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High-sensitivity, broadband-decoupled ^{13}C MR spectroscopy in humans at 7 T using 2D heteronuclear single-quantum coherence (HSQC)

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Abstract

Purpose— ^{13}C MRS has an intrinsically low NMR sensitivity which often leads to large acquisition volumes or long scan times. While the use of higher magnetic fields can overcome the sensitivity limitations, high RF power deposition associated with proton decoupling limits the achievable gain. 2D heteronuclear single quantum coherence (HSQC) MRS is a method that utilizes the high chemical specificity of ^{13}C MRS while retaining the high sensitivity of ^1H detection. Due to the 2D nature of the method, proton decoupled ^{13}C MR spectra can be obtained without the use of high-powered decoupling pulses.

Methods—A novel 3D localized 2D HSQC method based on 3D STEAM localization is presented and implemented at 7 T. The low RF power deposition of the method allows TR variation along the indirect dimension which, in combination with controlled aliasing, leads to an acceleration of 11.8 relative to a standard 2D NMR acquisition.

Results—Artifact-free, high-quality and high-sensitivity 2D HSQC spectra could be obtained on all subjects in 19 min from a small 9 mL volume placed in the leg adipose tissue. Complete proton decoupling was achieved along the indirect ^{13}C dimension despite the absence of broadband proton decoupling pulses. The high chemical specificity along the indirect ^{13}C dimension allowed the detection of 19 unique resonances from which the lipids could be characterized in terms of saturation and omega-6/omega-3 (ω_6/ω_3) ratio.

Conclusion—It has been demonstrated that high quality 2D HSQC NMR spectra can be acquired from human adipose tissue at 7 T. The HSQC method is methodologically simple and robust and is flexible regarding trade-offs between temporal and spectral resolution. 2D HSQC has a strong potential to become a default method in natural-abundance or ^{13}C -enriched studies of human metabolism *in vivo*.

Keywords

2D HSQC; carbon-13; adipose tissue; triglycerides; broadband decoupling

INTRODUCTION

Carbon-13 MR spectroscopy (MRS) is a unique technique to study metabolism non-invasively *in vivo*. Whereas natural abundance ^{13}C MRS can provide important information on, for example glycogen content (1–3), ^{13}C MRS becomes especially powerful when combined with intravenous ^{13}C -labeled substrate infusion to dynamically study metabolic pathways. In muscle and liver dynamic ^{13}C MRS has been used to detect glycogen and energy metabolism (4–6), whereas in brain ^{13}C MRS has revealed a quantitative relationship between energy metabolism and neurotransmission (see (7) for review).

Despite these powerful features and the potential for clinical relevance ^{13}C MRS is still limited to a small number of research groups. This is primarily due to the increased technical demands (second RF coil and transmit chain, broadband decoupling) and the intrinsically low sensitivity of ^{13}C MRS, which requires large voxels. There are several paths to higher sensitivity, each of which holds its own promises and challenges. Hyperpolarized ^{13}C MRS is a technique of great interest as it has the potential to increase the sensitivity several orders of magnitude by utilizing a non-equilibrium polarization (8). Besides the many technological challenges related to the generation and preservation of non-equilibrium polarization, hyperpolarized ^{13}C MRS is limited to the detection of very fast, dynamic metabolic pathways, thereby making it a complementary technique to conventional ^{13}C MRS. Indirect ^1H - ^{13}C MRS in which the protons attached to carbon-13 nuclei are detected has been used for sensitivity enhancement (9). Unfortunately, the higher proton sensitivity comes at the price of a greatly reduced spectral resolution due to the narrow (< 5 ppm) chemical shift dispersion. This has made the spectral separation of chemically similar compounds, like $[4-^{13}\text{C}]$ -glutamate and $[4-^{13}\text{C}]$ -glutamine unreliable at lower magnetic fields. Spectral editing or TE optimization could be used to detect specific resonances, like $[4-^{13}\text{C}]$ -glutamate (10), but comes at the cost of a reduced spectral information content. While the proton resonances of $[4-^{13}\text{C}]$ -glutamate and $[4-^{13}\text{C}]$ -glutamine can be uniquely identified at higher magnetic fields, a new problem arises in terms of increased RF power deposition related to broadband decoupling. The increased RF power deposition has largely prevented the use of conventional ^{13}C MRS methods at high magnetic fields of 7^+ T. Options to reduce the RF power deposition are provided by ^{13}C MRS without decoupling (11) or ^{13}C MRS of non-protonated carbon-13 positions with low or no decoupling power (12,13). However, these methods do not address the intrinsically low sensitivity of ^{13}C MRS and perhaps even compound the sensitivity issue due to the increased spectral complexity and reduced peak heights encountered without decoupling or by dealing with the lengthened T_1 relaxation time constants of non-protonated ^{13}C atoms.

Two-dimensional NMR techniques like heteronuclear multiple-quantum coherence (HMQC) and heteronuclear single-quantum coherence (HSQC) combine the desirable features of proton and carbon-13 MRS to give high-sensitivity proton detection with high carbon-13 spectral resolution. While 2D HMQC and HSQC are routine methods in high-resolution, liquid-state NMR (14,15) their use for *in vivo* applications has been limited (16–18). Proof-of-principles studies have been demonstrated on cat (16), monkey (17) and human (18) brain, but have never matured to mainstream indirect ^{13}C MR acquisition methods to be used in humans. A potential limitation of 2D HSQC is the intrinsically slow acquisition

process which may interfere with the time requirements posed by dynamic ^{13}C label turnover studies.

