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PRESS echo time behavior of triglyceride resonances at 1.5 T: Detecting ω-3 fatty acids in adipose tissue *in vivo*

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ABSTRACT

Aim: This study investigated the impact of fatty acid (FA) composition on the echo time behavior of triglyceride resonances in a clinical setting. The feasibility of ^{1}H NMR spectroscopy to detect these resonances was also evaluated in human adipose tissue *in vivo*.
Method: Ten edible oils chosen to cover a wide spectrum of FA compositions were used as phantom material. The detailed FA composition and intrinsic proton spectra of the oils were characterized by gas chromatography and high-resolution ^{1}H NMR spectroscopy (11.7 T), respectively. The detailed echo time behavior of the oils were subsequently measured by ^{1}H NMR spectroscopy in a clinical scanner (1.5 T) using PRESS. The effect of temperature was investigated in five oils.
Results: The olefinic (5.3 ppm) and diallylic (2.8 ppm) resonances exhibited distinct *J*-modulation patterns independent of oil FA composition. The methylene resonance (1.3 ppm) displayed an exponential decay, with the apparent T_2 showing a weak positive correlation with oil unsaturation ($R = 0.628$, $P = 0.052$), probably a result of changes in viscosity. For the methyl resonance (0.9 ppm), oils high in ω-3 FA displayed a markedly different *J*-modulation pattern compared to non-ω-3 oils. The characteristic *J*-modulation of the ω-3 methyl group could be attributed to the phase behavior of the ω-3 methyl triplet signal (all triplet lines in-phase at TE of 135 ms), a result of the ω-3 methyl end forming a first order spin system. The ω-3 methyl outer triplet line at 1.08 ppm of the TE = 140 ms spectrum was found to be useful for determining the ω-3 content of the oils ($R = 0.999$, standard error of estimate (SE) 0.80). The olefinic and diallylic proton resonance (measured at TE = 50 ms) areas correlated with the olefinic ($R = 0.993$, SE 0.33) and diallylic ($R = 0.997$, SE 0.19) proton contents calculated from the GC data. Information derived from long echo time spectra (TE = 200) demonstrated good correlations to GC data and showed no change with increasing temperature (and T_2). In ^{1}H NMR spectra (1.5 T) of adipose tissue in five healthy subjects, the analytically important olefinic and diallylic resonances were clearly resolved with a coefficient of variation of 1.6% and 8.4%, respectively, for repeated measurements. The characteristic phase behavior of the ω-3 methyl outer triplet line at 1.08 ppm could also be detected at very long echo times (470 and 540 ms).
Conclusion: Fatty acid composition has an impact on the echo time behavior of triglyceride resonances. Long TE spectra can resolve ω-3 FA in adipose tissue *in vivo*. These findings will benefit long TE studies of tissue lipids.

1. Introduction

Chromatographic methods have become the standard for analyzing fatty acid (FA) composition of triglycerides [1]. These methods are accurate and quantitative, but require sample extraction and preparation. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy have both been used to study the composition of triglycerides and edible oils [2–4].

Proton decoupled ^{13}C NMR spectroscopy has been shown to be useful in FA analysis of human adipose tissue triglycerides *in vivo* at 1.5 T [5,6]. However, the required instrument setup for ^{13}C spectroscopy is not normally available in clinical imagers. In addition, ^{13}C spectroscopy has not been reported to allow for detection of a separate ω-3 FA resonance at 1.5 T [6]. ^{1}H NMR spectroscopy, however, has become a routine clinical tool and is therefore readily available in most clinical 1.5 T imagers.

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In vivo ^{1}H NMR spectroscopy of triglycerides has mainly been applied to quantitate intrahepatocellular (IHCL) and intramyocellular lipid (IMCL) content at clinical field strengths [7,8]. However, relatively few attempts have been made to study the composition of these triglycerides. ^{1}H NMR spectroscopy has been used to measure polyunsaturated fatty acid (PUFA) maps of rat adipose tissue [9] and PUFA released during apoptosis in rats [10] at 4.7 T. In humans, the lipid content [11] and unsaturation [12] of bone marrow have been studied at 1.5 T by ^{1}H spectroscopy, with an unsaturation index derived from the olefinic proton resonance. Localized 2D-spectroscopic techniques have also been employed to study FA composition *in vivo*, but these techniques are not readily available [13,14].

It is not straightforward to apply ^{1}H spectroscopy to determine FA composition *in vivo*, since individual FA resonances are not well resolved. The intense water peak at 4.7 ppm may completely obscure the olefinic FA peak at 5.3 ppm and various tissue specific short-T_2 metabolites are present in short echo time spectra. A solution to this problem would be to use long echo times to suppress short-T_2 metabolites and water and to modulate the FA resonance lineshapes. Accordingly, long echo time spectroscopy has been shown to result in improved separation of the methylene (CH_2) and methyl (CH_3) resonances *in vivo* [15] and to provide a means to study the CH_2/CH_3 ratio (TE = 270 ms) in IMCL [16]. Recently, we suggested using long echo time (TE = 200 ms) spectroscopy to study IHCL unsaturation, as the olefinic resonance obtains a relative increase in intensity at TE = 200 ms due to *J*-modulation [17]. These studies suggest a possible role for long echo time spectroscopy in the *in vivo* analysis of triglyceride FA composition in muscle and liver.

Using long echo times, however, has the disadvantage of heavy T_2-weighting, which might skew the results due to differences in T_2-relaxation and *J*-modulation. We are not aware of any study reporting on the effects of fatty acid composition on the T_2-relaxation and *J*-modulation of triglyceride resonances. Clearly, there is a need to elucidate the echo time behavior of different fatty acids, such as saturated (SAFA), monounsaturated (MUFA), polyunsaturated (PUFA) and ω-3 FA.

The aim of this study is to determine the echo time behavior of the four major FA resonances that can be well separated at 1.5 T: the olefinic (**H**C=C**H**), diallylic (C=C–C**H$_2$**–C=C), methylene (C**H$_2$**), and methyl (C**H$_3$**) resonances. In order to examine the behavior of these resonances in triglycerides with different fatty acid compositions, we chose ten edible oils to be used as phantom material. The present study also explores the *in vivo* feasibility of long TE spectroscopy in characterizing human adipose tissue fatty acids.

