMR spectra acquired on product II, demonstrating that the manufacturers have not chemically modified their cod liver oil supplement or added synthetic DHA or EPA derivatives to it; i.e., all of the HUFAs therein are present as natural cod liver oil acylglycerol species (and/or those arising from any refinement process involved in their production). Electronic integration of these combined signals [the middle and higher field peaks of the triplets only and then multiplication by the factor 4/3, in view of the significant overlap of the lower field triplet peaks with the bulk chain (-CH_2-)$_n$ resonance centered at ~1.23 ppm], together with that representing the total fatty acids in spectra of product I [the combined intensities of the n-3 (*t*, δ = 0.940 ppm) and non-n-3 (*t*, δ = 0.846 ppm) signals], allows an estimation of the molar ratio (and hence percentage) of the synthetic ethyl ester derivatives of these highly unsaturated and other fatty acids to the total fatty acid content of this formulation. Using this method, the mean ± SE percentage of added ethyl esters (predominantly those of DHA and EPA) generated in or added to the cod liver oil in product I was found to be 62.57 ± 1.07 mol% of the total fatty acid content (n = 10), a figure higher than the total n-3 fatty acid content in this product (Table 1), demonstrating that non-n-3 fatty acids are also present as ethyl esters in this formulation. As expected, the ethyl group -CH_2OCOR signal centered at 4.09 ppm was also found to consist of two separate quartets, although, in view of its overlap with one of the triacylglycerol glycerol backbone-CH_2OCOR resonances located at 4.10 ppm, electronic integration of these was not possible. The differences between the chemical shift values of the F_α,F_β ^{1}H NMR resonances of the ethyl ester and glycerol-bound forms of DHA are expected to be extremely small and hence to exert no influence on the estimations of this HUFA made here. Indeed, the chemical shift range for these signals in methyl docosahexaenoate (2.31–2.38 ppm) (38) are very similar to those of the docosahexaenoylglycerol species present in the ^{1}H spectra of product II, which does not contain the ethyl ester.

^{1}H NMR analysis also revealed that deuterated methanol extracts of product I (obtained by the method described in Materials and Methods) contained high levels of the ethyl esters of these HUFAs and, as expected, only a very limited level of triacylglycerols (**Figs. 4**, **5**), an observation consistent with the high and low solubilities, respectively, of these species in this solvent system. Hence, these synthetic HUFAs are, at least in principle, recoverable from the bulk cod liver oil matrix using this extraction method. Interestingly, the very high intensity of the non-n-3-fatty acid F_ω signal (when expressed relative to those of all acylglycerol species present) confirms that at least some of the d_4-MeOH-soluble fatty acid ethyl esters are present in this form in the encapsulated product. Indeed, electronic integration of this resonance, together with those ascribable to the n-3 fatty acid F_ω and ethyl ester -CO.O.CH_2CH_3 groups, indicated that the great majority of fatty acids in this solution were present as ethyl esters (the combined F_ω signal intensity was only slightly greater than that of the lat-

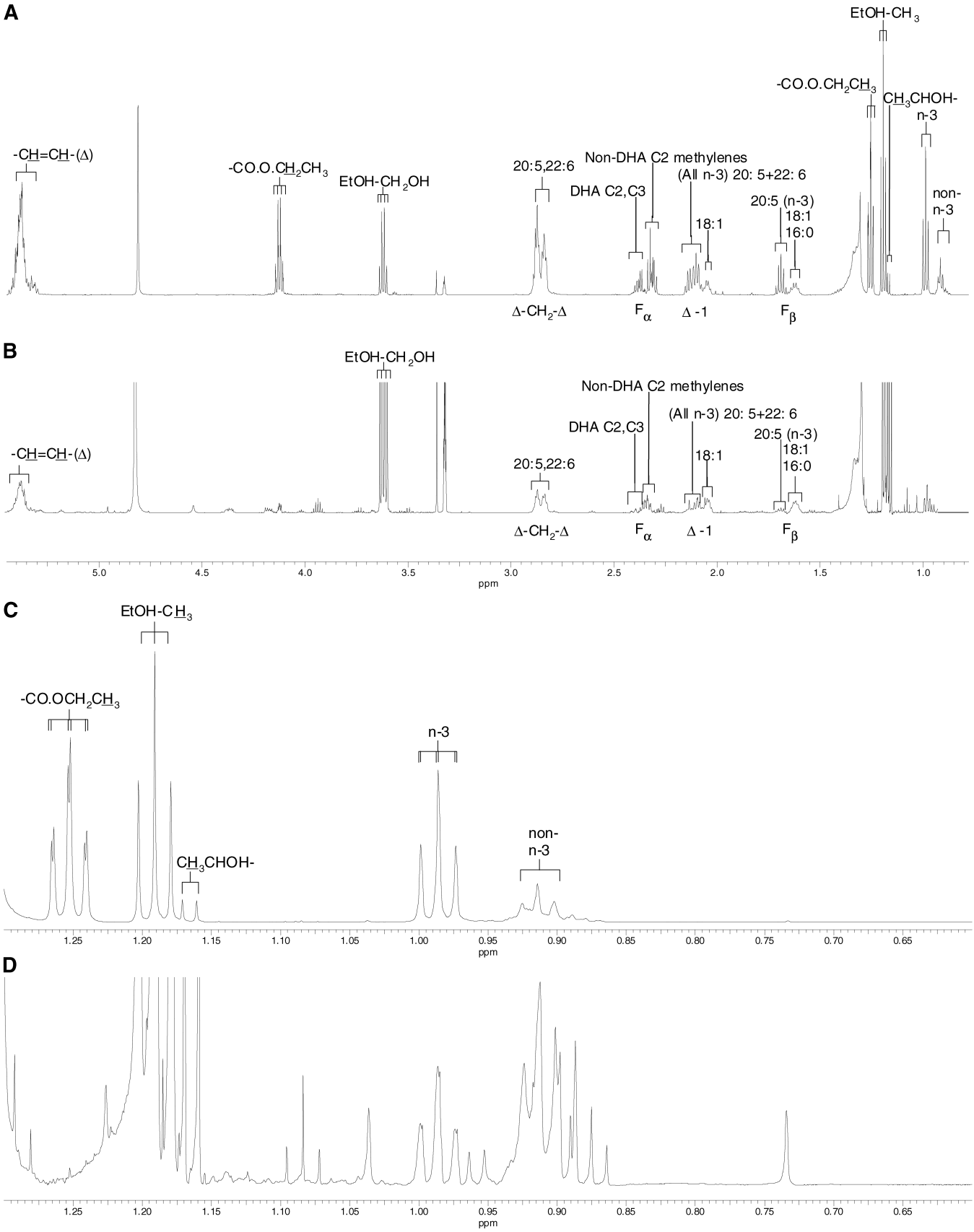


Fig. 4. The 600 MHz NMR spectra of d_4-methanol (d_4-MeOH) extracts of encapsulated marine oil products. A, B: 0.80–5.45 ppm region of the 600 MHz NMR spectra of a d_4-MeOH extract of products I and II, respectively; expanded 0.61–1.30 ppm regions of the partial spectra (A) and (B) are shown in C and D, respectively. EtOH-CH_3, ethanol-CH_3.