Here we demonstrate the use of a low-power, high-sensitivity 3D localized 2D HSQC method to characterize triglycerides in human adipose tissue *in vivo* at 7 T. The 2D HSQC data acquisition time has been accelerated 11.8 times without loss of sensitivity by controlled signal aliasing and repetition time variation. Fully decoupled ^{13}C MR spectra can be obtained as a summed 1D projection from the 2D HSQC data without the use of high-powered broadband decoupling. The high-quality, high-sensitivity 2D HSQC spectra allow a detailed characterization of triglycerides including the saturated and mono- and polyunsaturated fatty acids and omega-6/omega-3 ($\omega 6/\omega 3$) fatty acids. The sequence can be readily extended to allow the detection of ^{13}C -label turnover in muscle, liver or brain and holds promise to become a standard technique for dynamic and static ^{13}C MRS studies.

METHODS

General

MR measurements were performed on a 7 Tesla actively shielded, 68 cm inner diameter MR magnet interfaced to a Direct Drive spectrometer (Agilent, Santa Clara, CA) and equipped with an asymmetric gradient system (Agilent, Santa Clara, CA) capable of switching 50 mT/m in 512 μs . The gradient system included a full set of third order shim coils. All data were acquired using a single 80 mm diameter surface coil tuned to the proton frequency (298.1 MHz). Carbon-13 pulses were transmitted at 75.0 MHz through a pair of 160 mm diameter surface coils driven in quadrature (19).

Six human subjects (4 men, 2 women, age 38 ± 10 years) were scanned. All subjects were studied in accordance with Yale Institutional Review Board guidelines for research on human subjects. Subjects were placed supine on the bed going 'feet first' into the magnet. The right calf muscle was placed directly on top of the proton surface coil. Phantom studies were performed on store-bought vegetable (soybean) oil which, according to the nutritional facts label, contained 14% saturated fatty acids (SFAs), 21% mono-unsaturated fatty acids (MUFAs) and 65% poly-unsaturated fatty acids (PUFAs). The nutritional facts label indicated the absence of trans fatty acids and cholesterol.

Localized 2D HSQC pulse sequence

Figure 1 shows the pulse sequence used for all 2D HSQC studies. Spatial localization is achieved with a 3D STEAM sequence (20) provided by the three proton 90° pulses. During the delay $\text{TE}/2 = 1/(2^1J_{\text{HC}})$, where $^1J_{\text{HC}}$ is the one-bond heteronuclear scalar coupling between protons and carbon-13 nuclei, the transverse magnetization created by the first 90° pulse evolves into anti-phase coherence, half of which is subsequently converted to longitudinal scalar order by the second proton 90° pulse. The other half of the anti-phase coherence remains in the transverse plane and is destroyed by the crusher gradients immediately following the second proton 90° pulse (but prior to the first carbon-13 90° pulse) as is typical for STEAM (20,21). With appropriate coherence selection gradients this 50% could give rise to HMQC-based signals, but this feature was not further pursued in the

current study. The longitudinal scalar order can be excited to anti-phase coherence on the carbon-13 channel by a carbon-13 90° pulse. The ^{13}C coherence evolves under the influence of chemical shift, magnetic field inhomogeneity and homo- and heteronuclear scalar coupling during the evolution time $t_1/2$. As is typical for HSQC, a non-selective proton inversion pulse followed by an identical evolution time $t_1/2$ selectively refocuses the heteronuclear scalar coupling evolution. Linearly incrementing the evolution time t_1 will ultimately lead to a 2D HSQC spectrum in which the carbon-13 domain is characterized by ^{13}C chemical shifts and ^{13}C - ^{13}C homonuclear scalar coupling and the absence of ^1H - ^{13}C heteronuclear scalar couplings. In other words, perfect broadband decoupling has been achieved without the use of high-powered decoupling methods. At the end of the evolution period t_1 the coherence still resides on the carbon-13 channel and should be transferred to the proton channel for improved detection sensitivity. This is achieved by a second ^{13}C 90° pulse which stores the magnetization along the longitudinal axis as longitudinal scalar order. A final ^1H 90° pulse then generates anti-phase coherence on the proton channel, which evolves to in-phase coherence following a delay $\text{TE}/2 = 1/(2^1J_{\text{HC}})$. Protons not attached to carbon-13 nuclei are not affected by the two carbon-13 pulses and experience a conventional STEAM sequence (with an additional proton inversion pulse). As such the sequence described so far does not give an inherent separation between ^{12}C and ^{13}C -bound protons. This separation can be achieved through coherence selection accomplished by phase cycling or magnetic field gradients (22). Phase cycling has the advantage of retaining all coherences during t_1 , thereby maximizing sensitivity. However, phase cycling requires signal subtraction which may lead to subtraction artifacts when large undesired signals, such as 99% ^{12}C -bound lipid protons, must be removed *in vivo*. In addition, phase cycling requires a minimum of four scans to achieve frequency discrimination in the carbon-13 domain. Coherence selection by magnetic field gradients is a powerful alternative to phase cycling as it can achieve selection of ^{13}C -bound proton signals and ^{13}C frequency discrimination simultaneously in a single scan. However, using magnetic field gradients for coherence selection reduces the sensitivity by a factor of 2 relative to a phase-cycled method with frequency discrimination in the indirect dimension as only a single coherence pathway can be selected during the evolution period t_1 (22,23). Since the undesired ^{12}C lipid signal represents 99% of the total signal, coherence selection was achieved by magnetic field gradients as to minimize subtraction artifacts.