2. Experimental

2.1. Samples

Ten edible oils, acquired from local groceries, with varied compositions of different fatty acids were used as phantom material for the proton spectroscopic studies. The oils were: cod liver, olive, linseed, rapeseed, sunflower, frying (mixture), pumpkin, peanut, sesame, and walnut. Cod liver oil was chosen for its high content of long chain ω-3 PUFA and linseed oil for its high content of alpha-linolenic acid (18:3n-3),[1] also an ω-3 PUFA. Olive oil is high in oleic acid (18:1) or MUFA content and sunflower oil is high in linoleic acid (18:2n-6), a common ω-6 PUFA. Rapeseed and walnut oil both contain significant amounts of ω-3 PUFA (18:3n-3) but have very different proportions of MUFA and PUFA. Sesame seed, peanut, and frying oil have somewhat equal amounts of MUFA and PUFA. Pumpkin seed oil has a high content of saturated FA compared to the other edible oils. The detailed fatty acid compositions of the oils were determined by gas chromatography (Table 1).

Table 1
Detailed fatty acid composition of the edible oils as measured by GC. The individual saturated (SFA) and monounsaturated fatty acids (MUFA) are not shown.

Oil	SFA	MUFA	18:2n-6 (LA)	18:3n-3 (ALA)	20:5n-3 (EPA)	22:5n-3 (DPA)	22:6n-3 (DHA)
Cod liver	22.5	51.8	2.7	1.1	9.9	1.2	10.7
Frying	18.9	36.8	43.6	0.7	0	0	0
Olive	16.4	74.8	8.3	0.5	0	0	0
Groundnut	15.2	51.7	31.3	0	0	0	0
Linseed	7.9	14.4	15.7	61.9	0	0	0
Pumpkin	27.9	18.7	53.0	0.5	0	0	0
Rapeseed	8.0	61.6	21.1	9.3	0	0	0
Sesame	16.9	43.7	39.2	0.2	0	0	0
Sunflower	11.6	28.4	60	0	0	0	0
Walnut	12.3	4	70.2	13.6	0	0	0

2.2. ^{1}H NMR measurements at 1.5 T

The measurements were carried out on a clinical 1.5 T MR Imager (Avanto, Siemens, Erlangen, Germany). The body coil was used for transmitting and a flex-coil, placed above the oil container perpendicular to the main magnetic field, was used for receiving. The oils were measured at room temperature in their native containers, consisting of plastic or glass, to minimize oil oxidization from exposure to air. T_1-weighted localizer images were acquired in three planes. The ^{1}H NMR spectra of the oils were acquired with a PRESS sequence prescribing a 2.0 cm × 2.0 cm × 2.0 cm (8 ml) volume of interest (VOI) inside the oil containers. Automated shimming and transmitter gain adjustments were performed and the NMR signal was acquired with 1024 complex data points over a spectral width of 1000 Hz. Transmitter frequency was set on intense CH_2 resonance at 1.3 ppm. A repetition time of 1500 ms was used to match the relatively short T_1 of the large triglyceride molecules. To study T_2 relaxation and *J*-modulation of the resonances, a set of spectra were acquired with the echo times; 50, 80, 100, 120, ... 280, 300 ms (increments of 20 ms). The number of acquisitions was increased from 16 for TE = 50–100 ms to 32 for TE = 120–200 ms and 64 for TE = 300 ms. In order to measure the reproducibility of the method, the TE = 50 ms spectra were acquired twice with the oil containers removed from the magnet between measurements (i.e., new magnetic field homogeneity and transmitter power adjustments). Additionally, linseed oil was measured at TE = 135, 270, 405, 540, and 675 ms, with 128 acquisitions.

To investigate the echo time behavior at physiological temperature, five oils (cod liver, linseed, pumpkin, rapeseed, and walnut) were measured also at 35 °C. The oils were placed in a warm water bath preheated to 40 °C and allowed to cool down to 35 °C, isolated with a towel and placed in the magnet. A measurement setup similar to room temperature measurements was used, except that the acquisitions were dropped to 8 for all echo times. This was done to minimize the measurement time and the possibility of oils cooling down.

To assess the feasibility of the method *in vivo*, the dorsal waist adipose tissue of five volunteers were measured with a similar measurement setup. To avoid long measurement times, and there-

[1] *Abbreviations used:* 18:3n-3, ALA, alpha-linolenic acid, *all-cis*-9,12,15-octadecatrienoic acid; 18:2n-6, LA, linoleic acid, *all-cis*-9,12-octadecadienoic acid; 20:4n-6, ARA, arachidonic acid, *all-cis*-5,8,11,14-eicosatetraenoic acid; 20:5n-3, EPA, eicosapentaenoic acid, *all-cis*-5,8,11,14,17-eicosapentaenoic acid; 22:5n-3, DPA, docosapentaenoic acid, *all-cis*-7,10,13,16,19-docosapentaenoic; 22:6n-3, DHA, docosahexaenoic acid, *all-cis*-4,7,10,13,16,19-docosahexaenoic acid.

by movement artifacts, only five echo times were used; TE = 50, 80, 135, 200, and 300. For one subject, additional spectra were acquired at TE = 470 and 540 ms, with 64 and 96 acquisitions, respectively. Spectra with TE = 50 were repeated with new settings to determine reproducibility. The analysis of the short TE spectra (50) were perfumed as for the oils, except that the AMARES fitting now included also the water resonance, which is of appreciable amplitude in adipose tissue. For the long TE spectra (80, 135, 200, and 300) the AMARES fitting was modified to conform to the modulated lineshapes.

2.3. Analysis of 1.5 T ^{1}H spectra

All 1.5 T proton spectra were processed and analyzed with the jMRUI v3.0 software. Spectra were zero filled to 4096 data points and phased manually to obtain pure absorption spectra. Peak areas were determined with AMARES [18].

For two sets of echo times (TE = 50 and 200 ms) spectra, all resonances except the glycerol backbone (4.4–4.0 ppm) were included in the AMARES fit. The peak shapes of the FA resonances do not conform to pure Lorentzian or Gaussian lineshapes due to complex multiplet structure and minor shifts in resonance frequencies. Therefore, a combination of Gaussian and Lorentzian lineshapes were used for the fitting, with several peaks used for each resonance. All lineshapes were set at zero phase except for the one fitted to the ω-3 outer triplet, which was set to 180° phase. Prior knowledge was used to constrain the peak frequencies and linewidths. The zero order phase and begin time were set to zero in the AMARES algorithm. The individual resonance areas were divided by the sum of all peak areas to obtain a measure independent of transmitter and receiver gain, coil sensitivity, and oil density. For spectra with TE = 200 the methyl region was excluded from the normalization. The chemical shift scale was fixed by setting the methylene resonance to 1.3 ppm.