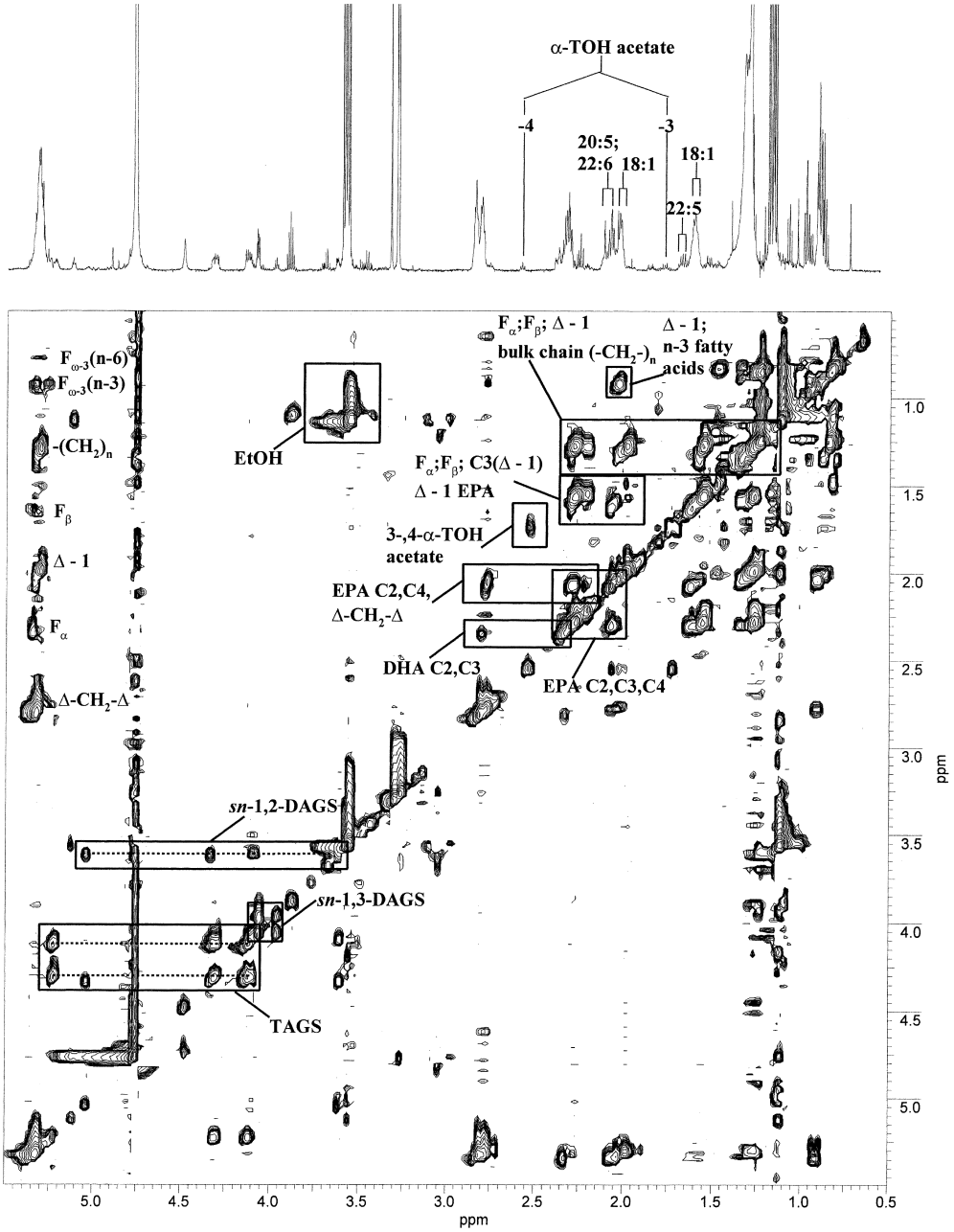


Fig. 5. The 600 MHz ^{1}H-^{1}H TOCSY spectrum of a d_4-methanol extract of product II. A typical spectrum is shown.

ter) and that ~70% of these were n-3 species. Consistent with the heterogeneity of the ethyl ester-CH_3 group resonance, the adjacent -CO.O.C$\underline{H}_2$CH$_3$ group (δ = 4.12 ppm) also comprised two (or more) distinct quartets, as noted above when in C^2HCl_3 solution. Of course, the fatty acid ethyl ester resonances were absent from ^{1}H spectra acquired on corresponding extracts of product II.

Also consistent with the chemical modification or fortification of product I with high levels of the methanol-extractable ethyl esters of DHA and EPA, the characteristic chain resonances of these fatty acids {notably EPA's F_β (1.70 ppm), DHA's F_α,F_β (2.378 ppm), and the Δ-1 signals of both of these HUFAs [F_ω-1 (Δ-1) at 2.10, and EPA's Δ-1 (C4) at 2.13 ppm]} were of a higher intensity than those ascribable to 18:1(n-9) (Δ-1 and F_β) and 16:0 (F_β), (Figs. 4, 5). However, the intensities of the characteristic 18:1 and 16:0 resonances were much higher than those of the glycerol backbone protons of acylglycerols present in this extraction solvent, an observation again confirming that these non-n-3 fatty acids are also present as ethyl esters in this product.

^{1}H-^{1}H TOCSY spectra of d_4-MeOH extracts of the products examined showed strong connectivities between the lipid chain resonances. Indeed, the vinylic ^{1}H signals (~5.4 ppm) were, as expected, linked to the Δ-CH_2-Δ, F_α, Δ-1, and F_β signals, and, with respect to n-3 and non-n-3 fatty acids, the F_ω triplets were located at 0.92 and 0.78 ppm,

respectively, in this medium. These spectra also showed clear connectivities between the Δ-CH_2-Δ, F_α, and F_β resonances of both DHA and EPA (the latter two signals centered at 2.378 ppm for DHA in this solvent system) and also links between these and the C4 (Δ-1) resonance of EPA (δ = 2.13 ppm). The identity and relative levels of many further, methanol-extractable components detectable in these spectra are outlined in detail below.

^{1}H NMR analysis also permitted the direct and rapid detection of diacylglycerols, together with the acquisition of data regarding the relative quantities of the *sn*-1,2- and *sn*-1,3-forms of these adducts, which arise from the incomplete biosynthesis of triacylglycerols, a limited activity of the lipase enzyme system, and/or lipolysis of triacylglycerols during processing, refinement, and/or storage of the products investigated. Indeed, *sn*-1,2-diacylglycerols were readily monitorable in the two marine oil formulations tested here by observation of a clearly resolved -CH_2OH, *sn*-3 doublet resonance located at 3.66 ppm (**Fig. 6**). Further *sn*-1,2-diacylglycerol signals detectable included those at 4.17, 4.31 (both -CH_2OCOR, *sn*-1), and 5.07 ppm (-CHOCOR, *sn*-2). Although some overlap between triacylglycerols and *sn*-1,3-diacylglycerol resonances is apparent in their glyceridic spectral regions, the latter's 4.03 ppm -CH_2OCOR, *sn*-1,3 multiplet signal was observable at an operating frequency of 600 MHz. 1D ^{1}H NMR and 2D ^{1}H-^{1}H NMR spectra of the d_4-MeOH extracts acquired on both products demonstrated that relatively high levels of *sn*-1,2 and -1,3 diacylglycerols were present therein (Figs. 4, 5). Indeed, the TOCSY spectra showed clear connectivities between the *sn*-1,2 signals located at 3.62 (-CH_2OH, *sn*-3), 4.08, 4.29 (-CH_2OCOR, *sn*-1), and 5.01 ppm (-CHOCOR, *sn*-2) and the *sn*-1,3 resonances at 3.96 (-CH_2OCOR, *sn*-1,3) and 4.02 ppm (-CHOH, *sn*-2) in this medium. Extraction of these cod liver oil products with methanol therefore serves as a partially selective means of isolating *sn*-1,2- and *sn*-1,3-diacylglycerols from triacylglycerols therein, insofar as these partial acylglycerols are much more soluble in this solvent system.

Interestingly, free glycerol was also detectable in both 400 and 600 MHz ^{1}H spectra acquired on product I (but not product II); i.e., characteristic ABX coupling pattern of resonances at 3.547 and 3.638 ppm (A and B protons, -CH_2OH groups) and 3.875 ppm (X proton, -CHOH group) as shown in Fig. 6, and although it is conceivable that some of it arises from tri- or di- or monoglyceride hydrolysis during periods of processing/refining by the manufacturer, or prolonged storage, its presence is probably largely ascribable to its dissolution from the capsule shell, which contains this agent, together with gelatin.

1D ^{1}H NMR and 2D ^{1}H-^{1}H NMR analysis of minor (low-level) components in marine oil supplements

The ^{1}H NMR technique also allowed the direct, noninvasive detection of components present at only low concentrations in the marine oil supplements examined. **Figures 7**