Proton excitation and spatial localization was achieved by a minimum-phase Shinnar-Le Roux (SLR) pulse ((24) pulse length, $T = 0.5$ ms, bandwidth, $\text{BW} = 13.72$ kHz, maximum RF amplitude, $\text{RF}_{\text{max}} = 3.33$ kHz, refocusing factor $= 0.1667T$). Proton inversion was achieved by a 1 ms adiabatic full passage (AFP) pulse ($\tanh/\tan$ modulation (25), $R = \text{pulse length} \times \text{frequency sweep} = 150$, $\text{RF}_{\text{max}} = 3.75$ kHz). Depending on the spectral range of interest and the available maximum RF amplitude, a number of RF pulse options exist for carbon-13 excitation. Excitation of the carbon-13 ‘fingerprint’ region between 10 and 40 ppm can be achieved with a 1 ms SLR pulse ($\text{BW} = 6.86$ kHz $= 91$ ppm, $\text{RF}_{\text{max}} = 1.5$ kHz, offset $= -5$ ppm). As the triglyceride resonances outside this window are not excited the spectral window in the indirect carbon-13 dimension can be reduced without consideration of spectral aliasing. A square pulse of 167 μs operates at the same maximum RF amplitude of 1.5 kHz, but has a less desirable excitation profile with non-uniform excitation within the

‘pass band’ and significant excitation of undesired signals in the ‘stop band’. With a maximum limit on the RF amplitude of 1.5 kHz it was not possible to create an amplitude-modulated RF pulse that could uniformly excite all triglyceride proton-bound carbon positions between 10 and 130 ppm. A 1 ms amplitude and frequency-modulated adiabatic full passage (HS4 modulation (26), $R = 20$) excitation pulse is able to uniformly excite more than 15 kHz (= 200 ppm) despite a limited maximum RF amplitude of 1.5 kHz. The increased bandwidth is accompanied by a non-linear, quadratic phase across the excitation profile. However, this is not a significant consideration when the 2D HSQC data are displayed in absolute-value.

The proton pulses and the carbon-13 AFP excitation pulses were centered in the middle of their spectral ranges at 3.1 ppm and 74 ppm, respectively. Signal acquisition in the direct, proton dimension was achieved as 512 complex points across a 3.0 kHz spectral width, whereas the indirect, carbon-13 dimension was sampled as 128 (*in vivo*) or 256 (*in vitro*) increments across a 2.0 kHz spectral width. The C2 carbon position in the α -chains was referenced to $\delta_H = 2.25$ ppm and $\delta_C = 33.85$ ppm, thereby generating the appearance that the ‘fingerprint’ region is not aliased. All data were acquired with eight averages employing a two-step phase cycle on the first ^{13}C excitation pulse (+x, -x) in concert with the acquisition (+x, -x).

STEAM TE crushers were executed as 1.5 ms 35 mT/m gradients along the spatial X dimension (Fig. 1). Coherence selection gradients were executed as 1.5 ms gradients along the spatial Y dimension with amplitudes 30, 30 and 15 mT/m. The relative gradient amplitude combination 2:2:1 is commonly used, but is only one of many possible combinations (22). The remaining gradient along the spatial Z dimension (1.5 ms 15 and 30 mT/m) was used to ensure that only longitudinal magnetization survives the period τ in between proton and carbon-13 excitation pulses. All data were acquired from a $3 \times 1 \times 3$ cm volume (XYZ) positioned between 2 and 4 cm below the gradient isocenter. No ^{13}C decoupling was applied during proton signal acquisition. With the outlined parameters (TR = 3000 ms) a time-averaged RF power level of 1.86 W was measured at the input of the RF probe. Assuming an effective mass of 1 kg and a 100% power deposition within the tissue this would give a time-averaged SAR of 1.86 W/kg, which is well within the FDA guidelines for extremities of 12 W/kg averaged over 15 minutes.

Scan time reduction

The ^{13}C spectral range for all proton-bound carbons in triglycerides spans approximately 120 ppm or 9 kHz at 7 T. An indirect ^{13}C acquisition window of 64 ms will then require 576 t_1 increments of 111 μs each, which with a repetition time of 3000 ms and 8 averages as proposed here, will result in an unrealistic measurement time of 230 min (or 29 min per average). Here two mechanisms are proposed to bring the measurement time down to less than 20 min, without sacrificing sensitivity or the quantitative nature brought by the long repetition time. The first mechanism has been used previously (27,28) and entails controlled aliasing of signals by carefully adjusting the spectral width and frequency offset in the indirect dimension. The ^{13}C ‘fingerprint’ spectral region between 10 and 40 ppm is relatively congested and is not ideal for additional aliasing, even with the improved spectral

dispersion provided by the ^1H dimension. However, the triglyceride glycerol and double bond moieties are well separated in the ^1H dimension, such that they can be aliased multiple times in the ^{13}C dimension without sacrificing information content. As spectral aliasing only leads to an apparent, lower sampling frequency, the sensitivity per unit time is not compromised in any way. With an indirect spectral bandwidth of 2 kHz, none of the resonances in the ^{13}C 'fingerprint' region alias, while accelerating the measurement time 4.5 times down to 51 min (or 6.4 min per average).

The second mechanism involves varying the repetition time TR as a function of the evolution time t_1 , with longer t_1 values being obtained with shorter TRs and thus greater T_1 saturation. While a similar approach has been used to speed up MR spectroscopic imaging (MRSI) by varying the TR as a function of k-space position (29), we are not aware of this mechanism being used to accelerate multidimensional NMR acquisition. TR variation in MRSI results in a slight broadening of the spatial point spread function, whereas TR variation in 2D NMR will lead to a small increase in line widths along the indirect dimension as discussed next.

The implementation of TR variation requires a number of parameters that control the exact function of the scan-to-scan TR variation, which in turn controls the amount of line broadening. A practical approach is to determine the longest and shortest repetition times, TR1 and TR2 respectively, as the longest TR determines the quantitative aspects of the scan, whereas the shortest TR is determined by experimental parameters like allowable RF power deposition and minimum TR due to the acquisition window. Once TR1 and TR2 are fixed, the temporal weighting function (e.g. exponential, Gaussian) remains as the only variable. The additional line broadening LW_{TR} due to an exponentially decreasing weighting function is given by

$$T_{2,\text{TR}} = \frac{1}{\pi LW_{\text{TR}}} = \frac{-t_{1,\text{max}}}{\ln\left(\frac{S_2}{S_1}\right)} \quad [1]$$

