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2 # Analysis of marine dietary supplements using NMR spectroscopy

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8

9 **Abstract**

10 The objective of this study is the qualitative and quantitative analysis of encapsu-

11 lated fish oil supplements by utilizing high-resolution multinuclear (^{1}H, ^{13}C, ^{31}P) and

12 multidimensional NMR spectroscopy. By employing sophisticated 2D NMR experi-

13 ments, such as HSQC-TOCSY, band-selective HSQC and semi-selective constant-

14 time HMBC we performed a systematic two-dimensional analysis of the various

15 components in fish oil. The present analysis offered a solid proof and confirms earlier

16 assignments based on model compounds. Moreover, this study revealed the presence

17 of *n*-1 acyl chains and *trans* fatty acids in concentrations ranging from 1.9-2.9% and

18 3.7 – 5.2%. Application of ^{31}P NMR spectroscopy to the analysis of micro-

19 constituents in fish oil supplements allowed the fast and accurate determination of ad-

20 ditional minor compounds in it. Quantitative determination of the various compounds

21 in the supplements was achieved upon integration of the appropriate NMR signals in

22 the relevant spectra.

23

24 *Keywords*: Fish oil supplements, 2D NMR, ^{31}P NMR, *trans* fatty acids, *n*-1 fatty acids

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1. Introduction

The consumption of *n*-3 fatty acids in a diet characterized by a low *n*-6/*n*-3 balance has several positive effects for the human health and appears to be beneficial against many diseases, including heart disorders[1,2,3], inflammatory diseases[4] and diabetes[5]. Since the Western diet is relatively poor in *n*-3 fatty acids, consumption of fish oil supplements is recommended in order for consumers to increase their *n*-3 intake, and hence to improve the *n*-6/*n*-3 balance in their nutritional diet.[1] The consumption of dietary supplements in the United States is high, and it is continuously increasing. At least 50% of the population claims that they have used dietary supplements,[6] and the data for their sales in 2013 were over $24.6 billion, according to Euromonitor International. Yet questions still remain about the quality, efficacy, and safety of some of the commercial dietary supplements. To evaluate the impact of these products on the public health, it is appropriate to document and quantify dietary supplements intakes, and to do this, the composition of dietary supplements must be known.[7]

Because dietary supplements have matrices, excipients and other materials that are different from original foods, the appropriateness of chemical methods developed for foods cannot automatically be assumed *a priori*. For that reason the Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH), and the Food and Drug Administration (FDA) have partnered with the Association of Official Analytical Chemists (AOAC) to improve methods for dietary supplement analyses.[7] Accordingly, the AOAC Official Methods program (AOAC, 2003)[8] has validated various analytical methods for a number of bio-constituents, such as β-carotene,

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chondroitin sulfate, glucosamine, terpenes, vitamins, omega-3 (*n*-3) fatty acids, and many others.

In the last decade, Nuclear Magnetic Resonance (NMR) spectroscopy has emerged as a rapid, efficient and reliable analytical method for the screening of multi-component matrices, such as pharmaceutical products[9] foodstuffs[10, 11] sports nutrition and dietary supplements.[12, 13] NMR spectroscopy has several advantages; it is a robust and quasi-universal detector, has a non-destructive nature and allows minimal or no sample preparation. It is intrinsically quantitative, thus overcoming the need to determine response factors required by other techniques, such as HPLC, whereas it needs no internal standards, which may not be available in the market. The NMR tubes are deep in length and have extremely small cross-sectional area, which can be treated as a small reactor with less contact to air. Since the volume of NMR tubes is very limited, less amount of sample is required, which is important for samples not easily available in large quantities, and, in addition, it uses tiny amounts of organic solvents, thus relieving the environment from dangerous wastes. Moreover, this technique provides spectra, which are highly specific, and it has the ability to provide global information about the sample in a single analysis, and, in addition, it offers multiple calibration options.

In cases where the complexity of the sample is so severe, causing extensive signal overlap in one-dimensional (1D) spectra, the arsenal of NMR spectroscopy provides a large number of analytical techniques starting from homonuclear and heteronuclear multi-dimensional NMR to its hyphenation with effective separation techniques, such as liquid chromatography (LC-NMR). Customary two-dimensional (2D) NMR techniques, such as COSY, TOCSY, NOESY, HSQC, etc., based on the inherent “communication” of nuclei with each other (through spin-spin and/or dipolar

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coupling), spread out the spectroscopic information in two dimensions, unraveling hidden nuclear connectivities and thus facilitating the structural characterization of the molecules in food samples. Whenever these conventional 2D NMR specialties fail to resolve the signal assignment in overcrowded spectra, more sophisticated 2D NMR methods, such as semi-selective constant-time 2D sequences, may be used effectively. Although NMR spectroscopy has been classified as a relatively insensitive methodology compared to HPLC or MS, recent advances in instrumentation and technology, such as shielded and increasingly stronger high-field magnets, cryogenic or multi-coil probes, solvent suppression techniques, advanced data processing, versatile pulse sequences and polarization transfer techniques, have increased the sensitivity up to the nanomolar range. Other improvements include small OD tubes, microtubes and microcoil technology. The high equipment price, which may increase the cost of the analysis, is counterbalanced by the long-lived NMR spectrometers and the many applications it offers, so that it lowers the cost of the analysis in the long run.

In this study we performed a comprehensive quantitative NMR analysis of commercially available fish oil supplements using three different magnetic nuclei (^{1}H, ^{13}C, ^{31}P) and several sophisticated two dimensional NMR techniques such as HSQC-TOCSY, band-selective HSQC and semi-selective constant-time HMBC, and ^{1}H Diffusion Ordered Spectroscopy.

Material and Methods

2.1. Dietary supplements

Two series of dietary supplements, which according to the producer contain fish oil and vitamin D3 (S1 and S2), and two series of dietary supplements containing fish oil, but not vitamin D3 (S3 and S4), were purchased from U.S. supermarkets.

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101 **2.2. Chemicals**

102 Pinacol, phosphorus trichloride, $Cr(acac)_3$, pyridine, cyclohexanol (99%), 103 dimethylsulfoxide (99.9%), deuterated chloroform, mono-, di- and triolein standards, 104 cholesterol, vitamin D3 and 7-dehydrocholesterol were obtained from Sigma-Aldrich 105 (St Louis, MO, U.S.). The synthesis of the phosphorus reagent was slightly modified[14] 106 from that described in the literature[15] in order to increase the yield of the product.

107 **2.3. Sample preparation for NMR experiments**

108 50 mg of fish oil obtained from the dietary capsules was dissolved in 500 uL of 109 $CDCl_3$ (0.01% TMS) and inserted directly in the 5-mm NMR tubes for recording one- 110 and two-dimensional ^1H and ^{13}C NMR spectra. For ^{31}P NMR experiments, samples 111 were prepared as follows: a stock solution (10 mL) composed of pyridine and $CDCl_3$ 112 in 1.6:1.0 volume ratio containing 0.6 mg of chromium acetylacetonate, $Cr(acac)_3$ 113 (0.165 μM), and 13.5 mg of cyclohexanol (13.47 mM) was prepared and protected 114 from moisture with 5A molecular sieves. 150 mg of the fish oil samples was placed in 115 a 5-mm NMR tube. The required volume of the stock solution (0.6 mL) and the 116 phosphorus reagent 2-chloro-4, 4, 5, 5-tetramethyl-3, 5-dioxaphospholane **I** (50 μL) 117 were added. The reaction mixture was left to react for ~15 min at room temperature. 118 Upon completion of the reaction, the solution was used to obtain the ^{31}P NMR 119 spectra.