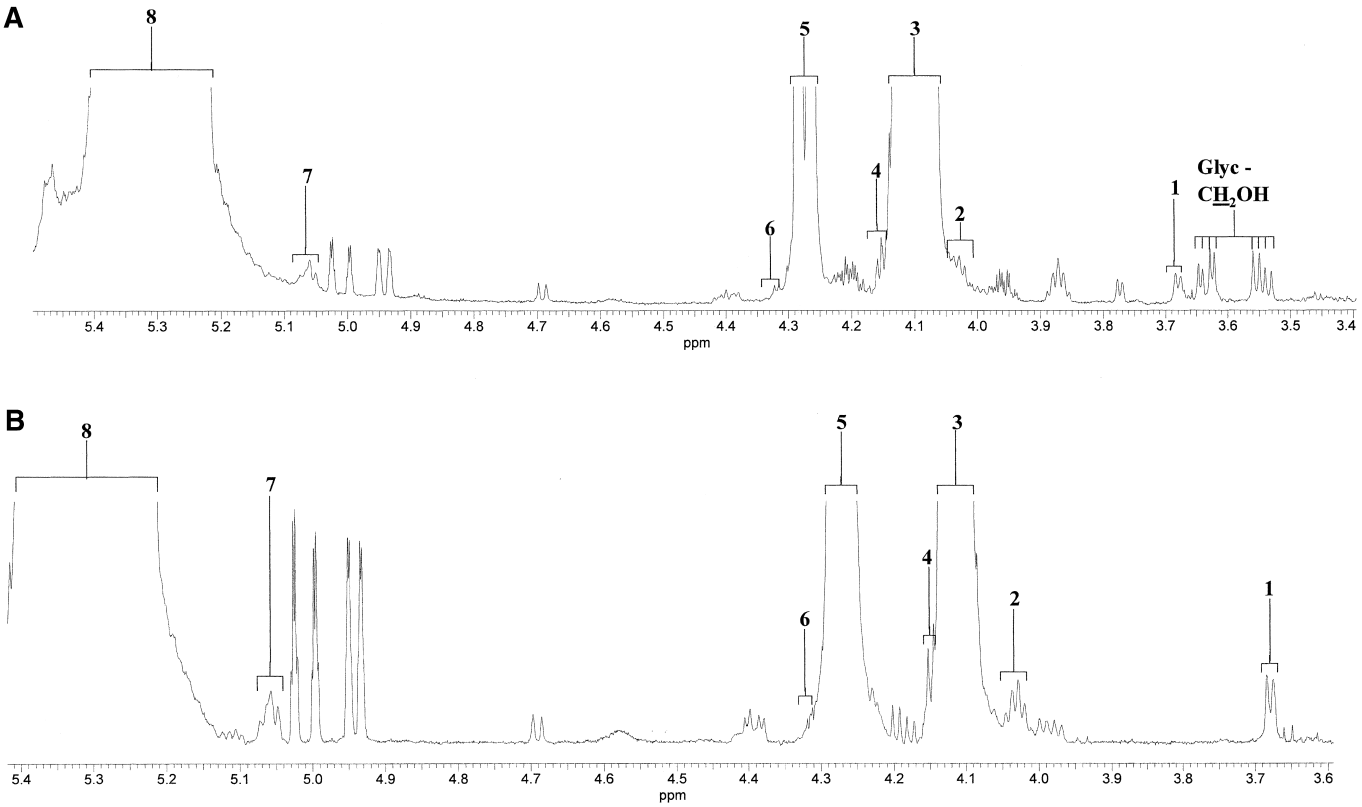


Fig. 6. The 600 MHz ^{1}H NMR spectra of encapsulated marine oil products in $CDCl_3$ solution. The 3.50–5.40 ppm (glyceryl proton) regions of the 600 MHz ^{1}H NMR spectra of (A) product I and (B) product II (typical spectra are shown). The -CHOH *sn*-2, *sn*-1,3-diacylglycerol signal (δ = 4.07 ppm) is obscured by the relatively intense triacylglycerol resonance located at 4.10 ppm.

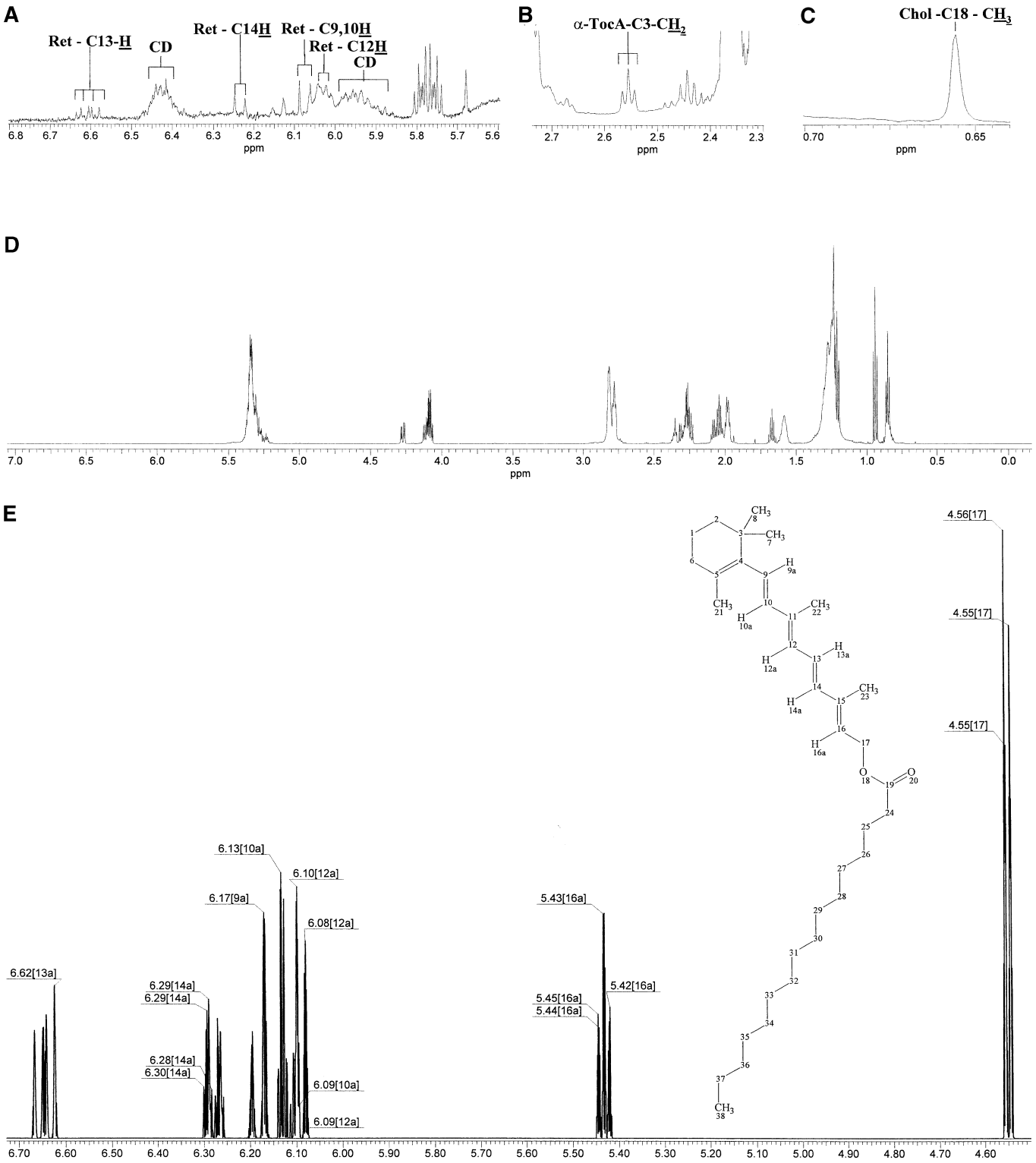


Fig. 7. ^{1}H NMR detection of α-tocopherol acetate, all-*trans*-retinol palmitate, and free cholesterol in product I ($CDCl_3$ solution). A–D: The 0.00–7.00, and expanded 0.64–0.70, 2.30–2.70, and 5.60–6.80 ppm regions of the 600 MHz 1D ^{1}H NMR spectrum of product I. E: Computer-simulated spectrum of all-*trans*-retinol palmitate generated from the software suite described in Materials and Methods. CD, conjugated diene region signals ascribable to one or more lipid oxidation products (LOPs), conceivably conjugated hydroxydienes; Ret, all-*trans*-retinol palmitate; α-TocA, α-tocopherol acetate; Chol, cholesterol.

and **8** display the expanded 0.00–7.00, 0.64–0.70, 2.30–2.70, and 5.60–6.80 ppm regions of the 600 MHz 1D ^{1}H spectra of the products investigated, which highlight selected minor component resonances that are either clearly or partially resolved from those of acylglycerol and/or alternative lipidic components present. These signals consist of those attributable to: *1*) α-tocopherol acetate [C7- and C8-position aromatic methyl substituents and the

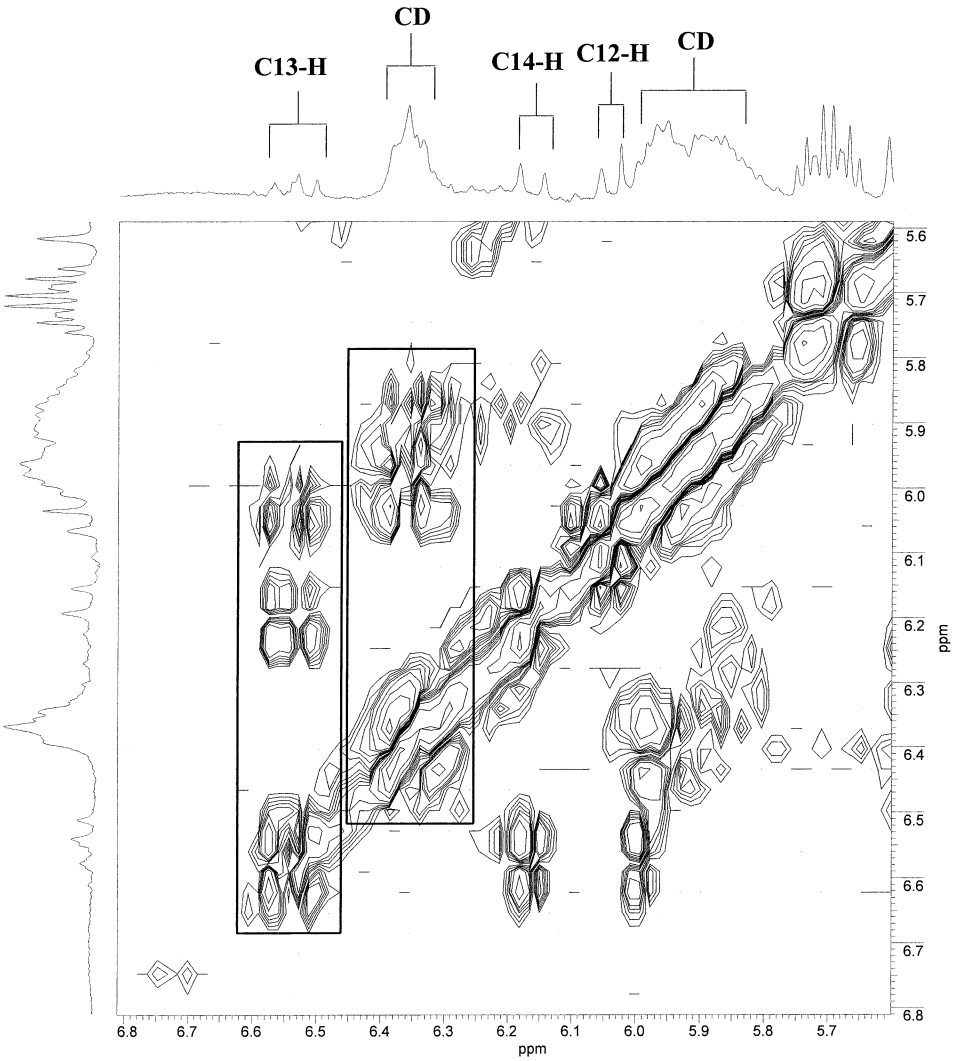


Fig. 8. The 400 MHz ^{1}H-^{1}H COSY spectrum of product I. A typical spectrum is shown. C12-H, C13-H, and C14-H, all-*trans*-retinol palmitate C12-, C13-, and C14-position protons.