where $S_1 = M_0(1 - \exp(-\text{TR}_1/T_1))$ and $S_2 = M_0(1 - \exp(-\text{TR}_2/T_1))$. The maximum t_1 increment, $t_{1,\text{max}} = (np_1 - 1)/sw_1$ where np_1 and sw_1 are the total number of t_1 increments (128 for *in vivo* data, 256 for *in vitro* data) and the spectral width of the indirect dimension (2 kHz). M_0 and T_1 are the thermal equilibrium magnetization and the longitudinal relaxation time constant for the resonance of interest. The t_1 -dependent TR variation needed to achieve exponential line broadening can then be calculated using the equation $(1 - \exp(-\text{TR}/T_1)) = \exp(-t_1/T_{2,\text{TR}})$ of which the solution is equal to

$$\text{TR} = -T_1 \ln \left[1 - \left(1 - e^{-\frac{\text{TR}_1}{T_1}} \right) e^{-\frac{t_1}{T_{2,\text{TR}}}} \right] \quad [2]$$

where t_1 runs from 0 to $t_{1,\text{max}}$. All studies presented here used $\text{TR}_1 = 3000$ ms and $\text{TR}_2 = 500$ ms, which for $T_1 = 1000$ ms leads to a 2.6-fold acceleration compared to a constant TR acquisition. Figure 2A shows the TR variation as a function of the evolution time t_1 for np_1

= 256 and $sw_1 = 2.0$ kHz. Figure 2B shows the line broadening caused by TR variation. When the carbon-13 line shape without TR variation is described by a Lorentzian line, the additional line broadening amounts to 2.2 Hz and retains the Lorentzian line shape. However, when the intrinsic ^{13}C line shape is Gaussian, the additional line broadening only amounts to 1.2 Hz as the Gaussian line is converted to a Voigt line shape. Compounds with a longer T_1 relaxation time constant will have slightly more line broadening, whereas shorter T_1 relaxation leads to dramatically reduced line broadening. Most importantly, as the initial t_1 increment is acquired with a long repetition time TR1 the integral of the 2D HSQC resonance should still be indicative of the fully-relaxed signal intensity. When the *in vivo* 2D HSQC acquisition parameters are employed with $np_1 = 128$, the line broadening for $T_1 = 1000$ ms is twice as large at 4.4 and 2.4 Hz for Lorentzian and Gaussian lines, respectively. Due to the complex T_1 dependency the line broadening for $T_1 < 1000$ ms and $T_1 > 1000$ ms will be more and less than twice, respectively. The exact line broadening is readily obtained by re-evaluating Eqs. [1] and [2] with the exact experimental parameters and T_1 relaxation time constants. Combining the two scan time reduction methods (i.e. controlled aliasing and TR variation) will lead to a total acceleration of 11.8 providing a final *in vivo* acquisition time of 19 min (or 2.4 min per average). With the parameters used for *in vivo* HSQC the sensitivity per time^{1/2} is essentially identical between variable and constant TR acquisitions.

Similar to ^{13}C MR spectroscopy, 2D HSQC spectra of triglycerides allow the determination of saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids. The fraction PUFAs can be determined from the ratio (H/I) or (G/I) whereby the label definition can be found in Fig. 4A and Table 2. The average of both ratios was used to determine the PUFA fraction. The UFA (= MUFA + PUFA) fraction can be determined as (F/I) or (E/2I). The average of both ratios was used to determine the UFA fraction. The MUFA and SFA fractions can be obtained from UFA – PUFA and $1 - \text{UFA}$, respectively. The ratio between omega-6 and omega-3 (ω_6/ω_3) fatty acids can be determined directly from the ratio (C_L/B_{Ln}) (see Table 2 for more details).

Data processing

All data were analyzed offline in home-made software written in Matlab 8.0 (The Mathworks, Natick, MA, USA). FIDs were truncated in the direct dimension at the point where the signal visually dropped below the noise level, zero filled to a $2,048 \times 2,048$ matrix, apodized and Fourier transformed. Unless mentioned otherwise, all data were processed and displayed in absolute value. *In vitro* data were not apodized, whereas *in vivo* data were processed (for display purposes only) with 15 Hz Gaussian and 25 ms shifted, 15 Hz Gaussian broadening in the direct and indirect dimensions, respectively, in order to reduce ‘spectral tailing’ common in absolute value representation.

Both *in vitro* and *in vivo* 2D HSQC resonances frequently displayed strong asymmetries (Fig. 3A) and spurious signals (Fig. 3B). Following a series of systematic experiments these were attributed to gradient-related temporal B_0 field distortions (exponentially damped eddy currents, as well as oscillatory, vibration-related effects) and long-term frequency drifts. These effects are commonly observed in localized ^1H MR spectroscopy and are typically removed by using a water reference scan (30). This approach is not an option for 2D HSQC,

as water is destroyed by the coherence selection gradients. After exploring several potential methods, Fig. 3 demonstrates the principle of the most successful method that has been employed for all data presented here. The primary assumption is that the 2D HSQC resonance of the triglyceride methyl group and its immediate surroundings can be described by a single singlet in the ^{13}C dimension and a single doublet in the ^1H dimension. Under high-resolution, liquid state conditions on pure oils multiple singlet resonances can be observed in the ^{13}C dimension, while the ^1H dimension displays fine structure due to proton-proton homonuclear scalar couplings. However, for the range of line widths encountered *in vivo*, the aforementioned assumption has been validated on a number of different oils. Inverse 2D Fourier transformation of the selected spectral region surrounding the methyl resonance gives a 'methyl-specific' FID signal (Fig. 3C). Comparing the phase of the experimental FID signal of Fig. 3C with a theoretical signal of a 2D HSQC methyl doublet signal with $^1J_{\text{HC}} = 126.6 \text{ Hz}$ provides a phase difference that is indicative of the temporal B_0 field distortions in both spectral dimensions (Fig. 3D). Multiplying the experimental FID with the negative of the phase in Fig. 3D restores the symmetrical line shape and removes all spurious signals (Figs. 3E/F). As the FID intensity modulates in the ^1H dimension due to the heteronuclear scalar coupling (see Fig. 3C), the phase is poorly defined when the FID intensity approaches zero (Fig. 3D). The phase in regions of low FID intensity could be restored by interpolation from the phase in regions of higher FID intensity. However, in practice no significant improvement could be detected in the final spectral quality, such that the phase as shown in Fig. 3D was applied directly to the data without interpolation. The explanation for this is found in the fact that most 2D HSQC resonances have low FID intensity around the same time because the heteronuclear scalar couplings are similar (Table 2).