120 **2.4. NMR Experiments**

121 ^1H and ^{13}C NMR experiments were conducted on a Bruker Avance III 122 spectrometer operating at 850.23 MHz and 213.81 MHz for ^1H and ^{13}C nuclei, 123 respectively, whereas those of ^{31}P NMR were carried out on a Bruker Avance III 124 spectrometer operating at 202.21 MHz. All experiments were performed at 25 ± 0.1

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125 ºC and the spectra were processed by the Topspin software package provided by
126 Bruker Biospin.

127 **2.4.1 One-dimensional (1D) NMR spectra.**

128 ^{1}H-NMR spectra were recorded using the standard "zg" pulse sequence with the
129 following acquisition parameters: 16 scans and 4 dummy scans, 64K data points, 90º
130 pulse angle (10 μs), relaxation delay 8 s to ensure quantitative results, spectral width
131 10 ppm. A polynomial fourth-order function was applied for base-line correction in
132 order to achieve accurate quantitative measurements upon integration of signals of
133 interest. The spectra were acquired without spinning the NMR tube in order to avoid
134 artifacts, such as spinning side bands of the first or higher order. Chemical shifts are
135 reported in ppm from TMS (δ = 0).

136 ^{13}C-NMR spectra were obtained with proton decoupling, using an inverse gated
137 decoupling method (zgig) to minimize NOE effects, and repetition delays between
138 consecutive 90º pulses equal to five times the longitudinal relaxation times measured
139 by the null method. ^{13}C spectra were recorded with spectral widths of 200 ppm, using
140 64K data points, a 90º excitation pulse (13 μs), acquisition time 0.8 s and relaxation
141 delay of 30 s in order to avoid signal saturation. 256 scans were collected and spectra
142 zero-filled to 128K. For all FIDs, line broadening of 1 Hz was applied prior to Fourier
143 transform. Chemical shifts are reported in ppm from TMS (δ = 0).

144 Typical spectral parameters for quantitative ^{31}P NMR experiments were as
145 follows: 90° pulse width, 12.5 μs, sweep width of 12 kHz, relaxation delay, 25 s,
146 memory size, 32K (zero-filled to 64K). Line broadening of 1 Hz was applied, and
147 drift correction was performed prior to Fourier transform. To suppress NOE effects,
148 the inverse gated decoupling technique was used. Polynomial fourth-order baseline
149 correction was performed before integration. For each spectrum, 128 transients were

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acquired. All ^{31}P chemical shifts reported in this paper are relative to the product of the reaction of **I** with water (moisture contained in all samples), which gives a sharp signal in pyridine/$CDCl_3$ at δ 132.18.

2.4.2 Two-dimensional (2D) NMR experiments.

Experimental details and pertinent references for most of the 2D pulse sequences used in this study can be found elsewhere.[16]

Gradient selected ^1H-^1H Correlation Spectroscopy (H-H-gCOSY) experiments were performed in the magnitude mode using 8 dummy scans, 8 scans and 256 increments using the standard Bruker pulse sequence cosyetgp. Spectral widths of 10 ppm were used in both dimensions, 2K data points in the *F*2 dimension and a relaxation delay of 1.5 s. The spectra were zero-filled to a final size of 2K × 2K prior to Fourier transformation.

^1H-^1H DQF (Double Quantum Filtered) COSY experiments were performed using the cosydfetgp.2 pulse sequence with 8 dummy scans, 32 scans and 256 increments. Spectral widths of 10 ppm in both dimensions, 2K data points in the *F*2 dimension and a relaxation delay of 1.5 s were used. The spectra were zero-filled to a final size of 2K × 2K prior to Fourier transformation.

^1H–^1H Total Correlation Homonuclear Spectroscopy (H–H-TOCSY). These spectra were acquired in the phase-sensitive mode with TPPI, using the DISPI2 pulse sequence (dipsi2etgp) for spin lock. Typically, 16 dummy scans, 32 scans and 512 increments were collected, with SWs of 10 ppm in both dimensions, 2K data points in the *F*2 dimension, spin-lock time of 80 ms, and a relaxation delay of 2.0 s. The data points in the second dimension were increased to 2 K real data points by linear prediction, and the spectra were zero-filled to a final size of 4K × 4K prior to Fourier transformation. A sine-bell squared window function was used in both dimensions.

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175 The *Gradient selected ^{1}H-^{13}C heteronuclear multiple bond correlation (gHMBC)*
176 experiment was performed using the hmbcgplpndqf pulse sequence with a low-pass *J*-
177 filter (3.4 ms) and delays of 65 and 36 ms to observe long-range C–H couplings
178 optimized for 3 and 7 Hz with 256 increments and 86 transients of 2048 data points.
179 The relaxation delay was 2.0 s. Zero-filling to a 2K × 2K matrix and π/2-shifted sine
180 square bell multiplication was performed prior to Fourier transform.

181 *The hybrid HSQC-TOCSY* experiment consists of the initial basic gradient
182 enhanced HSQC sequence, followed by a phase-sensitive TOCSY transfer step with
183 the TPPI method using the DISPI2 pulse train for the spin lock. The standard Bruker
184 pulse sequence hsqcdietgpsisp.2 was used. The experiment was conducted with 1K ×
185 256 complex points and a spectral width of 10 ppm for ^{1}H and 180 ppm for ^{13}C. 16
186 transients were collected for each point with 16 dummy scans. The mixing time was
187 80 ms and the relaxation delay 1.5 s. The spectra were zero filled to 2K × 2K and
188 processed with Qsine-square bell in both dimensions.

189 The combined experiment *Gradient selected ^{1}H-^{13}C multiplicity-edited*
190 *heteronuclear single quantum coherence (HSQC-DEPT or edited-HSQC)* was
191 performed using the hsqcedetgp pulse sequence with 512 × 512 complex points and
192 spectral widths of 180 ppm for ^{13}C (*F*1) and 10 ppm for ^{1}H (*F*2), 512 increments, 16
193 dummy scans and 16 scans for each increment according to the echo-antiecho
194 procedure, relaxation delay of 2 s; delays 3.45 ms (1/2 *J*) for multiplicity selection,
195 and 1.725 ms (1/4 *J*) for sensitivity improvement were used. Carbon decoupling
196 during proton acquisition was achieved by applying the GARP pulse train. Gradient
197 strengths were 20 and 5 G/cm. The data were multiplied in the ^{1}H time domain with a
198 sine weighting function. The ^{13}C time domain was doubled by forward linear
199 prediction prior to a cosine window function.

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200 *Band-selective Heteronuclear Single Quantum Coherence (HSQC)* spectra[17,18]
201 were recorded with 128 increments in *F*1 and 16 scans per increment, using the
202 standard shsqcetgpsisp.2 Bruker pulse sequence. Relaxation delays of 2 s and 2K data
203 points were used for spectral widths of 7 ppm in the proton dimension, whereas the
204 spectral width in the carbon dimension varied from 3 to 10 ppm. A shaped pulse of 2
205 ms was used in the ^{13}C channel for refocusing. Folded signals were suppressed using
206 digital quadrature detection (DQD). Carbon decoupling during proton acquisition was
207 achieved by applying the GARP pulse train. The data were multiplied in the ^{1}H time
208 domain with a sine weighting function. The ^{13}C time domain was doubled by forward
209 linear prediction prior to a cosine window function.