heterocyclic ring C3- and C4-position methylene groups (the latter -CH_2- group triplet being completely resolved)]; *2*) retinol palmitate (C9-, C10-, C12-, C13-, C14,- and C16-position chain vinylic protons in the 5.70–6.65 ppm chemical shift range, together with those arising from its C8- and C7-position methyl groups at 0.978 and 0.994 ppm, respectively); and *3*) free (non-esterified) cholesterol (very clearly resolved C18-methyl group singlet at 0.65 ppm, C26,27- and C19-methyl substituents, together with its $H_{3\alpha}$ proton multiplet). The identity of the form of α-tocopherol as its acetate ester in product II (unspecified by the manufacturers) was confirmed by comparisons of accurately determined ^{1}H chemical shift values of visible (resolved) resonances with those of an authentic standard in C^2HCl_3 solution [as expected, the phenolic-OH group resonance of α-tocopherol itself (δ = 4.63 ppm in C^2HCl_3 solution) was absent from spectra of either product].

Free cholesterol was readily determinable in the products tested because of the complete isolation of its C18-methyl group resonance from those of fatty acids. Indeed, estimates of the cholesterol content of products I and II were 5.19 ± 0.21 and 4.82 ± 0.19 mg·g^{-1}, respectively (mean ± SE values).

Our spectral prediction software (ACD, Inc.) served as a valuable means of facilitating and confirming our preliminary assignments for resonances of the retinol moiety of the added retinol palmitate; Fig. 7 shows the excellent agreement observed between such simulated and experimentally acquired spectra. Of course, resonances assignable to the palmitate moiety of this ester were masked by those of the high-level acylglycerol components in the products tested. 2D ^{1}H-^{1}H COSY spectra of the products confirmed clear connectivities between the chain vinylic proton resonances of the retinol portion of retinol palmitate; as an example, Fig. 8 displays a typical partial COSY spectrum that demonstrates linkages between the C13- (δ = 6.526 ppm), C12- (δ = 6.046 ppm), and C14-position (δ = 6.167 ppm) ^{1}H signals.

After consideration of the number of protons giving rise to the ^{1}H NMR signals of α-tocopherol acetate and retinol palmitate, the former were of a greater intensity than those of the latter, as expected from the relative

amounts of these vitamin adducts added to each supplement by their manufacturers (see Materials and Methods). Using the standard addition method outlined in Materials and Methods, we found that adding ~10 mg·g^{-1} of α-tocopherol acetate to product I doubled the intensity of its clearly visible 4-position -CH_2- group resonance, (subsequent to making allowances for very small differences in the between-replicate weights of α-tocopherol acetate added to this product and minor differences between those of the fish oil itself) a result consistent with its specified content (10 mg·1.050 g, or 9.52 mg·g^{-1}). However, for product II, these increases were much greater, and the estimated α-tocopherol acetate content of this formulation was 3.33 ± 0.07 mg·g^{-1}, i.e., approximately one-third that of product I.

In view of the very low content of vitamin A in products I and II (800 μg per 1.05 and 1.00 g respectively, and specified as all-*trans*-retinol palmitate in the former), and also of some overlap between its visible resonances and those of alternative agents [e.g., that involving its C13-position vinylic proton (*dd*, δ = 6.58 ppm)], only semiquantitative content data were provided by determinations of their intensities. As expected, vitamin D (specified as a D_3 preparation in product I) was not detectable by ^{1}H NMR spectroscopy.

Intriguingly, strong connectivities between two further resonances located at 6.38 (apparent *dd*) and 5.95 ppm (multiplicity unclear) were also discernable in these COSY spectra. As previously reported by our laboratory (39), these signals are in the spectral region that is characteristic of conjugated diene lipid oxidation products (LOPs), and careful consideration of their chemical shift values, and the coupling pattern of that at 6.38 ppm, indicates that they are attributable to one or more conjugated hydroxydiene species [e.g., 13-hydroxy-9-*cis*,11-*trans*-octadecadienoate derived from the peroxidation of the low levels of 18:2(n-6) present], the determination of which is known to be of practical value in the monitoring of fish and fish oil quality (40). Experiments to determine the nature and content of such LOPs in cod liver oil supplements are currently in progress in our laboratory, the results of which will be presented elsewhere.

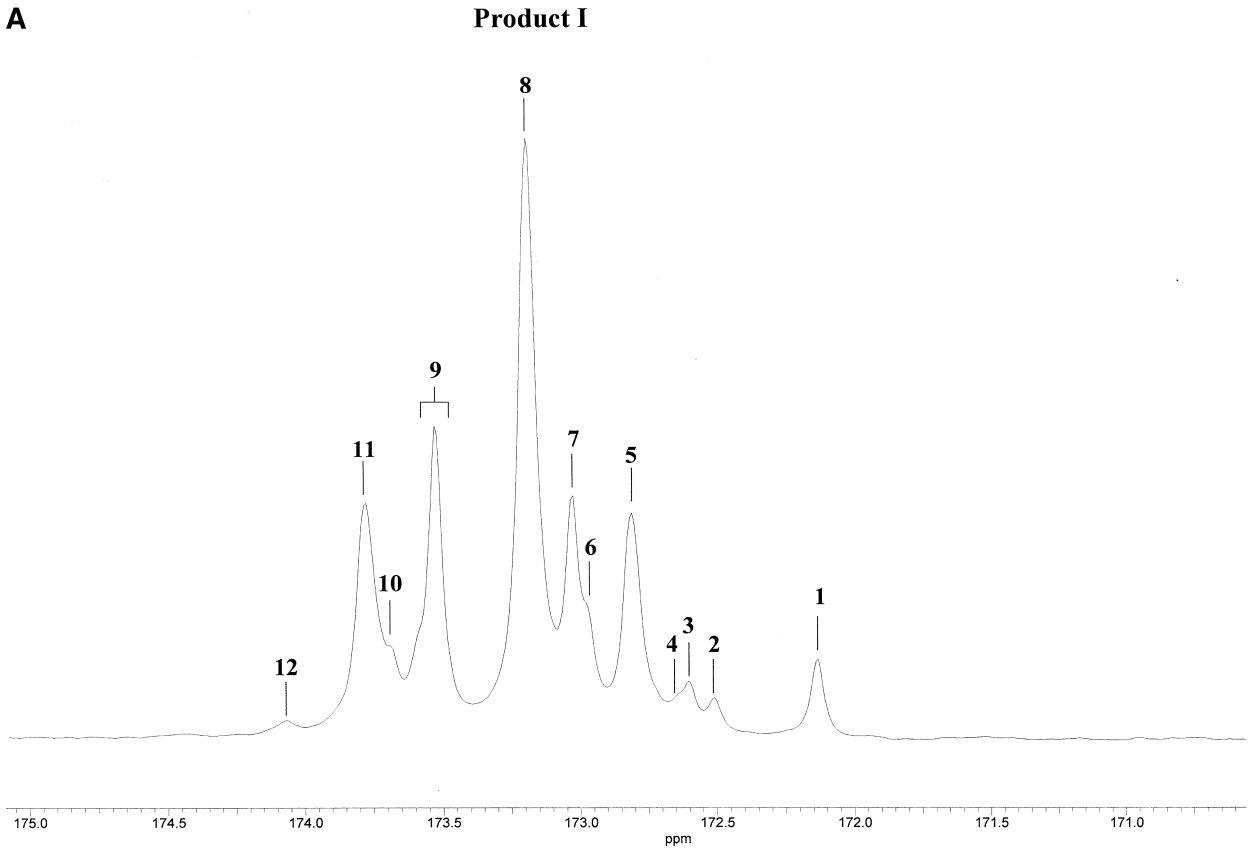
1D spectra acquired on deuterated methanol (d_4-MeOH) extracts of the fish oil products also confirmed the presence of cholesterol in these materials (Figs. 4, 5). Because the C19-position methyl group resonances for free and esterified cholesterol are readily distinguishable in this medium (41), it was possible to estimate the relative amounts of each component. Consistent with the ^{1}H spectra obtained in the C^2HCl_3 solution and also the ^{13}C NMR spectra detailed below, cholesterol was predominantly in the form of its free (unesterified) agent, with little or no cholesterol esters present.