RESULTS

Figure 4A shows a generic triglyceride with linoleic acid esterified in the β position. Figure 4B shows a 2D HSQC spectrum of vegetable oil obtained without TR variation ($\text{TR} = 3000 \text{ ms}$). Many different carbon atoms can be uniquely identified by their proton and carbon-13 chemical shifts. Figure 4C shows a 2D HSQC spectrum of the same vegetable oil sample obtained with TR variation ($\text{TR}_1/\text{TR}_2/\text{T}_1 = 3000/500/1000 \text{ ms}$). The 2.6-fold acceleration did not result in any visible artifacts or distortions. Figs. 4D/E shows the summed projections onto the carbon-13 axis of the 2D HSQC spectra shown in Figs. 4B/C, respectively. Only the methyl resonance at 14.2 ppm shows a small decrease in peak height in the TR-weighted acquisition. Table 1 gives a quantitative summary of the line broadening effects associated with TR variation. It follows that the line widths in the proton dimension (column 3) do not depend on the acquisition scheme, as expected. However, the line widths in the carbon-13 dimension (column 4) increase between 0.3 and 1.0 Hz when switching from constant to variable TR acquisition. The theoretically expected line width increases can be calculated by using the t_1 -dependent TR values and the measured proton T_1 relaxation times (column 5) and are summarized in column 6. When the native line shape acquired with constant TR is assumed to be Lorentzian, the expected line width increases are about twice as large as experimentally observed. However, with a native Gaussian line shape, the theoretically expected line width increases are in good correspondence to the measured

values. The final column shows the resonance integral obtained with TR variation as compared to that obtained with a constant TR. For all resonances the integrals are essentially identical, demonstrating that the quantitative nature of the measurement is preserved.

Figure 5A shows an anatomical MR image of the lower leg depicting the $3 \times 1 \times 3$ cm voxel placed within the subcutaneous adipose tissue. Figure 5B shows a 2D HSQC spectrum acquired with TR variation from the voxel depicted in Fig. 5A. Table 2 provides the proton and carbon-13 chemical shifts and heteronuclear scalar coupling constants for all resonances detected *in vivo*. The C2 carbon in the α -chains was used as a chemical shift reference assigned to $\delta_H = 2.25$ ppm and $\delta_C = 33.85$ ppm. This resonance was used as it provided a strong signal on all subjects and its chemical shift values are largely insensitive to the chemical composition of the fatty acid chain (31,32). Nineteen different resonances could be detected on all subjects. The chemical shift accuracy for proton and carbon-13 was typically better than 0.01 and 0.02 ppm, respectively. Increased chemical shift variability was observed for the linolenic C17 group due to a low signal-to-noise ratio and for the multiple $-(CH_2)_n-$ groups due to spectral overlap. Even though the *in vivo* 2D HSQC spectra only display three distinct $-(CH_2)_n-$ resonances, high-resolution 2D HSQC spectra reveals at least ten distinct $-(CH_2)_n-$ resonances (data not shown).

The peak assignments shown in Fig. 5B largely mirror those in Fig. 4B with the exception of the resonances appearing between the proton chemical shifts of 3.5 and 6.0 ppm. These resonances originate from the glycerol (K and L) and double-bond (F and G) moieties and appear at aliased carbon-13 chemical shifts, as indicated by the asterisk. Specifically, the α -glycerol resonance L is aliased once and appears at a lower frequency given by $62.06 - 26.68 = 35.38$ ppm, where 26.68 ppm is the carbon-13 spectral width. The β -glycerol resonance K is aliased twice from 69.21 ppm to 15.85 ppm and the double-bond resonances F and G are aliased four times, appearing 106.72 ppm below the real carbon-13 chemical shifts. The presence of spectral aliasing does not lead to assignment ambiguities due to the extra spectral resolution afforded by the proton dimension.

Increasing the vertical scale twenty-fold allows the detection of linolenic acid at $\delta_H = 2.04$ ppm and $\delta_C = 20.74$ ppm as shown in the inset of Fig. 5B. Figure 5C–E shows the summed projections onto the carbon-13 axis, revealing a fully decoupled ^{13}C NMR spectrum obtained without high-powered broadband decoupling. The projections in Fig. 5C and D were obtained by summing the 2D HSQC spectrum over the proton chemical shift ranges [0.0 ... 3.5] ppm and [4.5 ... 6.0] ppm, respectively. The projection in Fig. 5E required frequency-shifting the 2D HSQC data along the carbon-13 axis until glycerol resonances K and L were both aliased twice. The projection was then calculated over the proton chemical shift range [3.5 ... 6.0] ppm. While the projections closely resemble conventional ^{13}C -[1H] NMR spectra, there are a number of smaller resonances unique to 2D HSQC projections. Examples can be seen at carbon-13 chemical shifts 27.41 ppm (134.14 ppm aliased ^{13}C position) (Fig. 5C) and 128.16 ppm (21.44 ppm aliased ^{13}C position) (Fig. 5D) ppm. These signals arise from proton/carbon-13 interactions over more than one chemical bond. These so-called heteronuclear multiple-bond correlation (HMBC) signals are well-known from high-resolution NMR and can aid in the assignment of unknown compounds (33,34). The HMBC resonance in Fig. 5C corresponds to a correlation between carbon G and protons