210 *The band-selective constant-time Heteronuclear Multiple Bond Correlation*
211 *(HMBC)* experiments[18, 19] were acquired using the standard shmbcctetgpl2nd Bruker
212 pulse sequence with SWs of 7 ppm in ^{1}H dimension and 2-10 ppm in ^{13}C dimension,
213 with 2K × 128 data points. Selective excitation of ^{13}C was achieved using a 180º
214 Gaussian pulse. Pulsed field gradients were applied as half-sine shaped pulses. All
215 spectra were zero-filled to 4K × 512 data points for ^{1}H and ^{13}C dimensions,
216 respectively, and are presented in magnitude-mode.

217 *^{1}H Diffusion Ordered Spectroscopy (DOSY)* experiments were performed using
218 the STE bipolar gradient pulse pair (stebpgp1s) pulse sequence. 16 scans of 16 data
219 points were collected. The maximum gradient strength produced in the z direction
220 was 5.35 Gmm^{-1}. The duration of the magnetic field pulse gradients (δ) was optimized
221 for each diffusion time (Δ) in order to obtain a 2% residual signal with the maximum
222 gradient strength. The values of δ and Δ were 1.800 µs and 100 ms, respectively. The
223 pulse gradients were incremented from 2 to 95% of the maximum gradient strength in
224 a linear ramp. The temperature was set and controlled to 298 K with an air flow of

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225 670 l h^{-1} in order to avoid any temperature fluctuations due to sample heating during
226 the magnetic field pulse gradients.

227 **2. Results and discussion**

228 **3.1. Analysis of the NMR spectra**

229 **3.1.1 Fatty acids**

230 A commercial fish oil supplement is a mixture of several unsaturated (*n*-3, *n*-6, *n*-
231 9) and saturated fatty acids (SFA) in the form of triacylglycerols (TAG) and
232 diacylglycerols (DAG). The determination of the composition of these compounds,
233 and the evaluation of other important parameters, such as the positional distribution of
234 fatty acid chains on the glycerol skeleton and the determination of the *n*-6/*n*-3 ratio by
235 using NMR spectroscopy, rely on the correct assignment of the 1D ^{1}H and ^{13}C NMR
236 spectra. **Figures 1** and **2** illustrate the ^{1}H and ^{13}C NMR spectra of a commercially
237 available encapsulated fish oil supplement in $CDCl_3$ solution. Both spectra are
238 complex and have many additional signals compared to the spectra of edible oils of
239 plant origin, e.g. olive oil, sunflower oil. In contrast to these oils, which are dominated
240 by one or two types of fatty acids, fish oils contain a variety of different saturated and
241 unsaturated fatty acids in significant concentrations. Customarily, NMR assignment
242 of lipid constituents in fish oil is performed by using model compounds, in
243 combination with data from literature.[20,21,22] Also, T_1 relaxation times for the carbon
244 nuclei have been used for the NMR assignments.[20,21] Both methods are not considered
245 as fully reliable[23] due to the fact that the conformational behavior of model
246 compounds may be quite different relative to the counterparts in the complex food
247 matrix. It is known that chemical shifts and relaxation times are affected to some
248 extent by the conformational changes occurring especially in the saturated and
249 unsaturated fatty acid chains.[24] So far, there has been no systematic use of 2D NMR

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250 spectroscopy for the unambiguous assignment of the NMR chemical shifts of the lipid
251 content in fish oil supplements. This becomes crucial, when certain signals can be
252 used to obtain quantitative results and information about the positional distribution of
253 fatty acids on the glycerol moiety.

254 The total content of the unsaturated *n*-6, *n*-9, *n*-3 fatty acids, and the saturated
255 fatty acids (SFA) in fish oil could be determined from their terminal methyl protons in
256 the ^{1}H NMR spectrum of the supplement. Those of *n*-6, *n*-9 and SFA appear as
257 triplets in the region between δ 0.883 and δ 0.870 (**Figure 1**). On NMR spectrometers
258 operating at lower magnetic field strengths than the present instrument, the methyl
259 protons of *n*-6 fatty acid are usually overlapped by those of *n*-9 and the SFA. Their
260 discrimination could only be possible in commercial products containing *n*-6 fatty
261 acids at high concentration[25]. To unravel this ambiguity, ^{1}H NMR experiments were
262 conducted on an 850 MHz instrument. In the 850 MHz ^{1}H NMR spectrum, the methyl
263 protons of *n*-9 and the saturated fatty acids appear as a triplet at δ 0.880, whereas the
264 methyl protons of *n*-6 fatty acids resonate at δ 0.883 regardless of the low
265 concentration of *n*-6 acid (4% - 5%). To deal with the partial overlapping of these
266 triplets, a window function for resolution enhancement along with curve fitting was
267 applied resolving the two signals as shown in **Figure S1** (Figures and Tables denoted
268 by an S are provided as supporting material). *n*-3 fatty acids have distinct signal for
269 the terminal methyl protons in a ^{1}H NMR spectrum, which can be used for an easy
270 and fast determination of their total content in fish oil.[26] Because of the closer
271 proximity of these protons to olefinic carbons, they resonate at higher frequencies (δ
272 0.973) compared to *n*-6, *n*-9, and the saturated methyl protons. This is supported by
273 the corresponding cross peaks with the allylic protons at δ 2.072 in the COSY

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274 spectrum (**Figure S2A**), and the cross peaks with the bis-allylic protons at δ 2.812 and
275 the olefinic protons at δ 5.366 in TOCSY spectrum (**Figure S2B)**.

276 The COSY (**Figure S2A**) and TOCSY (**Figure S2B**) spectra are useful to assign
277 the unsaturated protons of the fatty acids. The allylic protons of Eicosapentaenoic acid
278 (EPA), which are close to the α and β carbonyl methylene protons (H_α and H_β), appear
279 at δ 2.109 and they have cross peaks in the TOCSY spectrum with the H_α and H_β
280 protons of EPA at δ 2.327 and 1.697 respectively. The allylic protons of all *n*-3 acyl
281 chains at the ω-2 position (next to the terminal methyl group) resonate at δ 2.072, and
282 they have cross peak in the COSY spectrum with the methyl protons at the ω-1
283 position. The allylic protons of monounsaturated *n*-9 fatty acids, mostly oleic acid
284 (OL), appear at δ 2.001, whereas the allylic protons of *n*-6 polyunsaturated fatty acids,
285 mostly linoleic (LO) and arachidonic (AA) acids resonate at δ 2.027 and have cross
286 peak in the TOCSY spectrum (**Figure S2B**) with the bis-allylic protons of *n*-6 fatty
287 acids, which resonate at δ 2.773. The bis-allylic protons of *n*-3 fatty acids form two
288 envelops at δ 2.845 and δ 2.810. The signal at δ 2.826 belongs to the bis-allylic of
289 Docosahexaenoic acid (DHA) (*n*-3), since it has a cross peak in the TOCSY with the
290 triplet at δ 2.391, which belongs to both H_α and H_β methylene protons of DHA. The
291 proton NMR chemical shifts of the present analysis for DHA and EPA as confirmed
292 by 2D NMR experiments are in agreement with those reported previously[20,22] and they
293 are summarized in **Table 1**.