^{13}C NMR analysis of marine oil supplements

A characteristic of the ^{13}C NMR spectra of DHA, EPA, their corresponding glycerides and other esters is the large number of vinylic and allylic carbon resonances present [e.g., DHA has 22 carbon atoms, of which 12 are vinylic and 7 are allylic, with only C1, C2, and C22 (the latter being F_ω) remaining], and the spectra acquired on the cod liver oil products examined reflected the high levels of these HUFAs present.

Reference to the extensive chemical shift data tabulated by Gunstone (43, 44), together with those of Sacchi et al. (45) (for both free fatty acids and their glyceridic and methyl esters) enabled the complete or partial distinction between individual fatty acids, the chemical nature of their esters (glycerides and ethyl esters of DHA and EPA), and the substitutional status of the glycerol backbone, together with the positional distribution of fatty acids on this moiety. The C1 (carboxyl) and vinylic carbon positions of triacylglycerols display small but significant chemical shift differences in relation to both the position of the fatty acid moiety on the glycerol backbone [i.e., *sn*-1(3) or -2 positions] and the positions and number of carbon-carbon double bonds in the fatty acid chain. The expanded carboxylate and vinylic carbon regions of typical spectra acquired on products I and II are displayed in **Figs. 9** and **10**, respectively.

The carboxylate region of spectra acquired on product II (i.e., that not chemically modified to or fortified with fatty acid ethyl esters) contains eight prominent resonances arrangable into four pairs with chemical shift differences of 0.38 or 0.39 ppm, and assignable to DHA (172.10 and 172.48 ppm), EPA (172.57 and 172.96 ppm), stearidonic acid [18:4(n-3)] (172.62 and 173.00 ppm), and a combination of 18:1(n-9), 16:0, and additional saturated fatty acids (172.78 and 173.16 ppm). The lower and higher chemical shift values in each pair correspond to the fatty acids bonded in the *sn*-2 (β) and *sn*-1(3) (α) positions, respectively, and hence the ^{13}C NMR technique offers unique regiospecific information regarding the positional distribution of these marine oil fatty acids on the glycerol backbone. The above chemical shift values are in excellent agreement with those of Gunstone and Seth (29), and the low chemical shift values for DHA are consistent with previous studies (42). The *sn*-1(3):*sn*-2 glycerol backbone position resonance intensity ratio for DHA (~2:3 for product II) was significantly different from those of all other acylglycerol fatty acids detectable (~2:1, corresponding to a random distribution between the two sites), confirming that this HUFA is highly concentrated in the *sn*-2 position, as previously noted by Gunstone and Seth (29). Although corresponding ^{13}C spectra of product I were very similar, they contained an intense resonance located at 173.49 ppm, which is assignable to the ethyl ester of DHA (and probably also that of EPA and further fatty acids therein), because the carboxyl group of DHA's methyl ester has the extremely similar chemical shift value of 173.45 ppm (38). Hence, multicomponent data obtained on product I was found not to be complicated by the presence of resonances attributable to the carboxyl carbon of these ethyl esters. Also notable was the observation that the relative intensities of the acylglycerol signals in spectra of product I differed from those of product II, most especially the much lower intensities of the docosahexaenoyl and eicosapentaenoylglycerol species [at both the *sn*-1(3) or -2 po-



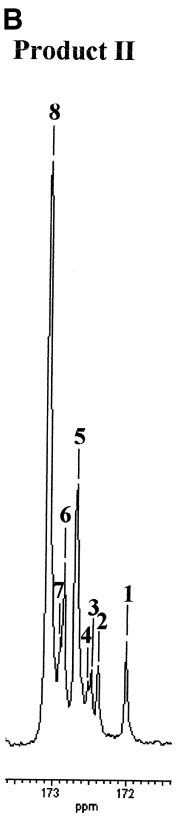


Fig. 9. Acylglycerol and ethyl ester C1 (carboxylate) region of the 150.93 MHz ^{13}C NMR spectra of products I and II ($CDCl_3$ solution) are shown in A and B, respectively. (Chemical shift assignments for the labeled resonances are given in Table 2.)

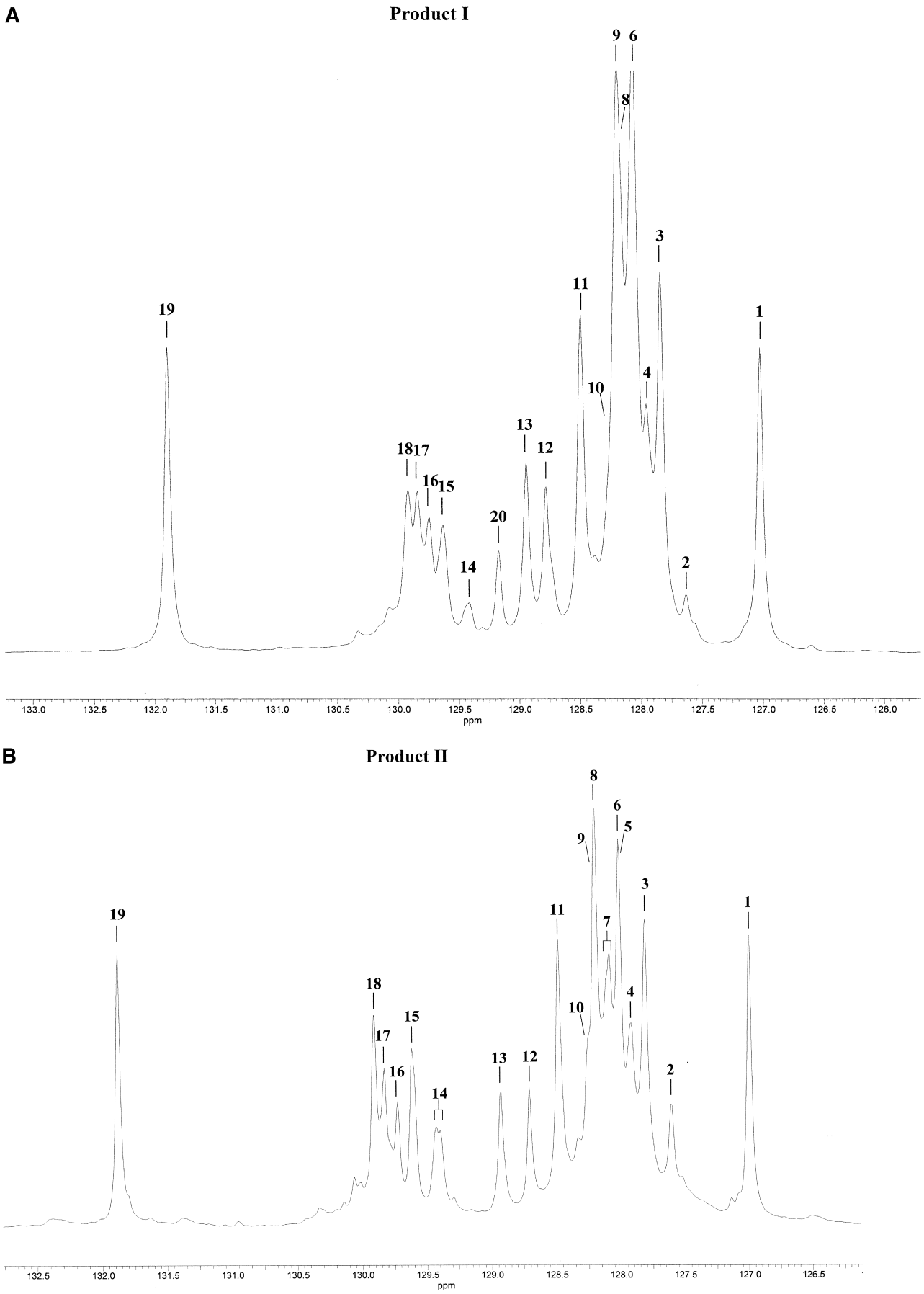


Fig. 10. Olefinic region of the 150.93 MHz ^{13}C NMR spectra of products I and II ($CDCl_3$ solution) are shown in A and B, respectively. (Chemical shift assignments for the labeled signals are given in Table 3.)

sitions], consistent with our results from ^{1}H NMR analysis that these HUFAs are predominantly in the form of ethyl esters in this formulation. ^{13}C NMR spectra of product I also contained a resonance at 173.74 ppm that was also noted by Gunstone and Seth (29) in corresponding spectra acquired on menhaden and other fish oils (172.72 ppm in their work). The chemical shift values of these carboxylate carbon resonances and their assignments are given in **Table 2**.