attached to the adjacent carbon H, indicated by G^*CH_H . The asterisk indicates again that the carbon-13 chemical shift is aliased. Whereas the actual GCH_H resonance appears at $\delta_H = 2.76$ ppm and $\delta_C = 128.16$ ppm, the aliased G^*CH_H resonance is visible at $\delta_H = 2.76$ ppm and $\delta_C = 21.44$ ppm (see inset in Fig. 5B). Similarly, the signal E^*CF_H visible in Fig. 5D originates from a correlation between carbon E and the protons attached to the adjacent carbon F. Particularly strong HMBC signals can be observed between carbon F and the protons on carbon E (FCE_H) and the carboxyl carbon and the protons on carbon J ($COOJ_H$). The HMBC signals are readily distinguished from HSQC signals by the heteronuclear scalar coupling in the proton dimension, which is typically < 10 Hz for HMBC signals. As the chemical structure and NMR assignment of triglycerides is well known, the HMBC resonances simply act as confirmatory signals. However, when studying unknown compounds, the HMBC signals may be emphasized by using a longer echo-time and can provide useful information for structure determination.

The amounts of SFAs, MUFAs and PUFAs could be determined on all subjects and averaged to 35 ± 4 %, 44 ± 5 % and 21 ± 2 % (mean $\pm$ standard deviation, $n = 6$), respectively. The omega-6/omega-3 ($\omega 6/\omega 3$) ratio could be determined on four subjects due to the limited SNR on the linolenic ' $\omega 3$ ' acid signal. The $\omega 6/\omega 3$ ratio was determined at 6.3 ± 2.9 (mean $\pm$ standard deviation, $n = 4$, range = 3.0 – 9.3).

DISCUSSION

Here we have presented a novel method for high-quality, high-sensitivity 2D HSQC studies on human adipose tissue *in vivo* at 7 T. Artifact-free 2D HSQC spectra could be acquired on all subjects, whereby the high spectral resolution of the indirect ^{13}C dimension enabled the detection of 19 resonances that could be assigned to unique chemical groups. Spectral lineshape distortions due the hardware imperfections (eddy currents, vibrations) were robustly eliminated by using the 2D methyl resonance as a reference. The indirect ^{13}C dimension was intrinsically proton-decoupled without the need for high-powered broadband decoupling methods, thereby greatly reducing the RF power deposition which, in turn enabled acceleration by TR variation. In combination with controlled aliasing a total acceleration of 11.8x could be achieved, leading to a reasonable scan time of 19 min.

The spectral information content of 2D HSQC spectra is not necessarily higher than that of direct ^{13}C MRS (35–38). However, 2D HSQC gains additional sensitivity and reduced RF power deposition while retaining a similar ^{13}C spectral resolution. Previous direct ^{13}C MRS studies of human adipose tissues have demonstrated the ability to characterize the triglyceride saturation profile (35–38) and even the $\omega 6/\omega 3$ ratio (39). However, to enhance the ^{13}C MR sensitivity most of these studies were performed essentially unlocalized without a clear definition of the spatial volume. As a result, significant amounts of intramuscular lipids could contribute to the observed ^{13}C MR spectrum. In contrast, the current study used a well-defined 9 mL volume placed in pure adipose tissue, thereby eliminating any partial volume effects. The SFA, MUFA and PUFA fractions obtained here are in reasonable agreement with previous values obtained by direct ^{13}C MRS studies (35–39). And despite the small detection volume (9 mL) small signals for linolenic acid could be reliably detected on four subjects, giving rise to a fairly large $\omega 6/\omega 3$ ratio range (3.0 – 9.3). The interpretation

of the quantitative lipid characterization should be done with caution as the current study employed a small, heterogeneous subject group. For example, it is known that the adipose lipid profile is dependent on diet (37,38). The current study did not control for diet, nor for age or gender. Nevertheless, the presented results demonstrate that 2D HSQC is capable of quantitative characterization of adipose lipids from small, well-defined volumes.

Phase-sensitive processing and display of 2D HSQC spectra acquired with a single coherence selection gradient combination (2:2:+1) will result in resonances exhibiting a phase-twisted line shape (23). The standard method to remove the phase-twist and obtain pure absorption line shapes is to acquire 2D HSQC with a coherence selection gradient combination (2:2:-1) that selects the alternate coherence pathway during the t_1 evolution time (22). Converting the phase-modulated 2D HSQC data to amplitude modulation will then give pure absorption line shapes. With the acquisition of eight averages, the current *in vivo* 2D HSQC protocol would lend itself to the acquisition of two data sets of four averages without a time penalty. However, this was not pursued as the minimum t_1 evolution period was rather long at 6.0 ms due to the coherence selection gradients and proton and carbon-13 RF pulses. A large initial t_1 evolution delay would lead to a very large linear phase roll across the spectrum which would lead to baseline problems with direct linear phase correction or unreliable results when using linear back projection over 12 data points (representing almost 10% of the total number of t_1 increments). In high-resolution, liquid-state NMR a non-negligible initial t_1 period is typically compensated with an additional carbon-13 refocusing pulse. However, provided that a low peak RF power (1.5 kHz), wide bandwidth (>120 ppm) refocusing pulse could be generated, the increased RF power deposition would overwrite the advantages of phase-sensitive detection. As a result, all 2D HSQC data reported here were processed and displayed in absolute value. This in turn opened the possibility of using wideband carbon-13 AFP excitation pulses for which the non-linear phase had no effect of the 2D HSQC spectral appearance. Absolute valued representation will decrease the spectral resolution due to significant tailing from the dispersive component. However, 2D HSQC spectra of triglycerides provided sufficient spectral resolution to avoid spectral overlap of all signals of interest. Absolute-valued processing was felt to be a disadvantage during signal integration to determine the various fatty acid fractions. As the spectral noise is also absolute-valued, integration starting from the zero baseline would lead to artificially high integrals which are dependent on the exact integration boundaries. For that reason signals were thresholded slightly above the spectral noise floor leading to consistent integrals. However, the choice of threshold level makes integration subjective. Work is currently in progress to perform 2D spectral fitting with simulated 2D basis sets on the phase-sensitive 2D HSQC data in order to achieve automated and objective quantitation. The presence of phase-twisted line shapes, higher-order phase rolls, (strong) ^1H - ^1H scalar coupling and ^{13}C - ^{13}C isotopomers can all quantitatively be accounted for, whereas the phase-sensitive noise prevents bias in the determined resonance intensities.