294 Next we consider the assignment of the ^{13}C NMR spectra of the dietary
295 supplements. This spectrum (**Figure 2**) is more informative compared to its ^1H NMR
296 counterpart. This is because of the increased resolution offered by the 850-MHz
297 spectrometer, though some signal overlapping still remains. The assignment of the
298 carbonyl carbons of *n*-3, *n*-6, *n*-9 and saturated fatty acids has been achieved by

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299 performing the band-selective constant-time HMBC experiment. This experiment is
300 more informative than the conventional *g*HMBC pulse sequence, since it provides
301 enhanced resolution in the F_1 indirect dimension, and thus confirms the positional
302 distribution of the *n*-3 fatty acids on the glycerol backbone. To the best of our
303 knowledge, this is the first time that this type of 2D NMR experiment is used in lipid
304 analysis, and the present results support the conclusion that this experiment can be
305 used as a fast screening tool for fish oil analysis. As a matter of fact, **Figure 3**
306 compares the normal, low-resolution *g*HMBC and the semi-selective constant-time
307 HMBC spectra in the carbonyl carbon region of fish oil. As can be seen, the normal
308 *g*HMBC spectrum (**Figure 3A**) is characterized by severe signal overlapping within
309 spectral window of less than 2 ppm, and hence it is unable to resolve any correlation
310 between carbonyl carbons and relevant protons two and three bonds away. On the
311 other hand, the band-selective constant-time HMBC spectrum (**Figure 3B**) of the
312 same fish oil sample, optimized for three-bond C–H coupling and recorded over a 2-
313 ppm spectral window, shows well-resolved cross peaks between the carboxyl carbons
314 of various fatty acids with the corresponding glyceridic protons at *sn*-1/*sn*-3, *sn*-2
315 positions (*sn* refers to Stereospecific Numbering). This finding is of specific
316 importance, because the carbonyl region is the most informative of the whole ^{13}C
317 NMR spectrum, and it can be used for the quantification of the different species, as
318 well as for the determination of the positional distribution of fatty acids on the
319 glycerol moiety. It is worth mentioning that the carbonyl region is the only NMR
320 pattern that can be used for the quantification of *n*-3 docosapentaenoic acid (DPA),
321 which is an elongated metabolite of EPA and an intermediary product between EPA
322 and DHA.[27] DPA shares most of the health benefits of EPA and DHA. Lately, it was
323 suggested that it may have additional health benefits, which are worthy of further

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324 research.[27] **Table 2** summarizes the chemical shifts of the carbonyl carbons of the
325 triacylglycerols of the fish oil supplement. These values are in agreement with those
326 reported in earlier studies.[28]

327 The carbonyl signals can be used to verify earlier assignments for the H_α and H_β
328 protons through the selective HMBC experiment, whereas carbons C2 and C3 of
329 DHA can be assigned using the regular *g*HMBC spectrum. The corresponding
330 spectrum in **Figure S3** shows relevant cross-peaks between protons at δ 2.391 and
331 carbons C2 and C3 of DHA at δ 34.02 and δ 22.60 respectively. The H_α methylene
332 protons of EPA resonate at δ 2.327 and have a cross peak in the selective HMBC
333 spectrum with the carbonyl carbon of EPA at δ 173.03 (*sn*-1,3) and 172.63 (*sn*-2),
334 whereas the H_β of EPA appear at δ 1.697, have a cross peak in COSY with H_α and
335 partially overlapped with the H_β methylene protons of *n*-6, *n*-9 unsaturated and
336 saturated fatty acids.

337 The use of selective HMBC and HSQC experiments, as well as the HSQC-
338 TOCSY experiment, provide valuable information about the olefinic and aliphatic
339 carbons. The HSQC - TOCSY spectrum in **Figure 4** shows connectivities between the
340 *J*-coupled protons in a spin network and each carbon involved in this network. For
341 example, the assignment of allylic and bis-allylic carbons of *n*-6 fatty acids at δ 27.05
342 and at δ 25.52, respectively, as well as the assignment of carbons C2, C3, C4, C5, C6
343 and C7 of EPA can be achieved using HSQC-TOCSY and selective HMBC. The
344 allylic protons of EPA, which are close to the H_α and H_β protons have a cross peak in
345 the selective HSQC spectrum with carbon C4 of EPA at δ 26.49 and in the selective
346 HMBC spectrum with carbons C5 and C6 at δ 128.94 and δ 128.76, respectively. In
347 addition, the bis-allylic protons of EPA at δ 2.807 have cross peak in the selective
348 HSQC spectrum with carbon C7 of EPA at δ 25.61, whereas the H_α and H_β protons of

EPA have cross peaks in the selective HSQC and HSQC-TOCSY with carbons C2 and C3 at δ 33.48 and 24.60, respectively. The combination of selective HSQC and HMBC experiments also confirmed the signals of carbons C6 and C7 of DHA at δ 25.60 and δ 128.08, respectively, which have cross peaks in these spectra with bis-allylic protons of DHA at δ 2.845. Another characteristic example of the effectiveness of the selective HMBC spectra in the NMR assignment of fish oil is for the clarification of the chemicals shifts of carbons C4 and C5 of DHA. There are controversial data about the chemical shifts of these carbons in literature.[20, 22] Examination of the selective HMBC spectrum provided the unambiguous assignment of carbons C4 at *sn*-1,3 positions and *sn*-2 position at δ 127.50 and δ 127.53 respectively, and of carbons C5 at *sn*-1,3 positions and sn-2 position at δ 129.54 and δ 129.47, respectively. These data are in agreement with data published by Aursand and Gradsdalen.[20]

Confirmation of the previous assignments and the establishment of new ones were achieved by conducting quantitative ^{13}C NMR experiments. By employing the inverse gated decoupling technique in order to suppress NOE effects on carbon signal intensities, and repetition time five times the longest T_1 values (the longest T_1 was found to be 6.32 s for carbonyl carbons) to avoid signal saturation, the signal integrals are representative measures of the concentration of the various lipid constituents in fish oils. The integrals can be further used to explore which signals belong to the same or different molecules. This method is a routine technique for ^{1}H NMR assignment, but it is rarely used in ^{13}C NMR because of the long duration of the experiment. Using the quantitative integrals of the carbonyl signals as a starting point, we were able to perform an extensive assignment in the olefinic and aliphatic carbons, validating thus the assignment *via* 2D experiments. This quantitative analysis allows the correct

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374 assignment of signals that may be overlapped in the F_1 dimension of the 2D spectra
375 such as the C3 carbons of Stearidonic acid (SDA) at *sn*-1,3 and *sn*-2 positions at δ
376 24.44 and δ 24.48, respectively. Combining the quantitative data with the HSQC-
377 TOCSY experiment we were able to assign carbons C4, C5 and C2 of SDA at δ
378 26.76, 28.93 and 33.84 which have cross peaks with the methylene protons of SDA at
379 δ 1.399. This observation is further supported by the fact that the signal at δ 1.399 has
380 a cross peak in COSY with H_β protons and thus it is concluded that this signal belongs
381 to the protons attached to the internal methylene carbons (C3 and C4) of SDA. The
382 quantitative ^{13}C experiment in combination with curve fitting also revealed for first
383 time that carbon C4 of EPA at sn-1,3 positions and carbon C4 of EPA at *sn*-2 position
384 have distinct NMR signals at δ 26.49 and δ 26.47, respectively. As it will be shown
385 below, the integral values will be used to quantify the fatty acid content in the fish oil.
386 The strength of quantitative ^{13}C NMR as an identification tool is shown in **Table S1**,
387 which displays the integrals of the ^{13}C signals uniquely attributed to EPA. As can be
388 seen, the integral values for both protonated and non-protonated (carbonyl) carbons
389 are the same within the experimental error, which means that signal saturation and
390 NOE effects are kept minimal. Another piece of evidence that corroborates the
391 accuracy of the quantitative ^{13}C NMR analysis is that the sum of the signal integrals at
392 and δ 131.85 and δ 131.92 which belong to the ω-3 carbon of linolenic acid and to the
393 ω-3 carbons of the remaining *n*-3 fatty acids, respectively, equals the sum of the
394 signal integrals of the carbonyl carbons of the respective *n*-3 fatty acids.