The vinylic regions of these spectra also provided valuable information concerning the nature of fatty acids present in the products tested, notably DHA and EPA (**Table 3**). In view of the large number of >C=C< bonds in these HUFAs, both DHA and EPA have a very large number of ^{13}C signals in this region. Acylglycerol oleoyl, DHA, EPA, and further fatty acids were readily assignable via comparisons with previously recorded data, and product I also contained two signals representative of simple alkyl esters of two (or more) of these fatty acids [C4 position resonances located at δ = 129.19 and 129.33 ppm, cf. that of methyl docosahexaenoate, 129.26 ppm (38)]. Indeed, well resolved C4,C5 and C5,C6 resonances of triacylglycerol DHA and EPA, respectively, were particularly notable, together with those arising from the C9 and C10 nuclei of oleoyl- [18:1(n-9)], and the C11 and C12 atoms of eicosenoylglycerols. Regiospecific (glycerol backbone position) chemical shift differences were detectable for selected DHA resonances (e.g., that of C4), but there was an incomplete resolution of these for the two EPA signals observed. Comparisons of the relative intensities of these *sn*-1(3)- and *sn*-(2)-glycerol backbone position DHA resonances confirmed that the latter contained a disproportionately high level of this HUFA (consistent with results attained from the carboxylate spectral region). The signal located at 131.92 ppm serves as a criterion for monitoring all the n-3 fatty acids present. Together with all of these resonances, ^{13}C NMR spectra acquired on product I also contained a DHA C4-position signal located at 129.14 ppm, which, by reference to previously published ^{13}C NMR data on the methyl esters of oleic acid, DHA, EPA, and further n-3 fatty acids in this spectral region, serves as a clear marker of synthetic ethyl esters present in this formulation, but nevertheless clearly distinguishes this product from the unadulterated product II.

Hence, the high level of distinction between the highly unsaturated and all other fatty acids in the products tested demonstrates the usefulness of this ^{13}C spectral region for the study of natural, refined, or chemically transformed or adulterated marine oil preparations.

The allylic (Δ-1 and Δ-CH_2-Δ) methylene group ^{13}C signals, which also offer a diagnostic capacity, were also examined in detail (**Fig. 11A**). The chemical shift values of these carbon atoms are critically dependent on whether they are *1*) mid-chain or near to the C1-position carboxyl or terminal-CH_3 group (F_ω), and *2*) adjacent to one or two double bonds. Moreover, the configuration of the adjacent vinylic groups (i.e., *cis*- or *trans*-) also exerts a major influence on the allylic carbon atoms' chemical shift value. These structure-specific allylic resonances served to detect either EPA alone (C4 group signal at 26.65 ppm) or a combination of DHA and EPA (mid-chain Δ-CH_2-Δ group carbons in the 25.5–26.1 chemical shift range). Although three or more F_ω resonances were present in the spectra acquired on product I (~14.2 ppm), one of these is clearly attributable to the -$OCH_2\underline{C}H_3$ group of the ethyl esters of DHA, EPA, and other fatty acids, including those of the n-6 and n-9 classes (Fig. 2). The $F_{\omega-1}$ (Δ-1) resonances of DHA, EPA, and stearidonate [20:4(n-3)] (~20.7 ppm) are clearly distinguishable from those of all other fatty acids present. Contributions toward this signal from linolenoylglycerols and all other n-3 fatty acids present are not considered problematic, in view of their very low contents in marine oils, [e.g., 0.4–0.9 mol% for linolenate

TABLE 2. ^{13}C NMR spectroscopic data for the C1 (carboxylate) signals of products I and II

Resonance Code	δ (ppm)	Assignment
1	172.10	DHA *sn* - 2
2	172.48	DHA *sn* - 1(3)
3	172.57	EPA *sn* - 2
4	172.62	SDA *sn* - 2
5	172.78	LA, OA, SA[a] *sn* - 2
6	172.96	EPA *sn* - 1(3)
7	173.00	SDA *sn* - 1(3)
8	173.16	LA, OA, SA[a] *sn* - 1(3)
9	173.49	Fatty acid ethyl esters[b]
10	173.69	Unassigned[b]
11	173.74	Unassigned[b]
12	174.05	Unassigned[b]

OA, oleic acid; SA, saturated fatty acid; SDA, stearidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid.

[a] All Δ9 (LA, OA, etc.) and saturated fatty acids, and possibly also Δ8 [i.e., 20:4 (n-3) and 20:3 (n-6)] and Δ7 [22:5 (n-3) and 22:4 (n-6)].

[b] Detectable in product I spectra only.

TABLE 3. ^{13}C spectroscopic data for the vinylic (>C=C<) resonances of products I and II

Resonance Code	δ (ppm)	Assignment
1	127.03	C19-DHA
2	127.64	C5-DHA
3	127.84	C16-DHA
4	127.951 [*sn*-1(3)]	
	127.963 [*sn*-2]	C8-DHA
5	128.04	C11-DHA
6	128.06	C13-DHA
7	128.120	Unassigned
	128.145	Unassigned
8	128.23	C14-DHA
9	128.25	C10-DHA
10	128.29	C7-DHA
11	128.52	C17-DHA
12	128.74	C6-EPA
13	128.96	C5-EPA
14	129.41 [*sn*-1(3)]	
	129.46 [*sn* - 2]	C4-DHA
15	129.65	C9-Oleoyl
16	129.76	C12-Eicosenoyl
17	129.86	C11-Eicosenoyl
18	129.94	C10-Oleoyl
19	131.92	C20: all n-3 fatty acids (including DHA)
20	129.19	
	129.33	Fatty acid ethyl esters[a]

[a] Detectable in product I spectra only.

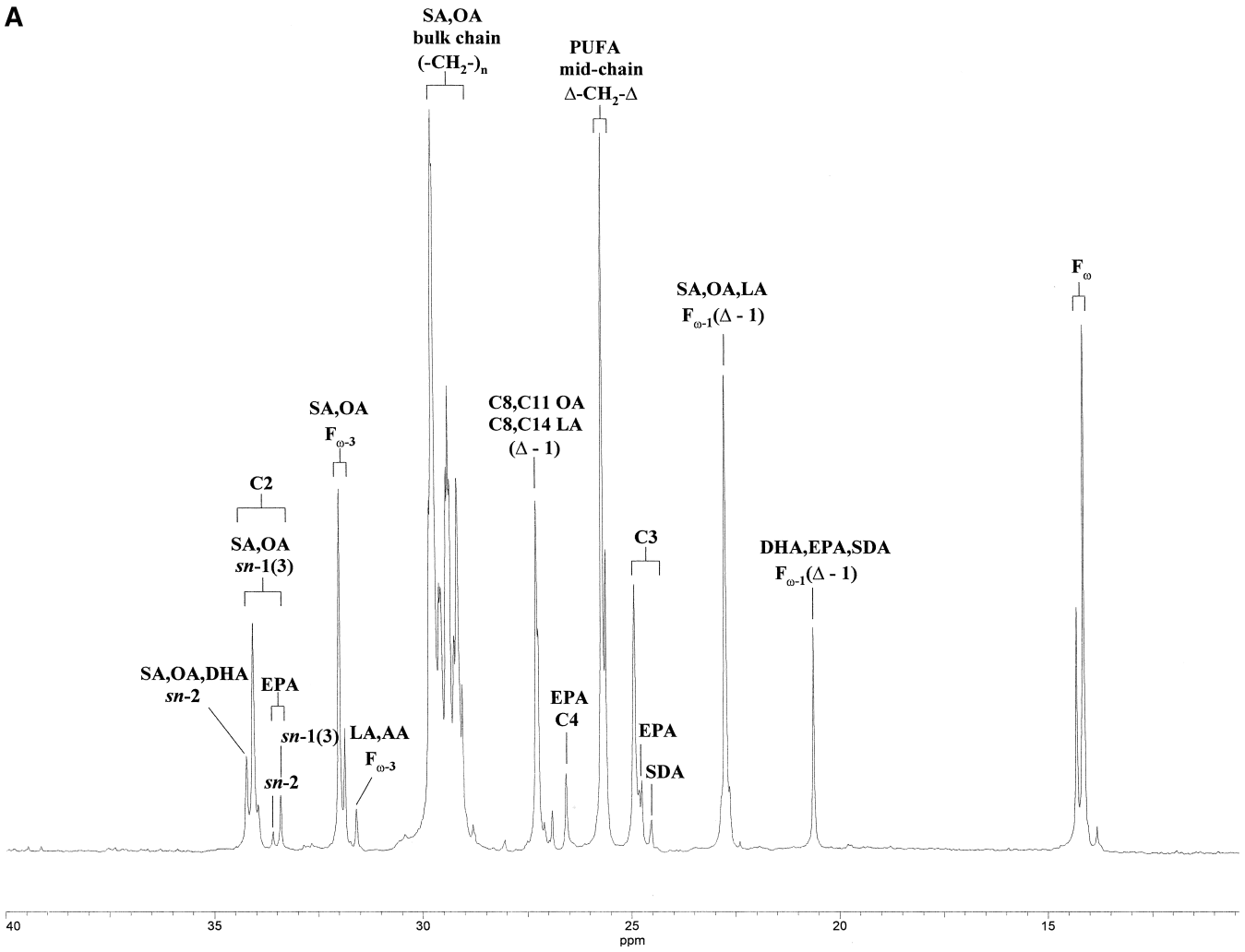


Fig. 11. 150.93 MHz ^{13}C NMR spectrum of product I ($CDCl_3$ solution). A: The 10–40 ppm region of the ^{13}C NMR spectra acquired on product I. B: Expanded 28.80–35.30 ppm region of the former spectrum. Typical spectra are shown. AA, arachadonic acid [20:4(n-6)]; *trans*-FA, *trans*-fatty acid; OA, oleic acid; SA, saturated fatty acid; SDA, stearidonic acid; EPA, eicosapentaenoic acid; LA, linoleic acid.