Several sources of signal loss can be identified and, depending on the application, eliminated. Firstly, the 2D HSQC pulse sequence achieves spatial localization based on 3D STEAM. It is well-known that 50% of the signal is lost by storing only half of the

magnetization along the longitudinal axis following the second 90° pulse. Ensuring signal refocusing prior to the second 90° pulse by employing a proton refocusing pulse will avoid the signal loss. However, besides the increased RF power deposition from the proton 180° RF pulse and the mandatory carbon-13 180° RF pulse needed to retain heteronuclear scalar coupling evolution, the spatial localization would lose its single-shot capability and become dependent on phase cycling. Another option would be to employ an alternative localization method such as PRESS (40), LASER (41) or semi-LASER (42) that inherently retain 100% of the available magnetization. Unfortunately, the proton 180° refocusing pulses would greatly increase the RF power deposition and demand the use of broadband carbon-13 180° inversion pulses to generate anti-phase coherence, all of which lead to a substantial lengthening of the echo-time TE. Whereas with the current surface coil setup a semi-LASER method required a minimum echo-time of 35 ms, the STEAM method was executed with TE = 7.8 ms (minimum TE = 6.0 ms). Given the short T₂ relaxation times and the extensive homonuclear scalar couplings of triglycerides (proton-only) STEAM provided 92% of the semi-LASER signal intensity of triglycerides in human leg *in vivo* (data not shown) thereby essentially nullifying the intrinsic signal loss. In addition the large bandwidth of 13.72 kHz achievable with 0.5 ms excitation pulses essentially eliminated chemical shift displacement artifacts. Furthermore, the low RF power deposition of STEAM allowed a six-fold variation of the repetition time, thereby indirectly increasing the sensitivity through more extensive signal averaging within the same experimental time.

Secondly, coherence pathway selection was achieved with magnetic field gradients leading to a 2-fold signal reduction compared to a phase-cycled variant. The primary reason for using magnetic field gradients is that desired signal selection (1% ¹³C natural abundance triglycerides) and undesired signal destruction (99% ¹²C triglycerides) are achieved simultaneously in a single scan. Phase cycling requires a minimum of two scans during which system, subject or other instabilities will lead to subtraction artifacts. The incoherent subtraction artifacts would lead to the well-known 't₁ noise' artifact, making detection of small 2D signals difficult or even impossible. With magnetic field gradients the t₁ noise was reduced to below the spectral noise floor, both *in vitro* and *in vivo*. Although not a strong consideration for the current application, coherence selection with magnetic field gradients would also provide an excellent, intrinsic water suppression. Given the large ratio between undesired and desired signal in the current study, the two-fold signal loss was well justified to minimize t₁ noise and obtain highly reproducible 2D HSQC spectra. However, in applications where the ¹³C enrichment has been increased, for example by intravenous infusion of ¹³C-labeled substrates, the use of phase cycling to increase sensitivity may be considered. Prime examples are the detection of ¹³C-labeled intermediates of energy and neurotransmitter metabolism, like glutamate and glutamine in the brain. Previous ¹H-[¹³C] ('POCE') MR studies have demonstrated that the brain is sufficiently stable to allow high-quality detection of ¹³C-labeled compounds through signal subtraction (9). In addition to the enhanced sensitivity, phase-cycling also provides the opportunity to acquire ¹H-[¹²C] data in addition to the ¹H-[¹³C] 2D HSQC data. The ¹H-[¹²C] data is useful in the determination of absolute metabolite concentrations and ¹³C fractional enrichments, but can also be used to monitor and correct any phase and frequency instabilities throughout the course of 2D HSQC signal acquisition.

Thirdly, the absence of broadband decoupling during acquisition leads to the formation of doublet signals along the proton dimension, thereby reducing the peak height two-fold and the (integrated) signal-to-noise ratio by 2. On lower magnetic fields and animal MR systems broadband decoupling can certainly be applied, thus simplifying the spectrum and enhancing the sensitivity. However, besides the obvious increase in RF power deposition, broadband decoupling comes with a number of considerations. In general, broadband decoupling leads to decoupling sidebands which become more pronounced at the lowest possible RF power settings typically used for *in vivo* applications (43). Besides reducing the apparent spectral signal-to-noise ratio, decoupling sidebands can be misinterpreted as genuine signal. In addition, broadband decoupling normally requires multiple filters on both the proton and carbon-13 channels, thereby complicating the experimental setup and potentially reducing the sensitivity. Finally, even on animal systems it is virtually impossible to achieve broadband decoupling over the entire ^{13}C spectral range due to limitations on the maximum available RF amplitude. Similarly to the arguments laid out for STEAM localization and magnetic field gradient coherence pathway selection, the advantages of broadband decoupling must be offset against the experimental complexity, RF power deposition and potential artifacts. At very high magnetic fields broadband decoupling (on the human head) is simply no longer an option as adequate performance cannot be achieved within the mandated SAR guidelines. For 2D HSQC the absence of broadband decoupling actually provided an unexpected advantage in that 2D HSQC resonances could be readily separated from 2D HMBC resonances based on the heteronuclear scalar coupling splitting along the proton dimension. In the presence of broadband decoupling 2D HMBC signals could have been incorrectly interpreted as 2D HSQC signals from low concentration compounds. The absence of broadband decoupling also allows the heteronuclear scalar coupling (Table 2) to be used as an additional means of peak identification.