For the echo time series (TE = 50–300), each of the four major resonances, olefinic (5.3 ppm), diallylic (2.8 ppm), methylene (1.3 ppm), and methyl (0.9 ppm), was quantitated separately. First all peaks, except the one to be analyzed, were removed with a Hankel–Lanczos filter (single variable decomposition method) [19]. The resulting echo time series spectra contained only the desired resonance. The spectra were apodized by an exponential function with a 5 Hz line-broadening factor and AMARES was used to quantify the echo time series resonance with a single Lorentzian lineshape. This was done to simplify the analysis of the large number of complex spectra. The T_2 of the methylene (CH_2) and methyl (CH_3) resonance was calculated by fitting a monoexponential function to the echo time series data. The T_2 of the ω-3 methyl group was estimated from the additional five echo times acquired for linseed oil. Now the methyl region was fitted with four Gaussian peaks fixed at 1.08, 0.97, 0.90, and 0.84 ppm. As all triglyceride resonances are *J*-coupled the decay rate of these groups should be viewed as an apparent T_2.

2.4. High-resolution ^{1}H NMR spectroscopy at 11.7 T

To study the original shape of the FA resonances with echo time of 0 ms and with good spectral resolution, ^{1}H spectra of all oils were measured at 27 °C using a Varian Unity Inova 500 NMR spectrometer (11.7 T, 500 MHz ^{1}H frequency) equipped with a 5 mm triple resonance probe head incorporating a *z*-gradient coil. The oils (30 μl) were dissolved in (650 μl) deuterated chloroform and ^{1}H NMR spectra were acquired using 8 repetitions, 8000 Hz spectral width, 16,000 complex data points and an 8 s relaxation delay. Time domain data was weighted by an exponential window function (3 Hz line-broadening factor) and zero filled up to 32,000 complex points prior to Fourier transformation. Two-dimensional COSY spectra were recorded from three oil samples: olive, linseed, and sunflower. In COSY, spectral width in both dimensions was 4473 Hz, number of acquired complex data points was 1342, number of increments was 350, and 2 repetitions were used. The time domain data matrix was apodized by sine function in both dimensions and the final data matrix size after Fourier transformation was 2048 × 2048. For the 1D ^{1}H spectra, the peak integration was performed for seven distinct FA resonances and three glycerol resonances. COSY spectra were used for resonance assignment purposes.

2.5. Fatty acid analysis by gas chromatography

In order to validate the measurements by ^{1}H NMR spectroscopy at 1.5 T, the total fatty acid compositions of the oils were determined independently by GC. The oils (50 μl) were diluted to 5 ml in 2:1 chloroform/methanol (v/v) and the total lipids from 100 μl portions extracted by Folch extraction. Fatty acid methyl esters (FAME) were prepared as previously described without separation of lipid classes [20]. Fatty acid composition was determined using an Agilent 5890 GC (Agilent Technologies, UK) equipped with a 30 m Rtx®-Wax capillary column with 0.53 mm ID and 1 μm film thickness (Thames Restek, Saunderton, UK). Fatty acids were identified with reference to lipid standards (Sigma–Aldrich, Gillingham, UK) and FAME mixtures of known composition (Thames Restek) were used as quality control material. Results were expressed as molar% (μmol/100 μmol fatty acids) and are presented in Table 1. From the GC data the proportions of olefinic and diallylic protons in the oils were calculated. All protons of the fatty acid chains were included and the glyceryl protons were excluded from the calculations.

3. Results

In the short echo time (TE = 50) spectra, four major proton resonances were clearly resolved: olefinic (**H**C=C**H**), diallylic (C=C—C**H$_2$**—C=C), methylene (C**H$_2$**), and methyl (C**H$_3$**). In contrast, the alpha-carbon (OC—C**H$_2$**—CH$_2$), allylic (C**H$_2$**—C=C), and beta-carbon (OC—CH$_2$—C**H$_2$**) proton resonances could not be resolved. The intensity of the glycerol backbone resonances (4.4–4.0 ppm) was insignificant, probably due to effects of *J*-modulation.

The original spectrum of cod liver oil is shown in Fig. 1, along with the peak fitting estimate and residue. The proportion of olefinic and diallylic protons in the oils measured by GC and proton MRS were correlated with $R = 0.993$ (olefinic) with a 0.33 standard error of estimate (SE) and $R = 0.997$ (diallylic) with a 0.19 SE. The slope of the linear regression line was 0.84 for the olefinic protons and 1.01 for the diallylic protons, indicating an overestimation of the olefinic protons by about 20%. For the repeated measurements, the olefinic proton content had a coefficient of variation (CV) of 2.5% and the diallylic proton content a CV of 7.8%. For the long echo time spectra (TE = 200) the correlation with GC results was $R = 0.983$ (olefinic) with a 0.52 SE and $R = 0.992$ (diallylic) with a 0.32 SE.

3.1. High-resolution NMR

The 11.7 T ^{1}H NMR spectrum of sunflower oil is shown in Fig. 2, with resonance assignments. From qualitative analysis of the 1D spectra, the detailed multiplet structures of the resonances could be inferred. The measurements confirmed the results of previous studies [21]. The olefinic resonance displayed a highly complex multiplet shape; only olive oil (high in MUFA) seemed to have an apparently simpler multiplet structure resembling a quartet. The diallylic resonances were found to present a triplet structure for

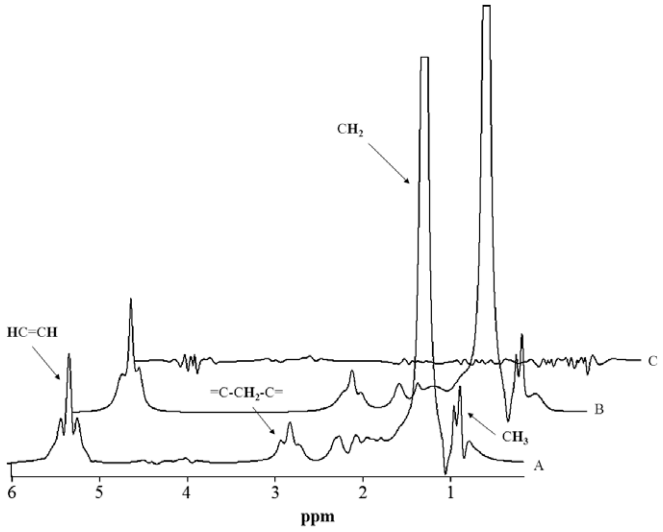

Fig. 1. The 1.5 T ^{1}H NMR spectrum of cod liver oil with TE = 50 ms (A), estimate (B), and residue (C) obtained with the AMARES algorithm. The four major fatty acid resonances, olefinic (**HC**=C**H**, 5.3 ppm), diallylic (=C–C**H$_2$**–C=, 2.8 ppm), methylene (C**H$_2$**, 1.3 ppm), and methyl (C**H$_3$**, 0.9 ppm) are indicated. The methyl group shows a double peak, originating from ω-3 and non-ω-3 methyl groups.