395 **3.1.2 *Trans* fatty acids**

396 ^{1}H NMR experiments conducted on the 850 MHz spectrometer reveals for the first
397 time the presence of a small triplet at δ 0.911, which belongs to the terminal methyl
398 protons of *trans* fatty acids. This assignment is supported by the fact that this triplet

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399 has a cross-peak in the semi-selective HSQC spectrum with the carbon signal at δ
400 13.82, which is characteristic for the methyl carbon of *trans* fatty acids[29] as shown in
401 **Figure 5**. It should be noted that the semi-selective HSQC experiment is much more
402 effective in terms of resolution compared to the conventional *g*HSQC, and offers
403 resolution comparable to that in the 1D ^{13}C spectrum. The signal at δ 13.82 in the ^{13}C
404 spectrum can be used as an extremely useful and rapid tool for the detection and
405 quantification of *trans* fatty acids in fish oils. The triplet at δ 0.911 in the proton
406 spectrum is equally useful for the quantification of *trans* fatty acids, although
407 instruments operating at Larmor frequencies higher that 600 MHz are required to
408 obtain adequate resolution.

409 Because of the damaging effect of high doses of *trans* fatty acids on human's
410 health,[30] further attempts were made in the present study to assign the type of the
411 *trans* *n*-3, *n*-6 or *n*-9 fatty acids. This requires a closer scrutiny of the COSY (**Figure**
412 **S2A**) and TOCSY (**Figure. S2B**) spectra to assign the pertinent proton signals that
413 can be used for identification and quantification of the various *trans* fatty acids. The
414 triplet at δ 0.911 doesn't have a cross peak with the allylic protons in the COSY
415 spectrum, and hence this triplet does not belong to *n*-3 *trans* fatty acid. This finding is
416 in contrast to other studies which indicated the presence of *n*-3 *trans* fatty acids in fish
417 oil.[31, 32] In addition, the same triplet has a cross peak with the allylic protons in the
418 TOCSY spectrum, which means that it does not belong to *n*-9 *trans* fatty acids either.
419 On the other hand, these *trans* FA are not in the form *n*-6 FA either, because in
420 contrast to the methyl groups of *n*-6 (and *n*-9) fatty acids which don't have cross peak
421 with olefinic carbons in the HSQC-TOCSY spectrum, the methyl group at δ 0.911 has
422 cross peaks with olefinic carbons at δ 129.93 and δ 127.98 in this spectrum. These
423 signals may belong to olefinic carbons Cω4 and Cω5 of fatty acids. The triplet at δ

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424 0.911 has cross peaks with the multiplet at δ 1.382, which in turn has cross peaks with
425 the allylic protons δ 2.069 and olefinic protons at δ 5.353 in the COSY and TOCSY
426 spectra. Inspection of the selective HSQC (**Figure 6**) and HSQC-TOCSY (**Figure 4**)
427 spectra reveals connectivities between the proton signals at δ 1.382 with carbon at ω-2
428 position at δ 22.74. These ^{1}H and ^{13}C spectroscopic arguments corroborate with the
429 presence of *n*-4 *trans* fatty acids in the fish oil supplements. However we were not
430 able to observe a distinct signal in the carbonyl carbon area for the *trans* fatty acids
431 and thus further investigation about their chemical structure is required.

432 **3.1.3 *n*-1 acyl chains**

433 Small but considerable amounts of *n*-1 acyl chains were found in fish oil
434 supplements. This finding is in agreement with a recent study[32] which revealed the
435 presence of *n*-1 acyl chains in fish oil supplements using gas chromatography (GC).
436 The presence of *n*-1 acyl chains in trout extracts has been also recently reported.[29] The
437 following observations in the NMR spectra consolidate the presence of the *n*-1 acyl
438 chains. The terminal olefinic protons of the *n*-1 acyl chains form an AB system at δ
439 4.989 and δ 5.052 (**Fig 1**) and have cross peak in the COSY spectrum with proton at
440 ω-2 position at δ 5.818. The integral ratio between these protons is 2:1, as expected.
441 Also, the signal at δ 5.052 is in *trans* configuration with the neighboring ω-2 proton
442 since it has the characteristic *trans* J coupling of 17 Hz, whereas the olefinic ω-1
443 proton at δ 4.989 is in *cis* configuration with the ω-2 proton at δ 5.818 since it has a
444 characteristic *cis* J coupling of 10 Hz with it. The HSQC-DEPT spectrum (**Figure S4**)
445 gives further supports for the presence of the *n*-1 acyl chains. Methyl carbons in this
446 spectrum appear as positive signals, whereas methylene and methyl carbons emerge
447 as negative signals. The negative carbon signal of the terminal ω-1 olefinic carbon at
448 δ 114.71 shows one-bond correlation with ω-1 protons at δ 4.989 and δ 5.052