(28)]. For product I, the intensity of this signal was approximately two-thirds that of the $F_{\omega-1}$ resonances of all other fatty acids (at ~22.7 ppm), consistent with our ^{1}H NMR determination of the mol% of n-3 fatty acids in this supplement (~40%, Table 1).

Particularly useful as an index for the DHA and EPA contents of the products tested were their C2 (F_α) position signals at 33.87 [*sn*-1(3)-position for DHA] and 33.35 and 33.54 ppm [*sn*-1(3)- and -2 positions, respectively for EPA], also shown in Fig. 11A. Indeed, with the exception

of an unassigned signal at 33.74 ppm, these resonances were clearly upfield and isolated from those corresponding to all other fatty acids therein (>34.0 ppm for both *sn*-1,3- and -2-position glycerides).

The C3 position signals were able to distinguish EPA from stearidonic acid (Fig. 11A), and two further (unassigned) resonances were located at 24.73 and 24.85 ppm, in agreement with the ^{13}C NMR data of Gunstone and Seth (29).

Chemical shift values of and assignments for resonances present in the glyceryl carbon region of the ^{13}C spectra acquired are given in **Table 4**. Consistent with our ^{1}H NMR data (Fig. 6), ^{13}C NMR spectroscopy confirmed that these particular marine oil supplements contain several glyceridic ester species, in addition to the predominant triacylglycerols, specifically *sn*-1-monoacylglycerols, and *sn*-1,2- and *sn*-1,3-diacylglycerols. As expected, the intensities of the two resolved *sn*-1,2-diacylglycerol signals were equivalent, as were those of the three separate *sn*-1-monoacylglycerol resonances; the intensity of the *sn*-1,3-diacylglycerol glyceryl-CH_2 signal was twice that of its glyceryl-CH resonance. The *sn*-1-mono-, *sn*-1,2-di- and *sn*-1,3-diacylglycerol content of one of the marine oil supplements (product I) was found to be 41%, 11%, and 48%, respectively, of the total nontriacylglycerol glycerides, and 2.7%, 0.7%, and 3.2% of the total glycerides present.

Free fatty acids, which have ^{13}C resonances located in the 176–178 ppm chemical shift range and provide a quantitative index of free acidity in culinary oils (46), were undetectable in spectra obtained on both of the products tested here. However, in ^{13}C spectra acquired on product I (but not product II), low-intensity but clearly visible resonances in the 32.5–32.7 ppm region, which serves as an extremely useful and selective, but narrow, spectral "window" for the detection of *trans*-fatty acids (*trans*-allylic methylene group signals) (47), confirmed the presence of one or more of these agents (e.g., elaidate) in this supplement; i.e., two signals located at 32.59 and 32.67 ppm were observed (Fig. 11B). A further resonance located at 32.79 ppm may also arise from a *trans*-fatty acid adduct. However, partial *trans*-isomers of DHA and EPA, specifically those with the *trans*-configuration at selected >C=C< positions (i.e., the 17-*trans* fatty acid, and the 11- and 11-,17-*trans* methyl esters of EPA, and the 19-*trans* fatty acid of DHA), are documented to have *trans*-allylic -CH_2- group ^{13}C resonances located in the 30.30–30.74 ppm chemical shift range for the above EPA derivatives, and at 30.83 ppm for the DHA isomer when in the form of its free fatty acid (43).

In view of the large amounts of the ethyl esters of DHA and EPA detectable in product I (~65% of the total fatty acid content), its ^{13}C NMR spectrum contained intense resonances at 60.06 and 60.25 ppm assignable to the ethyl group $CH_3\underline{C}H_2OCOR$ atom of each species (together with that at 14.27 ppm for their terminal-$\underline{C}H_3$ group carbons).

The ^{13}C NMR spectroscopic technique also had the ability to detect the low-level (minor) components α-tocopherol acetate and free cholesterol in the cod liver oil products examined here (**Fig. 12**). Indeed, 17 out of a possible 26 free cholesterol resonances were detectable (i.e., those assignable to C3, C4/13, C5, C6, C9, C10, C11, C12, C14, C17, C18, C20, C21, C22, C23, and C24); the remainder overlapped with those of acylglycerols and, when present, ethyl esters of various fatty acids (including DHA and EPA). The spectra obtained also confirmed that little or no cholesterol ester species were present in either product. For α-tocopherol acetate, however, we found that all of the aromatic ring ^{13}C resonances were visible, as were those arising from the C2- heterocyclic ring and the C11′-chain position -CH_2- groups.

In view of its extremely low added content in both fish oil preparations, ^{13}C NMR analysis was unable to detect retinol (as retinol palmitate), unlike ^{1}H NMR spectroscopy, which offers a much greater sensitivity.

TABLE 4. ^{13}C chemical shift values (δ) of glycerol carbons in different classes of glycerides detectable in marine oil supplements

Resonance Code	δ (ppm)	Carbon Atom	Glyceride
1	61.32	-CH_2, *sn*-2	*sn*-1,2-Diacylglycerols
2	62.04	-CH_2, *sn*-1	*sn*-1,2-Diacylglycerols
		-CH_2, *sn*-1,3	Triacylglycerols
3	63.34	-CH_2, *sn*-3	*sn*-1-Monoacylglycerols
4	64.99	-CH_2, *sn*-1,3	*sn*-1,3-Diacylglycerols
5	65.15	-CH_2, *sn*-1	*sn*-1-Monoacylglycerols
6	68.14	-CH, *sn*-2	*sn*-1,3-Diacylglycerols
7	68.93	-CH, *sn*-2	Triacylglycerols
8	70.19	-CH, *sn*-2	*sn*-1-Monoacylglycerols
9	72.13	-CH, *sn*-2	*sn*-1,2-Diaclyglycerols

DISCUSSION

Application of both 1D ^{1}H NMR and 2D ^{1}H-^{1}H NMR spectroscopic techniques to the analysis of commercially available fish oil supplements provided a high level of data regarding the identification and, where appropriate, quantification of a wide range of key fatty acid components (as triacylglycerols or otherwise) in encapsulated marine oil supplements. Further components detectable included *sn*-1-monoacylglycerols, and *sn*-1,2- and *sn*-1,3-diacylglycerol adducts, together with a range of minor (low content) agents such as free glycerol and cholesterol, and vitamins E and A (as α-tocopherol acetate and all-*trans*-retinol palmitate, respectively). The above techniques were very useful for monitoring the DHA content of the products examined as $mg \cdot g^{-1}$, $mol \cdot kg^{-1}$, and mol% units (a consequence of its unique, clearly resolved F_{α},F_{β}-position ^{1}H resonances), together with the total n-3 fatty acid content expressed as a percentage of the total fatty acids present. Because the DHA content of marine oils serves as a valuable criterion for determining their quality (47), its rapid and nondestructive analysis by ^{1}H NMR spectroscopy clearly offers many advantages. Moreover, in 1992, the British Nutrition Foundation recommended that DHA and EPA (combined) should represent 0.5% of the total energy of an average population's dietary intake (48), and hence, specific and selective methods for their determination are required. EPAs as acylglycerol species were also distin-