all PUFA types, with the chemical shifts characteristic to PUFA type, as previously described [21]. The methylene resonance formed a complex pattern with three overlapping peaks, which have been previously assigned to SAFA, MUFA, and PUFA. There was also indication of a multiplet structure underlying the three main peaks. This structure was more pronounced for oils high in PUFA, but the origin of the structure remains unclear. The methyl resonances indicated little difference for SAFA and MUFA methyl groups, while linoleic (ω-6) methyl obtained a 0.01 ppm shift and ω-3 FA a 0.09 ppm shift downfield. The *J*-coupling constants for the different methyl groups were 7.05 Hz for SAFA and MUFA, 6.73 Hz for ω-6 PUFA and 7.50 Hz for ω-3 PUFA. The 2D COSY clearly showed that ω-3 methyl protons are coupled with allylic CH_2 protons at 2 ppm, while SAFA, MUFA, and ω-6 PUFA methyl protons couple with methylene protons at 1.3 ppm.

Table 2
Proton MRS and GC results: apparent T_2 of the methyl and methylene resonance, diallylic, and olefinic proton content of the oils, and the ω-3 PUFA content of the oils.

Oil	Methyl T_2[a] (ms)	Methylene T_2 (ms)	Diallylic protons (%)		Olefinic protons (%)		ω-3 PUFA (%)	
			GC	MRS	GC	MRS	GC	MRS
Cod liver	–	85.0	6.1	6.5	10.7	12.4	23.0	2.3
Frying	294	75.4	2.6	2.9	7.4	9.4	0.7	0
Olive	227	73.6	0.5	0.9	5.4	6.9	0.5	0
Peanut	238	74.1	1.8	2.0	6.7	8.1	1.8	0
Linseed	478[b]	78.8	8.7	8.8	14.4	17.9	61.9	6.8
Pumpkin	286	74.6	3.2	3.5	8.0	9.9	0.5	0
Rapeseed	–	73.8	2.3	2.7	7.7	9.6	9.3	1.1
Sesame	244	72.6	2.3	2.5	7.2	9.1	0.2	0.1
Sunflower	294	74.2	3.5	4.0	8.8	11.1	0	0
Walnut	–	76.0	5.9	5.8	11.2	13.1	13.6	1.3
Mean	264[c]	75.8	3.7	4.0	8.8	10.8	11.2	1.2
SD/mean	0.12[c]	0.05	0.67	0.60	0.30	0.29	1.74	1.84

[a] Influenced by *J*-modulation.
[b] ω-3 middle triplet line when in-phase.
[c] Non-ω-3 oils only.

3.2. Echo time series

The TE series spectra revealed an exponentially decaying methylene resonance and a decay dominated by *J*-modulation for the olefinic, diallylic, and methyl resonances. The methylene resonance (Table 2) displayed a trend towards longer T_2 with increasing double bond content of the oil ($R = 0.628$, $P = 0.052$). The monoexponential fit had a correlation of $R > 0.995$ for all oils. Visual inspection indicated that the data agrees well with the fit, although multiexponential behavior cannot be ruled out. When increasing temperature to 35 °C the methylene T_2 increased from 77.6 ± 4.5 to 91.1 ± 3.8 ms (mean ± SD). The T_2 estimated from the two distinct temperature measurements correlated with $R = 0.978$ and the olefinic and diallylic resonances estimated from the TE = 200 spectra correlated with $R = 0.997$ (0.97 slope of the line) and $R = 0.999$ (1.01 slope of the line).

The olefinic peak TE series for six oils are shown in Fig. 3A; the four remaining oils do not differ from the presented oils in their behavior. All oils exhibit a local maximum at TE of 180–200 ms. The olefinic peak intensity ratio TE_{200}/TE_{50} is highly correlated

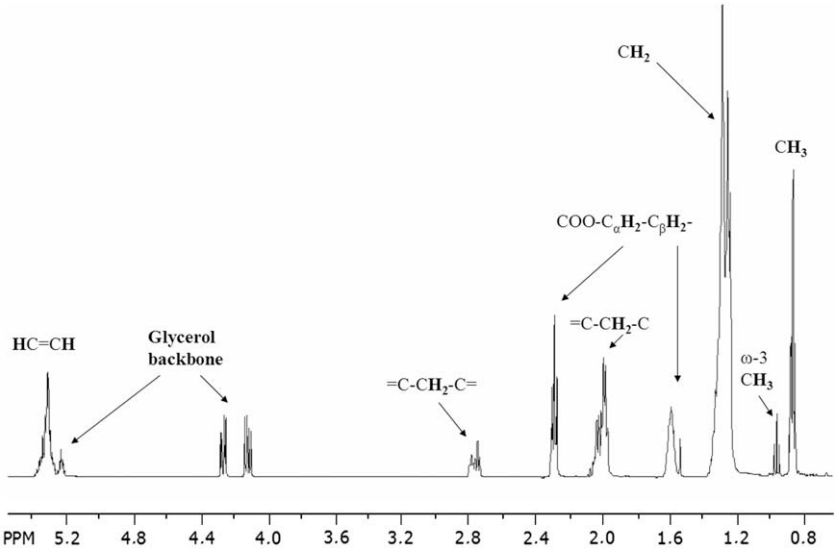

Fig. 2. The 11.7 T ^{1}H NMR spectrum of rapeseed oil (9.3% ω-3 FA) with all resonances assigned. The resonances originating from ω-3 and non-ω-3 methyl groups are clearly resolved.

with the total olefinic proton content of the oils (determined by GC) with $R = 0.985$.

The diallylic peak TE series for the six oils with the highest content of diallylic protons are shown in Fig. 3B. All oils exhibit a local maximum at TE = 180 ms and the intensity of this maximum is higher for linseed and cod liver oil, which are high in ω-3 PUFAs.

The TE series of the methyl group was found to be markedly different for oils containing significant amounts of ω-3 PUFA, (i.e., linseed, cod liver, walnut, and rapeseed) compared to non-ω-3 oils (i.e., sunflower, pumpkin, frying, peanut, sesame, olive). The methyl TE series for two high ω-3 PUFA oils (linseed and cod liver) and two non-ω-3 oils (sunflower and olive) are shown in Fig. 3C, showing that the ω-3 methyl groups obtain very large increases in their peak intensity compared to other oils. When comparing oils consisting mainly of non-ω-3 PUFA (sunflower) and MUFA (olive), there was no difference in their methyl group TE series behavior. Although there was heavy *J*-modulation of the methyl group, the monoexponential fit provided a gross estimation of T_2. There was a clear increase in the methyl T_2 for oils high in ω-3 (linseed and cod liver), with T_2 more than twice that of non-ω-3 oils. For the non-ω-3 oils there was a trend towards longer T_2 with increasing PUFA content.