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3 449 confirming the existence of methylene olefinic protons, and the positive carbon signal
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5 450 at ω-2 position at δ 136.80 shows a one-bond correlation with the *n*-1 protons at δ
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7 451 5.818. The appearance of conjugation in the *n*-1 chains is demonstrated by the fact
8
9 452 that the olefinic protons at ω-1 and ω-2 positions show cross peaks in TOCSY and
10
11 453 COSY spectra with the bis-allylic protons at ω-4 position which appear at δ 2.828 and
12
13 454 the olefinic protons at ω-4 position which appear δ 5.436. Apart from signals at δ
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15 455 136.80 and δ 114.71, the distinct signal at δ 31.40, which belongs to the bis-allylic
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17 456 carbon C3, as found from the HSQC-TOCSY spectrum, can be used as an index for
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19 457 the quantification of the *n*-1 acyl chains. This is fortunate because the bis-allylic
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21 458 protons at position ω-3 of *n*-1 acyl chains, which appear at δ and 2.828 overlap with
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23 459 those of *n*-3 fatty acids and cannot be used for quantification purposes.
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25 460 By means of diffusion ^{1}H NMR experiments, we were able to derive some
26
27 461 qualitative information about the degree of the glycerol esterification by the *n*-acyl
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29 462 and *trans* acyl chains. The diffusion coefficient corresponding to signals at δ 4.146
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31 463 and/or δ 4.297 which belong to all TAG molecules (approximate average MW of 850)
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33 464 in the sample, was measured to be 2.48 10^{-10} m^2 s^{-1}. This value is comparable to the
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35 465 diffusion coefficient of the signal at δ 4.989 and/or the signal at δ 5.052 (2.54 10^{-10} m^2
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37 466 s^{-1}), which belong to the olefinic protons of the *n*-1 acyl chain at ω-1 position, and
38
39 467 similar to the diffusion coefficients of the *n*-3 fatty chains (2.49 10^{-10} m^2 s^{-1}) and *trans*
40
41 468 fatty chains (2.39 10^{-10} m^2 s^{-1}). The lighter cholesterol molecule (MW 386) has a
42
43 469 faster diffusion coefficient of 3.22 10^{-10} m^2 s^{-1} (calculated from the signal at δ 0.677).
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45 470 These data indicates that the *n*-1 acyl chains as well as *trans* fatty acids presumably
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47 471 exist in the form of TAG.
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49 472 **3.1.4 Other minor compounds**
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473 ^{31}P NMR Spectroscopy will be used for the determination of the remaining micro-
474 constituents in fish oil supplements. **Figure 7** compares the ^{31}P NMR spectra of two
475 phosphitylated samples of marine supplements with and without added vitamin D3.
476 Both spectra show the signals of the phosphitylated mono- and diacylglycerols.[14] The
477 signals at δ 147.42 and at δ 146.33 are attributed to the primary and secondary
478 phosphitylated hydroxyl groups of free glycerol, respectively.[33] The signals at δ
479 139.08 and δ 138.28 belong to the biphenols hydroxytyrosol and tyrosol, respectively,
480 whereas vitamin E (tocopherol) resonate at δ 144.39. The signal at low frequencies (δ
481 134.73) represents the phosphitylated carboxyl groups of all free fatty acids in the
482 supplements[14] and can be used for the determination of the free acidity in fish oils.
483 The signal at δ 145.20 is due to the internal standard cyclohexanol used for
484 quantitative analysis. The signal at δ 144.92, which belongs to cholesterol, has an
485 increased intensity in the supplement containing vitamin D3. It is tempted to assume
486 that the phosphitylated hydroxyl group of vitamin D3 resonates at the same frequency
487 with that of cholesterol. Nevertheless, addition of commercial vitamin D3 to the
488 phosphitylated sample results in a new signal at δ 145.31. This finding indicates that
489 the producer of the supplements does not add pure vitamin D3, but probably its
490 precursor 7-dehydrocholesterol. This compound is transformed into vitamin D3 in the
491 human body, and its phosphitylated hydroxyl group has the same chemical shift with
492 that of cholesterol. Addition of commercial pre-vitamin in the supplement with no
493 vitamin resulted in a signal that overlapped the signal of cholesterol at δ 144.92
494 supporting our conclusion. It should be noted that 1,2-DAG and 1,3 DAG can be also
495 quantified in fish oil from their characteristic peaks in the ^{1}H NMR spectrum at δ
496 3.728, 4.238 and 4.329 and at δ 4.084, 4.171 and 5.085 respectively. By using an 850
497 MHz instrument, even 1-monoglycerides, (1-MAG) can be quantified from their H3'

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498 signal at δ 3.593. Cholesterol can be also determined from its signal at δ 0.677 which
499 belongs to methyl carbon C18. Further, 7-dehydrocholesterol can be detected from its
500 signal at δ 0.679 after the application of a window function for resolution
501 enhancement. The ^{13}C spectra can be used only for the determination of 1,3 DGs. The
502 signal to noise ratio of the signals at δ 61.98 of C1', of 1,2-DG at δ 65.00, of C3' of 1-
503 MG and the signal at δ 13.851 of C18 of cholesterol (as found from the selective
504 HSQC experiment) is very low and cannot provide reliable quantitative results.

505 **3.2. Quantitative Analysis of marine Supplement**

506 Both proton and carbon NMR spectra can be used for the quantitative
507 determination of the major constituents of fish oil supplements. The signals that can
508 be used for that purpose are denoted by asterisks in **Tables 1** and **2**. The triplet at δ
509 0.974 can be used for the determination of the total content of the *n*-3 fatty acids
510 according to the relationship $C_{n\text{-}3} = I/S$, where I is the integral of the methyl protons of
511 *n*-3 and S is the sum of integrals of the methyl protons of SFA, *n*-6, *n*-9, *n*-3, *trans*
512 fatty acids, and the normalized integral (multiplied by 3/2) of the terminal protons of
513 *n*-1 acyl chains. Nevertheless, this triplet cannot be used for the determination of
514 DHA, EPA and the other individual fatty acids. The concentration of DHA can be
515 calculated from the intensity K of the triplet at δ 2.391 according to the relationship
516 $C_{DHA} = ¾\ K/S$. The quantitative determination of EPA is determined using the
517 equation $C_{EPA} = 3/2\ G/S$, where G is the integral of the signal at δ 1.697. The content
518 of the remaining *n*-3 fatty acids can be determined by subtracting of EPA and DHA
519 values from the total *n*-3 content. *n*-6 fatty acids are determined from the relationship
520 $C_{n\text{-}6} = 3/2\ I_{n\text{-}6} / S$, where $I_{n\text{-}6}$ is integral of the bis-allylic protons at δ 2.773. *n*-9 fatty
521 acids (mainly oleic acid) can be quantified according to the equation $C_{n\text{-}9} = (3/4\ I_{n\text{-}9}$
522 where $I_{n\text{-}9}$ is the integral of the allylic protons of *n*-9 at δ 2.010. However, in

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3 523 instruments operating in Larmor frequencies lower than 800 MHz, the equation $C_{n\text{-}9}$ =
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5 524 $(3/4\ Q - 3/2\ I_{n\text{-}6})\ /\ S$, should be used. Q is the integral of the allylic protons of n-6 and
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7 525 n-9 at δ 2.010 which partially overlap. n-1 acyl chains can be determined using the
8
9 526 relationship $C_{n\text{-}1} = 3I_{n\text{-}1}$ / S, where $I_{n\text{-}1}$ is the integral of the signal at δ 5.818, whereas
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11 527 trans fatty acids can be calculated from the equation $C_{trans} = I_{trans}$ / S, where I_{trans} is the
12
13 528 integral of the signal at δ 0.911. The concentration of saturated fatty acids (SFA) can
14
15 529 be calculated from the equation $C_{SFA} = S - C_{n-3} - C_{n-6} - C_{n-9} - C_{n-1} - C_{trans}$.
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17 530 The nutritional index $C_{n\text{-}6}/C_{n\text{-}3}$ appears to be an important index and is critical for
18
19 531 the human health. Deviation of this ratio from a critical value may induce several
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21 532 diseases.[1] Unfortunately, this index is lower than it should be in western diets because
22
23 533 of their poor content in n-3 fatty acids. More specifically, it is suggested that an ideal
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25 534 $C_{n\text{-}6}/C_{n\text{-}3}$ ratio should be around unity, or lower, whereas in western diets the ratio is
26
27 535 about 15.[1, 34] This nutritional index can be easily calculated from the ratio of the
28
29 536 normalized intensities of the triplet at δ 2.773 (n-6 fatty acids) over the triplet at δ
30
31 537 0.973 (n-3 fatty acids) from $C_{n\text{-}6}/C_{n\text{-}3} = 3/2\ I_A/I_B$, where I_A and I_B are the integrals of
32
33 538 the signals at δ 2.773 and δ 0.973, respectively.
34
35 539 Fatty acids concentration can be obtained using ^{31}C NMR spectroscopy as well
36
37 540 upon integration of the appropriate aliphatic, olefinic and carbonyl signals (denoted
38
39 541 by asterisks in **Table 2**). As noted before, the carbonyl signals can be used for the
40
41 542 determination of the acyl chain positional distribution on the glycerol skeleton. **Table**
42
43 543 **3** contains the quantitative results for the fatty composition of four samples, of
44
45 544 encapsulated fish oil as determined by ^{1}H and ^{13}C NMR. These values are the average
46
47 545 of three measurements for each sample and represent relative (%) concentrations for
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49 546 fatty acids. By knowing the exact amount of the sample in the NMR tube the relative
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547 concentration can be easily converted to absolute ones with a very good accuracy,
548 since these components consist of >99% of fish oil total concentration.