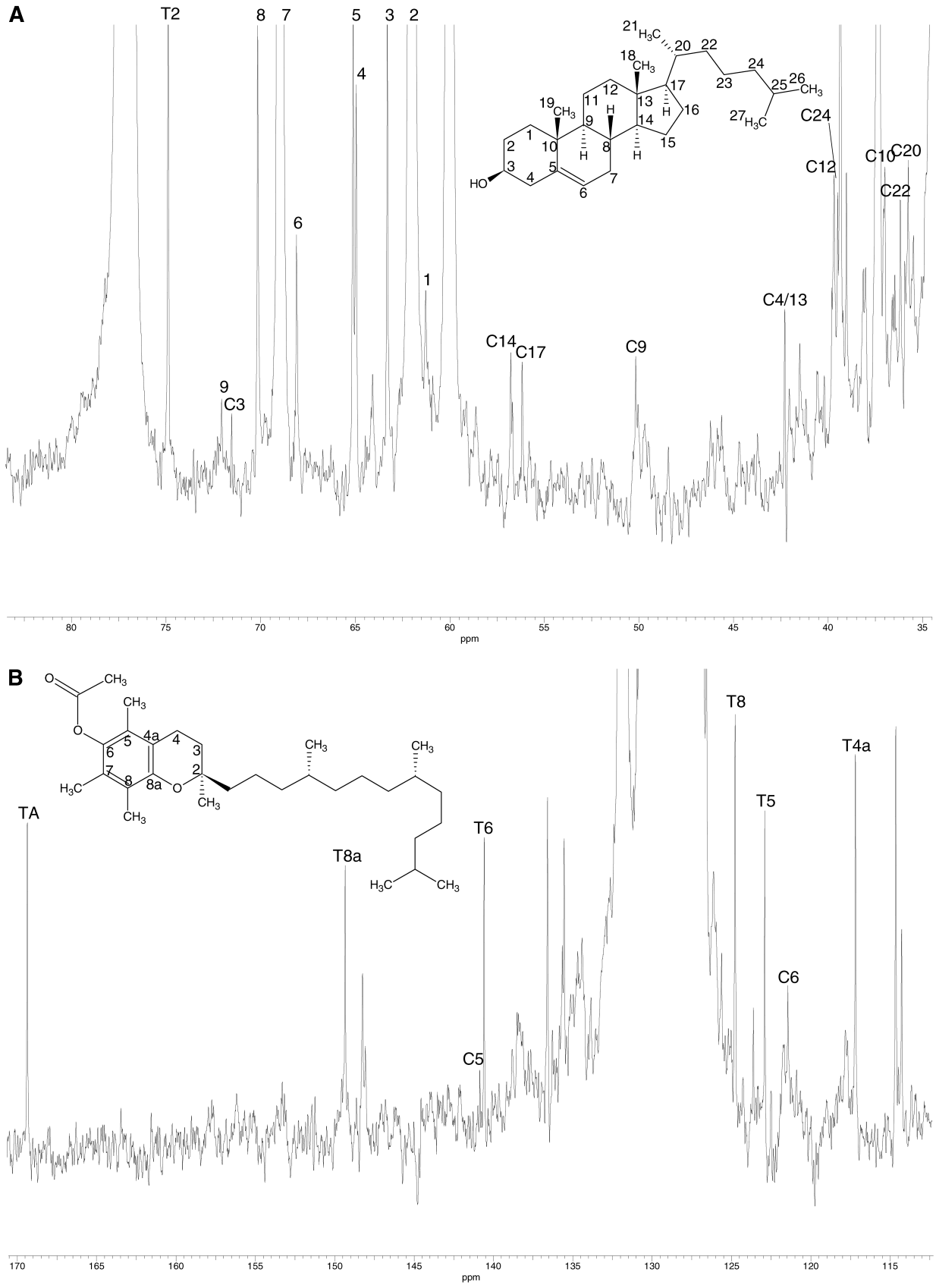


Fig. 12. Expansion of selected regions of the 150.93 MHz ^{13}C NMR spectrum of product I ($CDCl_3$ solution). A, B: Expanded 34.50–83.50 and 112.0–170.50 ppm regions, respectively, showing signals ascribable to low-level (minor) components. A typical spectrum is shown. C3–C24 and T2–T8a, selected cholesterol and α-tocopherol acetate resonances, respectively, detectable in the spectra acquired, and corresponding to the carbon nucleus numbering system in the structures depicted. TA represents the acetate group carboxylate resonance of α-tocopherol acetate; 1–9 represent resonances arising from glyceryl carbons in different classes of glycerides and correspond to those listed in Table 4.

guishable from other glycerol-bound fatty acids in the 1D ^{1}H NMR spectra acquired, and the 2D ^{1}H-^{1}H COSY technique facilitated its detection by virtue of strong connectivities between its F_{α},F_{β} and C4(Δ-1) signals, and also the very low content of potentially interfering fatty acids.

The 2D ^{1}H-^{1}H techniques employed here also facilitated the detection of synthetic ethyl esters of both DHA and EPA, together with lower levels of those of non-n-3 fatty acids, in one of the products tested (product I), a very important observation in view of the current demand for organic, unadulterated food products. Hence, high-field NMR spectroscopy serves as an extremely useful and rapid means of distinguishing natural sources of cod liver and other fish oils (i.e., DHA and EPA in acylglycerol form) from those that have been fortified with synthetic derivatives such as the ethyl esters identified here. It is understood that these ethyl esters are generated in or supplemented to selected encapsulated cod liver oil products as a means of overcoming adverse taste and aftertaste effects associated with the ingestion of these formulations. (However, the manufacturers of product I specify that their capsules contain "Cod liver oil enriched with fish oil Omega-3′".) Because a significant (albeit relatively small) fraction of these ethyl esters are non-n-3 fatty acids (Fig. 4), it appears that the manufacturers have subjected a cod liver oil preparation to a direct esterification process rather than conducting this step subsequent to separation and isolation of the individual fatty acids from the matrix.

Presumably, the manufacturers of product I have added the ethyl rather than methyl esters of these HUFAs, because hydrolysis of the latter in the gut or, alternatively, via the actions of esterase enzymes in vivo will liberate methanol, the toxicological properties of which are well known and extensively documented (49).

As observed here, d_4-MeOH proved to be an effective extraction medium for the ethyl esters of both n-3 and non-n-3 fatty acids present in product I that were acquired by ^{1}H NMR spectra, demonstrating that little or no triacylglycerols [as revealed by the low intensity of their glyceridic ^{1}H resonances, together with that of the bulk chain $(-CH_2-)_n$ groups, which partially overlaps that of the ethyl esters' $-C\underline{H}_2OCOR$ group in spectra of the untreated oil in C^2HCl_3 solution] were extractable from this product under these conditions.

In the areas of lipid chemistry and technology, a high level of valuable NMR applications are provided by ^{13}C NMR spectroscopy, a phenomenon generally attributable to the much wider chemical shift range of these nuclei. Indeed, resonances assignable to the highly variable carbon environments and also to the substitutional status on the glycerol backbone have much diagnostic value. Such chemical shift data have been extensively reviewed and tabulated by Gunstone and Seth (29, 43, 44) for a range of acylglycerol and free fatty acid species. As demonstrated here, the chemical shift values and relative intensities of methylenic, vinylic, and ester carboxyl resonances are suitable for the direct, rapid, and virtually noninvasive analysis of fatty acid components in commercially available fish oil supplements.

The ^{13}C NMR technique is particularly useful in distinguishing between mono-, di- and triacylglycerols, and also the particular substitutional isomers (e.g., *sn*-1,2- and *sn*-1,3-diacylglycerols) therein. Indeed, mono-, di- and triacylglycerols have characteristic chemical shift values for not only the three glycerol backbone carbons but also the C1 and C2 carbons in each acyl chain, and such data can, in theory, give rise to both quantitative and semiquantitative information concerning a whole series of these glycerol esters present in fish oil supplements such as those tested here.

Such information is likely to be of much value in distinguishing natural fish oils from those that have been subjected to industrial refining processes. Moreover, these data may also be valuable in determining the shelf life of fish oil products, because in virgin olive oils, *sn*-1,3-diacylglycerol levels increase after prolonged storage, a consequence of either lipolysis or 1,2/1,3-diacylglycerol isomerism (46). Similarly, information acquired regarding the positional distribution of individual fatty acids on the glycerol backbone of acylglycerols may also serve to provide a discriminatory index concerning the nature, refinement, and/or adulteration of these products.

Although free fatty acids (which provide a valuable measure of the acidity of culinary oil products) were not detectable in the preparations investigated here, NMR-detectable levels of *trans*-fatty acids were found in one of the fish oil supplements by virtue of a highly specific, albeit narrow, ^{13}C chemical shift "window." Consumption of these agents by the human population purportedly gives rise to a wide range of adverse health effects, e.g., the risk of coronary heart disease (50) and low birth weight (51). Interestingly, partially hydrogenated marine oils represent a major source of dietary *trans*-fatty acids in some countries (51).

Because fish oil supplements have been reported to elevate the concentrations of LDL cholesterol in some individuals (52), the presence of this agent in the products examined here could be considered a contributory factor, although the levels detected were low ($\sim$5 mg·g^{-1}).

With regard to LOPs, Igarashi et al. (28) found that the peroxide values of bonito, salmon, and tuna fish oils ranged from 0.6–10.6 meq·kg^{-1}, and therefore the ^{1}H NMR detection of conjugated diene signals tentatively assignable to one or more PUFA-derived hydroxydiene species is not unexpected.

A combination of high-resolution NMR data acquired on fish oils with multivariate statistical analysis techniques, such as principal component analysis, may have the ability to provide evidence for the source (i.e., species of fish) and possibly geographic origin of the species from which these products were collected.

Although it is generally accepted that DHA and EPA (together with further n-3 fatty acids), either individually or in concert, are responsible for the health benefits offered by marine oil supplements, these properties are also expected to be dependent on the precise molecular nature of these agents [e.g., as ethyl esters detectable in product I, as partial or triacylglycerols, and their substitu-

tional status (positional distribution) with respect to the glycerol backbone], their relative concentrations, and, indeed, the chemical nature and levels of all other components therein (e.g., vitamin A and E derivatives). The detection of relatively low concentrations of components that are known, or likely, to exert adverse effects on human health, such as LOPs (53), *trans*-fatty acids (50, 51), and, to a much lesser extent, cholesterol, is also an important factor for consideration. Therefore, we conclude that multicomponent monitoring of these products by 1D and/or 2D NMR spectroscopic techniques provides a wealth of molecular information that is likely to be of much value to those conducting clinical studies on the therapeutic actions putatively offered by these materials.

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