3.3. ω-3 PUFA

The ω-3 methyl resonance at 0.96 ppm could be identified from the spectra (TE = 50) of oils containing significant amounts of ω-3 PUFA (linseed, cod liver, walnut, rapeseed). Upon closer examination of linseed oil (62% ω-3) spectra, it was found that the splitting of the ω-3 methyl group resulted in triplet peaks at 1.08, 0.96, and 0.84 ppm, while the non-ω-3 methyl groups resonated at 0.90 ppm. The triplet structure of the non-ω-3 FA disappears rapidly with increasing echo time. The TE series also revealed that the outer triplet lines of the ω-3 methyl group were modulated by the echo time, alternating between negative and positive phases. To investigate the phase modulation, the linseed oil TE series spectra were analyzed again by AMARES. This time the phase of the peaks fitted to the ω-3 methyl outer triplet lines were left unrestricted and estimated by the AMARES algorithm. The resulting phase behavior of the ω-3 outer triplet lines is shown in Fig. 4. The phase behavior demonstrates that the ω-3 outer triplet lines can be estimated to be in-phase with the middle triplet line every 135 ms. Similar phase behavior was also observed for the three other oils high in ω-3 PUFA (cod liver, rapeseed, walnut). Fig. 5 shows the methyl region (1.2–0.6 ppm) of linseed and olive

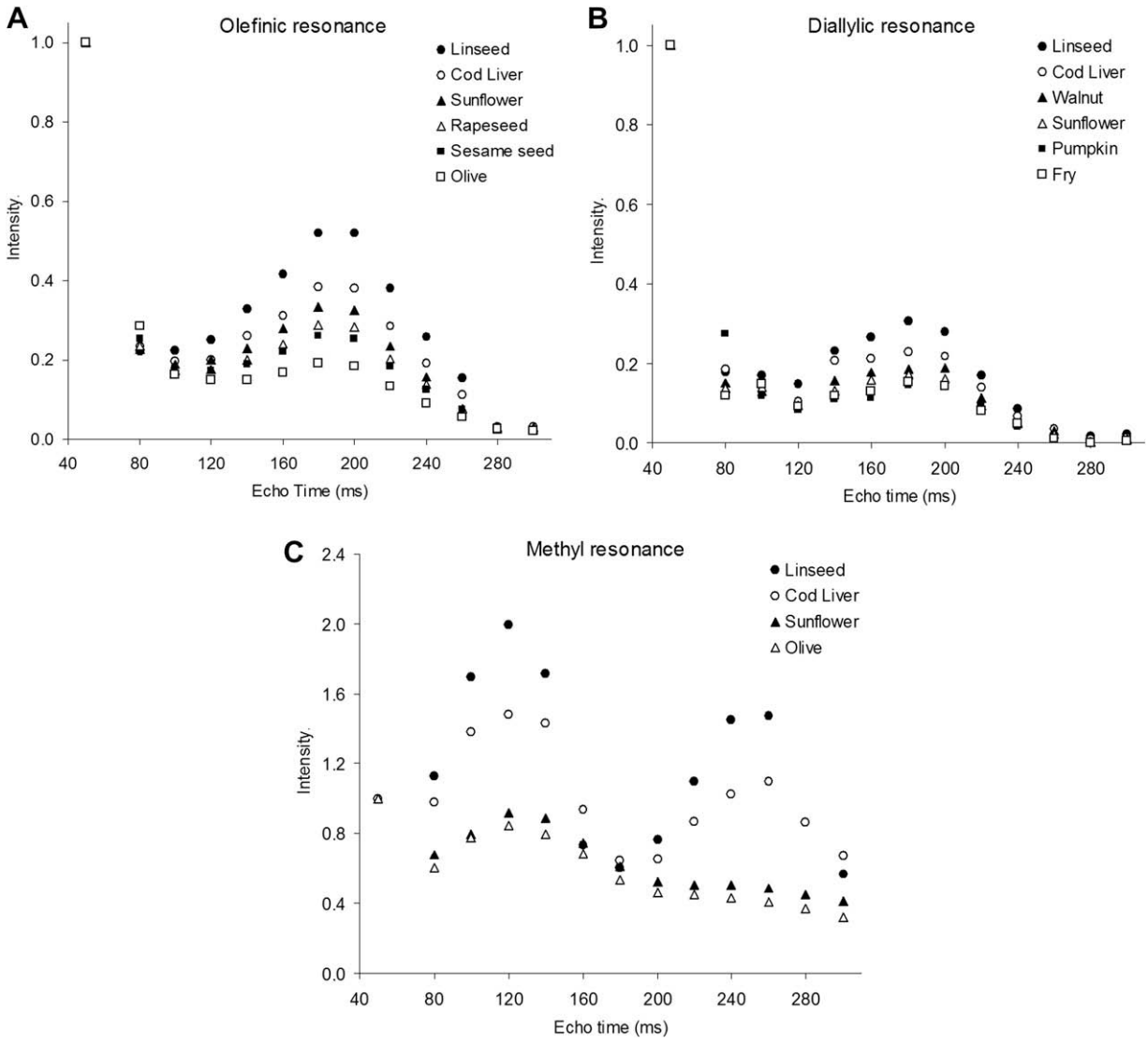

Fig. 3. The echo time series of the olefinic (A), diallylic (B), and methyl (C) resonance intensity (normalized to TE = 50). The two oils containing the highest contents of ω-3 methyl protons are shown in (C); linseed (closed circle) and cod liver (open circle), along with two non-ω-3 oils; sunflower (closed triangle) and olive (open triangle).