549 In general, the results obtained by ^{1}H and ^{13}C NMR agree within the experimental
550 error ($R^2 = 0.994$) and are within the concentration range expected for fish oil. A few
551 differences may arise from the fact that the NMR signals of SFA, *n*-6 and *n*-9 fatty
552 acids, overlap with the signal of the methyl groups of other compounds that appear in
553 fish oil, other than fatty acids, such as cholesterol, tocopherols etc.

554 The regio-specific analysis indicated the preference of DHA to the *sn*-2 position,
555 whereas EPA tended to be associated with the *sn*-1,3 positions. Concentration of
556 monounsaturated *n*-9 fatty acids are higher in *sn*-1,3 positions, whereas SFA seem to
557 be randomly distributed on glycerol skeleton. DPA is likely to have a preference for
558 *sn*-2 position, whereas Stearidonic (SDA) and Eicosatetraenoic acids (ETA) acids
559 have a preference for *sn*-1,3 positions. Recent studies[35] have shown that the variation
560 of the positional distribution of fatty acids depends on the origin of the fish oil. In this
561 respect, the present analysis can be a very effective tool for the authentication of fish
562 oil supplements as well as the detection of the adulteration.

563 The use of known quantity of the internal standard allowed the quantification of
564 all minor compounds found in the present supplements using ^{31}P NMR spectroscopy.
565 The quantitative data is summarized in **Table 4**, whereas they are compared with the
566 corresponding ^{1}H and ^{13}C data for minor compounds in **Table 5**.

567 **4.0. Conclusions**

568 ^{1}H, ^{13}C and ^{31}P NMR spectroscopy was proved to be a powerful tool for
569 qualitative and quantitative determination of the composition of fish oil supplements.
570 The quantitative analysis was achieved by the integration of the appropriate NMR
571 signals after the secure assignment of the corresponding signals by using extensively

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2D pulse sequences, such as HSQC-TOCSY, band-selective constant-time HMBC, band-selective HSQC, etc. Our results indicate that the supplements consist of the major compounds triacylglycerols (TAG) of *n*-3, *n*-6, *n*-9 fatty acids, and *n*-1 acyl chains. In addition, they contain approximately 4-5 % of *trans* fatty acids. Monoacylglycerol (MAG), diacylglycerol (DAG), free glycerol and 7-Dehydrocholesterol (precursor of vitamin D3) were the dominant minor species in the supplement as detected by ^{31}P NMR spectroscopy. ^{31}P NMR is the best method for determining the partially esterified glycerol species, as well as other minor compounds bearing free hydroxyl groups such as 7-dehydrocholesterol despite the fact that the phosphitylation reaction destroys the sample and lengthens the duration of the analysis. On the other hand, ^{1}H NMR is much more rapid compared to other methods as the spectrum can be recorded in less than one minute, in contrast to ^{13}C NMR analysis, which lasts 30 min and ^{31}P which lasts 25 min. However, the carbon NMR spectrum is much more informative and it can provide quantitative data for more individual fatty acids such as SDA, DPA, ETA, Ln *n*-3, AA etc, and in addition it can be used for determining the positional distribution of various fatty acids on glycerol skeleton.

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3 697 **Table 1.** ^{1}H-NMR chemical shifts of fish oil fatty acids in $CDCl_3$ solution. * denote
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5 698 signals that can be used for quantitation puproses
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Signal	*δ* ppm	Proton	Compound
1*	0.677	CH_3 (18)	Cholestarol
2*	0.678	CH_3 (18)	7-dehydrocholesterol
3*	0.880	$CH_2C\underline{H_3}$ (t), $J_{\omega1,\ \omega2}$= 7.27 Hz	*n*-9, SFA acyl chains
4*	0.883	$CH_2C\underline{H_3}$ (t), $J_{\omega1,\ \omega2}$= 7.08 Hz	*n*-6 acyl chains
5*	0.911	$CH_2C\underline{H_3}$ (t), $J_{\omega1,\ \omega2}$= 7.65 Hz	*Trans* acyl chains
6*	0.973	$CH_2C\underline{H_3}$ (t), $J_{\omega1,\ \omega2}$= 7.63 Hz	*n*-3 acyl chains
7	1.230-1.347	–(CH2)n- (envelope)	*n*-6, *n*-9, SFA acyl chains
8	1.382	–(CH2)n- (envelope)	*Trans* acyl chains
9	1.399	–(CH2)n- (envelope)	SDA acyl chain
10	1.616	OCOCH2C$\underline{H2}$ (envelope)	*n*-6, *n*-9, SFA, SDA acyl chains
11*	1.697	OCOCH2C$\underline{H_2}$ (t) $J_{H\alpha,\ H\beta}$= Hz	EPA acyl chain
12	2.010	$CH_2CH{=}CH$ (m)	*n*-9 acyl chains
13	2.035	$C\underline{H_2}CH{=}CH$ (m)	*n*-6 acyl chains
14	2.072	$CH_3C\underline{H_2}CH{=}CH$	*n*-3 acyl chains
15	2.311	OCOC$\underline{H_2}$CH2 (t) $J_{H\alpha,\ H\beta}$= Hz	All acyl chains except DHA
16*	2.391	OCOC$\underline{H_2}$C$\underline{H_2}$ (t)	DHA acyl chain
17*	2.772	$CH{=}CHC\underline{H_2}CH{=}CH$	*n*-6 acyl chains
18	2.810	$CH{=}CHC\underline{H_2}CH{=}CH$	*n*-3 acyl chains
19*	3.593	3'a-C$\underline{H_2}$OCO	Glycerol of 1-MAG
20	3.722	3'a, 3'b-C$\underline{H_2}$OCO (br)	Glycerol of 1,2-DAG
21*	4.073	2'-C$\underline{H}$OH (br)	Glycerol of 1,3-DAG
22	4.146		Glycerol of TAG
23	4.173	1'b, 3'b-C$\underline{H_2}$OCO (dd)	Glycerol of 1,3-DAG
24	4.292		Glycerol of TAG
25	4.238	1'a-C$\underline{H_2}$OCO (dd)	Glycerol of 1,2-DAG
26*	4.329	1'b-C$\underline{H_2}$OCO (dd)	Glycerol of 1,2-DAG
27*	4.989	-CH=C$\underline{H_2}$ *cis* (dd)	*n*-1 acyl chains
28*	5.052	-CH=C$\underline{H_2}$ *trans* (dd)	*n*-1 acyl chains
29*	5.082	2'-C$\underline{H}$OCO	Glycerol of 1,2-DAG
30	5.268	2'-C$\underline{H}$OCO	Glycerol of TAG
31	5.297-5.401	CH=CH (m)	All acyl chains
32*	5.436	CH=CHC$\underline{H_2}$CH=CH2	*n*-1 acyl chains
33*	5.818	-C$\underline{H}$=CH_2	*n*-1 acyl chains

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712 **Table 2.** ^{13}C-NMR chemical shifts of fish oil fatty acids in $CDCl_3$ solution. * denote
713 signals that can be used for quantitation puproses
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Signal number	*δ* ppm	Carbon
	Carbonyl Carbons	
1*	173.24	C1 SFA (*sn*-1,3)
2	172.21	C1 OL, LO (*sn*-1,3)
3*	173.16	C1 ETA (*sn*-1,3)
4*	173.13	C1 DPA (*sn*-1,3)
5*	173.03	C1 SDA (*sn*-1,3)
6*	172.97	C1 EPA (*sn*-1,3)
7	172.82	C1 SFA (*sn*-2)
8	172.79	C1 OL, LO (*sn*-2)
9*	172.73	C1 ETA (*sn*-2)
10*	172.69	C1 DPA (*sn*-2)
11*	172.61	C1 SDA (*sn*-2)
12*	172.56	C1 EPA (*sn*-2)
13*	172.48	C1 DHA (*sn*-1,3)
14*	172.08	C1 DHA (*sn*-2)
	Olefinic Carbons	
15*	136.80	Cω1, *n*-1
16	131.92	Cω3 all *n*3 except LN
17*	131.85	Cω3 LN
18*	130.37	C15 AA
19*	130.11	C9 LN
20*	130.06	C13 LO
21	129.90	C10 OL
22	129.82	C12 20:1, C14 22:1
23	129.72	C11 20:1, C13 22:1
24	129.60	C9 OL
25*	129.54	C5 DHA *sn*-2
26*	129.47	C5 DHA *sn*-1,3
27*	128.94	C5 EPA
28*	128.76	C6 EPA
29*	128.45	C17 *n*-3
30	128.08	C7 DHA
31*	127.71	*n*-3
32*	127.53	C4 DHA *sn*-2
33*	127.50	C4 DHA *sn*-1,3
34*	126.86	Cω4, all *n*-3
35*	114.71	Cω2, *n*-1
	Aliphatic Carbons	
36	34.02	C2 DHA
37	33.84	C2 SDA
38*	33.48	C2 EPA *sn*-2
39*	33.30	C2 EPA *sn*-1,3
40*	31.40	C3 *n*-1
41	28.93	C4 SDA
42*	27.05	Allylic *n*-6
43	26.76	C5 SDA
44*	26.49	C4 EPA *sn*-1,3
45*	26.47	C4 EPA *sn*-2
46	25.60	C6 DHA
47*	24.60	C3 EPA

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48*	24.48	C3 SDA *sn*-1,3
49*	24.44	C3 SDA *sn*-2
50	22.74	Cω2 Trans FA
51	22.60	C3 DHA
52	20.47	Cω2 EPA, DHA, SDA
Methyl Carbons		
53*	14.27	Cω1, all *n*-3
54*	14.13	Cω1, SFA
55*	14.11	Cω1, OL
56*	14.07	Cω1, LO
57*	13.80	Cω1, *trans* FA

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755 **Table 3.** Fatty acid composition (%) of four fish oil samples determined by ^{1}H and
756 ^{13}C NMR.
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Samples	S1[a]	S2[a]	S3[b]	S4[b]
^{1}H NMR				
n-3	35.5	36.3	35.4	36.8
EPA	20.1	19.7	20.5	20.8
DHA	7.0	7.5	7.2	7.9
Other *n*-3	6.4	6.1	5.7	6.1
n-9	18.9	16.9	17.3	18.2
n-6	3.5	3.9	4.4	5.6
SFA	35.9	37.2	36.4	32.7
Trans	4.1	5.2	4.3	4.2
n-1	2.5	1.9	2.5	2.9
n-6 / *n*-3	0.10	0.11	0.12	0.15
n-6 / *n*-9+SFA	0.90	0.10	0.10	0.10
^{13}C NMR				
Carbonyl carbons				
EPA sn-1,3	16.2	15.9	14.8	14.1
EPA sn-2	3.1	3.7	2.9	3.3
DHA sn-1,3	2.2	2.6	2.3	2.7
DHA sn-2	4.1	5.0	4.5	4.6
ETA sn-1,3	1.8	1.6	1.6	Not detected
ETA sn-2	0.2	0.4	0.2	Not detected
DPA sn-1,3	1.3	1.4	2.9	1.5
DPA sn-2	1.7	2.0	1.5	2.1
SDA sn-1,3	4.6	4.3	4.9	5.1
SDA sn-2	1.4	1.8	2.6	2.3
n-9 + *n*-6 sn-1,3	17.0	19.1	17.3	20.4
n-9 + *n*-6 sn-2	4.8	5.2	5.22	6.9
SFA sn-1,3	23.1	21.8	22.4	18.9
SFA sn-2	13.3	13.7	12.5	13.3
n-3	36.6	38.7	37.9	35.7
Olefinic Carbons				
n-1	2.0	1.9	2.2	2.6
n-3	34.9	35.3	39.0	33.3
Ln *n*-3	0.8	0.7	0.65	0.9
AA	0.9	1.1	1.1	1.2
LO	3.1	3.3	3.0	2.9
OL	19.9	16.8	13.0	16
DHA sn-1,3	2.2	2.6	2.6	5.5
DHA sn-2	4.8	4.9	4.7	3.1
EPA	19.5	18.5	19.8	19.0
Other *n*-3	6.9	6.7		5.7
Methyl Carbons				
n-3	35.2	37.4	36.2	36.1
n-9	17.5	16.1	14.0	14.9
n-6	3.2	3.6	3.9	4.8

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SFA	37.9	37.6	37.0	38.6
Trans	4.2	4.0	3.7	4.1
n-6 / *n*-3	0.09	0.10	0.10	0.13
n-6 / *n*-9+SFA	0.07	0.08	0.08	0.08

[a]sample without vitamin D3
[b]sample with vitamin D3 added

Table 4. Quantitative data (absolute values) of minor compounds of fish oil samples as determined by ^{31}P NMR.

^{31}P NMR	S1[a]	S2[a]	S3[b]	S4[b]
1-MAG (mg/100g)	15.88	18.47	67.19	105.31
1,2-DAG (g/100g)	0.76	0.82	1.08	1.21
1,3-DAG (g/100g)	1.67	1.88	2.68	2.84
Glycerol (mg/100g)	3.59	5.58	5.97	6.00
Cholesterol + 7-dehydrocholesterol (mg/100g)	412.60	430.82	19.02	24.00
Vitamin E (mg/100g)	19.59	19.10	12.46	30.00
FFA (g/100g)	0.04	0.05	0.17	0.13
Hydroxytyrosol (mg/100g)	14.81	14.81	17.28	22.92
Tyrosol (mg/100g)	9.45	9.87	11.42	15.04

[a]sample without vitamin D3
[b]sample with vitamin D3 added

Table 5. Composition (%) of minor compounds of four fish oil samples as determined by multinuclear (^{1}H, ^{13}C, ^{31}P) NMR spectroscopy.

Components/Samples	S1[a]	S2[a]	S3[b]	S4[b]
^{1}H NMR				
1-MAG	0.07	0.06	0.23	0.32
1,2-DAG	1.12	1.19	2.10	2.38
1,3-DAG	2.64	2.73	3.91	4.71
^{13}C NMR				
1,3-DAG	2.51	2.62	3.32	3.99
^{31}P NMR				
1-MAG	0.05	0.05	0.19	0.31
1,2-DAG	1.13	1.22	1.61	1.80
1,3-DAG	2.49	2.80	3.99	4.23
Cholesterol + 7-dehydrocholesterol	0.62	0.65	0.03	0.04

[a]sample without vitamin D3
[b]sample with vitamin D3 added

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