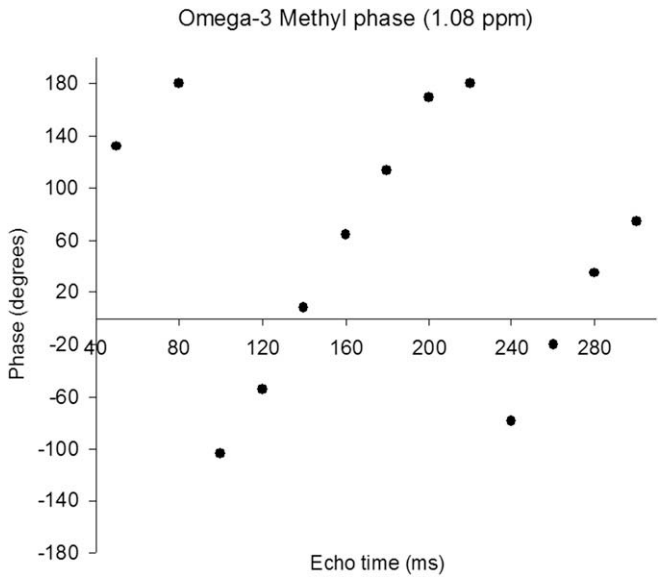

Fig. 4. The echo time series of the phase behavior of the ω-3 methyl group left outer triplet line at 1.08 ppm in linseed oil.

oil with TE = 140 ms (upper trace) and TE = 200 ms (lower trace). For linseed oil the ω-3 methyl outer triplet lines are in-phase at TE = 140 and anti-phase at TE = 200 with the ω-3 middle triplet line. For olive oil, no triplet is visible as the outer triplet lines are not present at TE > 100 ms. The apparent T_2-relaxation of the ω-3 methyl triplet was estimated from the five echo times (135, 270, 405, 540, and 675) acquired from linseed oil, as the ω-3 triplet is in phase. The outer methyl triplet line (1.08) showed a faster decay with T_2 = 297 ms, while the middle triplet line (0.97 ppm) had T_2 = 478 ms.

The oil spectra recorded at TE = 140 ms were used to test the ability of proton NMR spectroscopy to measure ω-3 PUFA levels. The analysis was performed as previously described for the TE = 50 ms oil spectra, with four peaks used to estimate the methyl resonances and six additional peaks used for the remaining resonances. The ω-3 methyl outer triplet line at 1.08 ppm, which is free from overlap, provided the best estimate for the ω-3 level of the oils, correlating with the GC determined ω-3 content with R = 0.999 and 0.80 SE.

3.4. In vivo feasibility

The subcutaneous adipose tissue of the volunteers had (mean ± SD); 6.70 ± 0.40% olefinic and 0.49 ± 0.11% diallylic proton content as determined from the TE = 50 ms spectrum. The TE = 50 ms spectrum along with the peak fitting estimate and residue for adipose tissue is shown in Fig. 6. The coefficient of variation was 1.6% and 8.4%, for olefinic and diallylic resonances, respectively. The T_2 of the methylene resonance averaged 91.3 ms with a 1.25 ms standard deviation. All monoexponential fits had correlation of R > 0.998. From the TE = 470 and 540 ms spectra in a single patient, the anti-phase and in-phase ω-3 outer triplet line at 1.08 ppm was clearly observed with phase behavior corresponding to oil studies. The spectra are displayed in Fig. 7, with the ω-3 outer triplet line in phase at TE = 540 ms and anti-phase at TE = 470 ms. When comparing the short (TE = 50) and long (TE = 200) echo time spectra, the olefinic content correlated with R = 0.826 and the diallylic with R = 0.987, and the inter-individual variance decreased from 6.4% to 3.0% (SD/mean) for the olefinic and from 17.9% to 8.8% for the diallylic content.

4. Discussion

In *in vivo* spectroscopy, the use of long echo times produce spectra with well resolved peaks, a less intense water signal and a flat baseline. In order to apply long echo time spectroscopy to study fatty acid composition, a better understanding of the T_2 relaxation effects and *J*-modulation for different FA are needed. Ideally, this would be investigated by measuring the behavior of pure triglycerides, necessitating large amounts of expensive synthetic compounds. As the characteristics of the PRESS sequence [22] and the strength of the magnetic field [23] will influence the observed T_2 relaxation and *J*-modulation, the measurements should be performed with an *in vivo* instrument used for human studies. There-

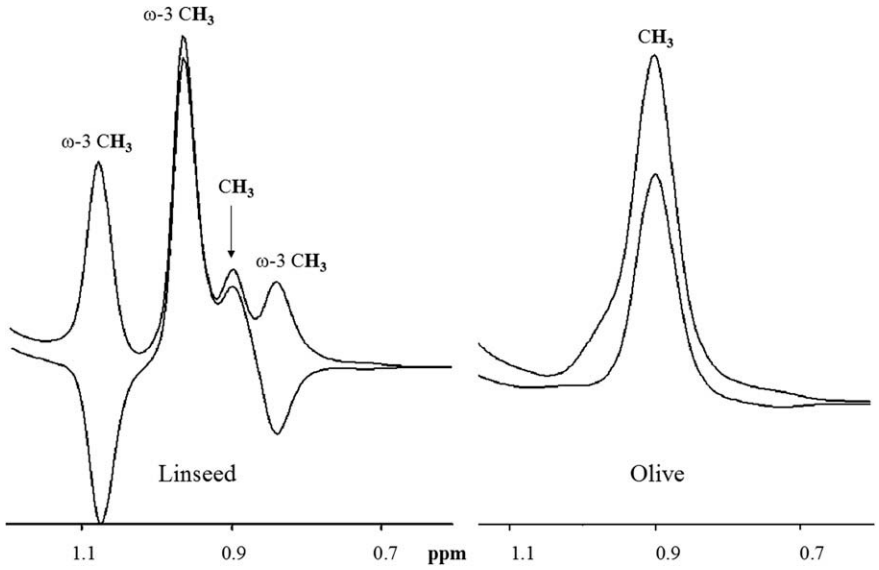

Fig. 5. Methyl region ^{1}H NMR spectra of linseed oil (left, 60% ω-3 FA) and olive oil (right, 0.5% ω-3 FA) at 1.5 T with TE = 140 ms (upper trace) and 200 ms (lower trace). Olive oil shows only one non-ω-3 CH_3 peak (0.90 ppm) and no triplet structure. Linseed oil shows four peaks; the ω-3 CH_3 triplet (1.08, 0.96, and 0.84 ppm) and the non-ω-3 CH_3. The ω-3 CH_3 triplet peaks are in-phase at TE = 140 ms (outer triplet peaks up) and anti-phase at TE = 200 ms (outer triplet peaks down).

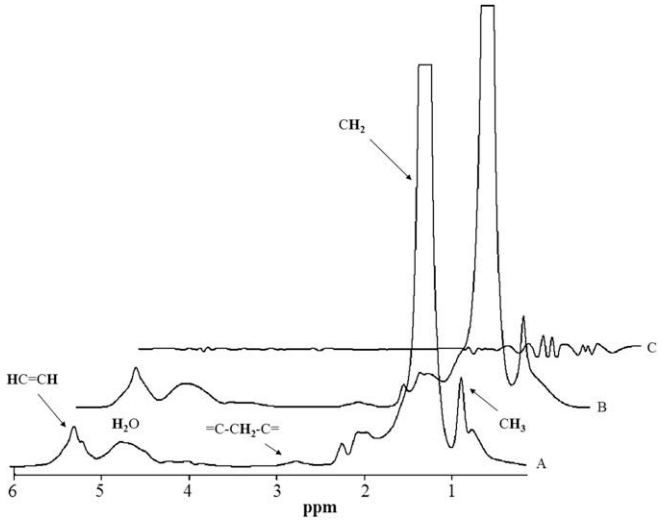

Fig. 6. The *in vivo* ^{1}H NMR spectrum of subcutaneous adipose tissue at 1.5 T with TE = 50 ms (A), estimate (B), and residue (C) obtained with the AMARES algorithm.

fore, we elected to use edible oils with different FA compositions as phantom material.

We chose a 50 ms echo time, as our preliminary measurements had shown that the minimum 30 ms echo time spectra exhibited problems with the baseline and peak fitting. We attempted to correct the resonance intensities for *J*-modulated and T_2 relaxation, but this was not possible, as the decay rate is not clearly defined. However, the linear correlation between the GC and proton spectroscopy was very good ($R > 0.99$) without relaxation correction. The olefinic proton content was, however, systematically overestimated by about 20% in the proton spectrum, which probably is a result of the used echo time. By normalizing the olefinic and diallylic proton content, we partially correct for the T_2 relaxation and *J*-modulation effects of the 50 ms echo time. For the repeated measurements, the olefinic and the diallylic proton content had a CV of 2.5% and 7.8%, respectively, indicating good reproducibility. The good correlations with GC results remained at TE = 200 with olefinic ($R = 0.983$) and diallylic ($R = 0.992$) protons, although the *J*-modulation resulted in systematically overestimated values.

The *in vivo* measurements of subcutaneous adipose tissue showed olefinic and diallylic proton content comparable to that of olive oil, the least unsaturated oil. The *in vivo* spectra showed slightly broader linewidths, but the four major resonances were still well resolved. Repeated measurements indicated good reproducibility (CV 1.6–8.4%) *in vivo*. Resonances in long echo time spectra were better resolved and had lower inter-individual variance for the estimated olefinic and diallylic contents. This suggests that the better resolved long TE spectra produce a more reliable fit. Using long TE spectroscopy for characterizing adipose tissue triglyceride composition still needs to be validated.

4.1. Apparent T_2-relaxation

Using longer echo times improves spectral quality, but the measurements also become sensitive to variations in the T_2 of the molecules. The measured T_2 values of the CH_2 resonance in our oils showed surprisingly little variation (standard deviation of 5%) considering the large differences in oil compositions. There was a positive trend towards longer T_2 with increasing olefinic proton content, although cod liver oil was an outlier with exceptionally long T_2. The long chain PUFA (EPA and DHA) of cod liver oil do not contribute to the methylene resonance (none exist in the fatty acid chain), which would indicate an indirect influence on T_2 relaxation. These results indicate that the fatty acid composition is not a direct determinant for the T_2 relaxation of the methylene resonance.

The apparent T_2 values of the methyl group, on the other hand, were much longer for the ω-3 oils, partly due to the higher mobility of these methyl groups. *J*-modulation also contributes to the observed difference in T_2 values of non-ω-3 vs. ω-3 oils. However, the T_2 of ω-3 methyl triplet lines in linseed oil, obtained from in-phase spectra, were still quite long (middle triplet line $T_2 = 478$ ms). There was also some indication of longer T_2 for ω-6 PUFA compared to SAFA and MUFA. These differences in T_2 relaxation have

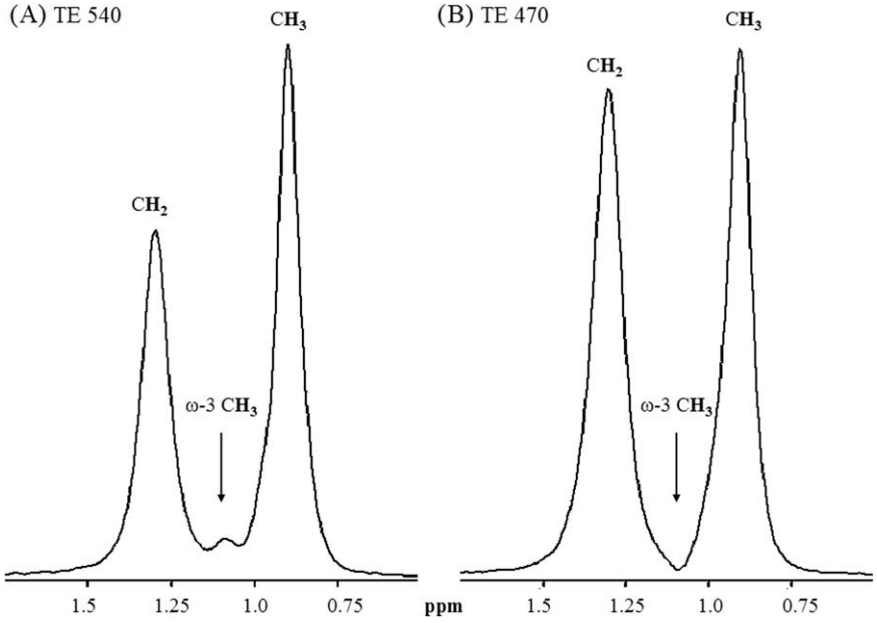

Fig. 7. Adipose tissue *in vivo* spectra with (A) the ω-3 outer triplet in-phase (TE = 540) and (B) anti-phase (TE = 470).

to be considered when evaluating the CH_2/CH_3 ratio from long echo time spectra.

The longitudinal relaxation time T_1 has been linked to the unsaturation level, temperature [24], and viscosity of non-polar hydrocarbons [25]. Of these, unsaturation and temperature both influence the viscosity of the sample. However, analysis of different fatty acids diluted in $CDCl_3$, and therefore lacking characteristic viscosity, show little differences in T_1 and T_2-relaxation times [26]. As dipole–dipole interaction, one of the principal relaxation mechanisms, is linked to the mobility of the spins, it is reasonable to assume that the T_2-relaxation of the methylene group in oils will reflect their characteristic viscosity rather than FA composition. The methyl group T_2 relaxation on the other hand, seems to be heavily influenced by the ω-3 content of the oil.

Reported T_2 of the methylene resonance, measured from bone marrow *in vivo* with PRESS, are in the range 73–90 ms [11], comparable to the average T_2 we observed in adipose tissue (91.3 ms). The standard deviation determined from five subjects was only 1.25 ms, indicating very small differences. No relationship was found between the methylene T_2 and olefinic or diallylic content in the subjects.

Five oils were also measured at a temperature of 35 °C in a warm water bath. As no other temperature control was used, the methylene T_2 derived from these spectra were considered more prone to temperature variation than room temperature measurements. However, a clear increase in methylene T_2 was observed with increasing temperature, which resulted in T_2 comparable to the T_2 *in vivo*. Despite the considerably longer T_2 of the methylene resonance, the olefinic and diallylic content estimated from TE = 200 spectra showed no difference between measurements done at room and physiological temperatures. This shows that information on triglyceride fatty acid composition derived from long echo time spectra is insensitive to changes in the T_2 of the triglycerides.

4.2. J-modulation

The TE series of the oils showed that the four major FA resonances (olefinic, diallylic, methylene, and methyl) had characteristic responses to increasing TE-values. The TE behavior of the olefinic and methylene resonances were similar to those reported for bone marrow triglycerides [11]. The TE behavior of the diallylic resonance has not been previously reported.

The olefinic proton signal of all oils exhibited a local maximum around 180–200 ms, which had an intensity proportional to the olefinic content of the oils ($R = 0.985$). The reason for the higher maximum for high olefinic oils arises from differences in T_2-relaxation and *J*-modulation. The olefinic protons of the PUFA chains are coupled to diallylic protons as well as allylic protons, as opposed to the olefinic protons of MUFA chains, which are only coupled to allylic protons. The shape and occurrence of the local maximum is, however, well preserved for all oils, considering their different FA compositions.

The TE series of the diallylic protons showed a local maximum at 180 ms. Oils with longer PUFA chains exhibited stronger intensities. The stronger local maximum in the diallylic protons of long chain PUFA compared to short chain PUFA might be a consequence of characteristic ω-3 PUFA *J*-couplings. Changes in the T_2-relaxation of the diallylic resonance might also be a factor. The diallylic peak is of low intensity compared to the other three major resonances and presents a challenge for *in vivo* detection, as the PUFA content of tissue TG is low [27]. The small peak may be lost due to high noise level or become overlapped by neighboring resonances due to inhomogeneity. From visual inspection of the spectra, it was observed that the diallylic peak was well resolved in the 120–200 ms TE region.

The TE series of the methyl protons showed clear differences between ω-3 PUFA oils and non-ω-3 oils. In contrast, no clear differences existed for oils high in ω-6 PUFA, SAFA, and MUFA. The behavior of the ω-3 methyl group was traced to the ω-3 methyl outer triplet lines, which showed an in-phase anti-phase behavior (with respect to the middle line) with a period of 135 ms.

4.3. ω-3 PUFA

The left methyl triplet line (1.08 ppm) at TE = 140 ms was used to quantify the amount of ω-3 PUFAs. The obtained values correlated well with the GC results ($R = 0.999$), but were systematically underestimated by a factor of 10, as the method does not take into account all ω-3 protons and is subject to T_2 relaxation. Most of the "non-ω-3" labeled oils contained small amounts (around 1%) of ω-3 PUFA (18:3n-3), which is near the likely ω-3 PUFA content in human adipose tissue [27]. When visually inspecting the spectra (TE = 140 ms), the characteristic outer triplet line of ω-3 at 1.08 ppm could not be clearly identified for the non-ω-3 oils due to the intense methylene resonance. For olive oil and frying oil, 0.5% and 0.7% ω-3 PUFA, respectively, a small peak corresponding to the ω-3 outer triplet line at 1.08 ppm could be identified in the TE = 280 ms spectra.

The detection of the ω-3 peak *in vivo* is hampered by the strong methylene resonance and low ω-3 content. Studies on oils indicated that the ω-3 peak is better resolved at increasing TE multiplets of 135 ms. Therefore, additional *in vivo* spectra were obtained with very long echo times (470 and 540). The outer methyl triplet line of ω-3 PUFA was clearly observed at 1.08 ppm in two spectra with different TE (470 and 540), with characteristic phase behavior (inverted peak at TE = 470). To our knowledge this is the first reported detection of ω-3 PUFA in adipose tissue *in vivo*. The ω-3 resonance has since been observed in several other subjects and research is underway for validating these findings. Initial findings from a larger *in vivo* study ($N = 15$) showed a 0.014 ± 0.008 (mean ± SD) normalized intensity of the left methyl triplet line in adipose tissue from TE = 540 spectra, comparable to the intensity in olive and frying oil TE = 540 spectra, with 0.011 (0.5% ω-3) and 0.015 (0.7% ω-3), respectively [own unpublished data].

The stark differences in PRESS echo time behavior of ω-3 and non-ω-3 fatty acids are due to strong coupling, i.e., second order effects, at 1.5 T field strength for non-ω-3 fatty acids. The chemical shifts of the non-ω-3 FA chain end (—$\mathbf{CH_2}$—$\mathbf{CH_2}$—$\mathbf{CH_3}$) are very close to each other (∼0.9 ppm for methyl and ∼1.3 ppm for methylene protons) compared to coupling constants of ∼7 Hz, making the spin system strongly coupled. The resonances at the end of the ω-3 FA chain (=$\mathbf{CH}$—$\mathbf{CH_2}$—$\mathbf{CH_3}$), however, have a relatively large chemical shift spread (5.3, 2.0, and 0.9 ppm), leading to a first order spin system. The behavior of a second order spin system of saturated FA chains in the course of the PRESS pulse sequence results in the methyl group collapsing to a resonance line resembling a broad singlet at long TE. These effects and the influence of detailed PRESS sequence parameters would best be investigated by simulation runs, and therefore are not discussed here. The *J*-coupling and resulting echo time behavior of triglyceride resonances is a complex issue that requires further research.

The incidence of obesity, insulin resistance, type 2 diabetes and non-alcoholic fatty liver disease is growing rapidly and adds to the burden of atherosclerosis and its consequences [28]. These conditions are associated with abnormal quantity and distribution of adipose tissue depots. Long TE spectroscopy has shown potential in investigating lipid composition in skeletal muscle [16], liver [17], and now adipose tissue. The noninvasive nature of the method allows repeated longitudinal studies on multiple tissue types for investigation of the role of lipid composition in the pathophysiology and treatment of disorders related to lipid accumulation.

5. Conclusions

The FA composition of triglycerides has a weak impact on the T_2-relaxation of the methylene resonance, probably resulting from changes in viscosity. The outer methyl triplet line of ω-3 FA (1.08 ppm) exhibits a characteristic echo time dependence, which can also be observed in adipose tissue *in vivo*. The olefinic and diallylic resonances can be estimated from long echo time (TE = 200) spectra as differences in echo time behavior for different fatty acids appear negligible. Information on triglyceride FA composition derived from long echo time spectra is not influenced by the T_2 of the triglycerides. This study is an important step in applying long echo time spectroscopy to investigation of adipose tissue, muscle, and liver lipid composition at clinical field strengths.

Acknowledgments

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