The sensitivity of the current 2D HSQC data was sufficiently high to detect the small signal of linolenic acid at $\delta_{\text{H}} = 2.04$ ppm and $\delta_{\text{C}} = 20.74$ ppm on all subjects from a relatively small 9 mL voxel. No signals from trans fatty acids could be detected on any subject, nor on the summed 2D HSQC spectrum from all subjects. It should be noted that the currently used proton surface coil was not optimized for adipose tissue surrounding the calf muscle. A phased array of multiple, small (4 cm diameter) surface coils would likely lead to a substantial sensitivity increase. Under idealized conditions the relative sensitivities for direct ^{13}C MRS, polarization transfer-enhanced ^{13}C MRS and indirect ^1H - ^{13}C MRS are 1, 4 and 32, respectively (44). Even with the sensitivity losses due to STEAM localization and gradient coherence selection the sensitivity for HSQC would still be superior to direct ^{13}C MR detection with the added advantage of a greatly reduced RF power deposition. However, under realistic conditions the sensitivity is affected by, among many other parameters, multiplicity, T_1 and T_2 relaxation and sequence timings. In addition, sensitivity should be evaluated in concert with RF power deposition, spectral resolution and information content. While these comparisons are beyond the scope of the current manuscript, they are actively pursued in our laboratory.

Despite the widespread use of 2D NMR methods in high-resolution, liquid-state NMR, the popularity of 2D NMR for *in vivo* applications has always been limited. One of the primary

reasons for this is the fact that traditional 2D NMR is time-consuming, requiring many t_1 increments to ensure adequate spectral resolution in the indirect spectral dimension. Both the MRI (45,46) and 2D NMR (47) fields have seen the development of a number of acceleration methods aimed at reducing the total experiment duration. Regardless of the exact details all these methods are fundamentally based on a trade between sensitivity and temporal resolution. Unfortunately, as most *in vivo* ^{13}C MRS applications are sensitivity limited the achievable acceleration will be limited. Here we have demonstrated novel acceleration methods that are based on a trade between spectral and temporal resolution. Controlled aliasing is a method that has been previously used to achieve acceleration factors up to five times (27,28). The primary requirement for controlled aliasing is that the information content of the spectrum is roughly known such that the indirect spectral width can be chosen as to minimize or avoid spectral overlap due to aliasing. For most *in vivo* applications the spectral peak positions are well known, making controlled aliasing a straightforward acceleration method. Controlled aliasing can become problematic when aliasing of unknown signals (e.g. due to disease) leads to unwanted spectral overlap. In this case the indirect bandwidth can be changed slightly to change the aliased frequency or selective ^{13}C excitation pulses can be used to completely eliminate spectral aliasing. Decreasing the repetition time TR with increasing t_1 evolution times was used as a secondary acceleration method. Previously, 2D MR spectroscopic imaging implemented with a decreasing TR for increasing k-space coordinates has been described (29). The increased T_1 -weighting for higher k-space coordinates would lead to a small increase in the spatial MRSI voxel size ('point spread function'). Similarly, the increased T_1 -weighting for longer t_1 evolution delays as employed here led to a small, T_1 -dependent line broadening. A 2.6-fold acceleration led to relatively small (3–14%, Table 1) line width increases while still retaining the quantitative nature of the scan. Additional scan time reduction can potentially be found in more optimal (possibly non-linear) t_1 sampling and the use of heavily truncated t_1 sampling in combination with 2D spectral fitting.

2D HSQC has been used to study the triglyceride profile in adipose tissue at ^{13}C natural abundance. However, 2D HSQC is by no means limited to natural abundance studies and has the potential to enhance studies where the metabolic fate of ^{13}C -enriched substrates are detected. For example, the intravenous infusion of $[1-^{13}\text{C}]$ -glucose allows the detection of $[4-^{13}\text{C}]$ -glutamate, which is indicative of TCA cycle activity. Detection of $[4-^{13}\text{C}]$ -glutamine in the human brain is indicative of a glutamate-glutamine neurotransmitter cycle (7). Whereas ^1H - $[^{13}\text{C}]$ 'POCE' MRS (9) has been used to enhance the detection sensitivity, the spectral resolution of ^1H NMR is typically not sufficient to reliably separate glutamate from glutamine. 2D HSQC utilizes the high chemical specificity of ^{13}C MRS to separate $[4-^{13}\text{C}]$ -glutamate from $[4-^{13}\text{C}]$ -glutamine while still retaining the high proton detection sensitivity. The combination of high sensitivity and high specificity can make 2D HSQC the default method for the study of ^{13}C -labeled compounds *in vivo*, at natural abundance or during the infusion of ^{13}C -labeled substrates. Low field applications can benefit from the high sensitivity and resolution, whereas high field (7^+ T) applications can, in addition, benefit from the low RF power deposition. Direct ^{13}C MR methods would only be required for specialized applications that require, for example the detection of bicarbonate (48) or unprotonated carbon positions (12,13).

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Figure 1.

3D localized 2D HSQC pulse sequence. The high bandwidth (13.72 kHz) ^1H 90° pulses achieve 3D STEAM localization with minimal chemical shift displacement, whereas the ^1H 180° pulse refocuses ^1H - ^{13}C scalar coupling evolution during t_1 thereby leading to broadband ^1H decoupling. A wide range of ^{13}C 90° pulses are possible for broadband (>200 ppm) to narrowband excitation. The coherence selection gradients (gray, 2:2:1 ratio) achieve single-scan suppression of unwanted signals. At 7 T broadband decoupling during acquisition is not possible due to excessive RF power deposition. However, it is an option at lower magnetic fields to increase the sensitivity by an additional factor 2. Note that when the two 90° pulses are merged into a single 180° inversion pulse ($t_1 = 0$, no coherence selection gradients) the sequence reduces to a two-step proton-observed, carbon-edited (POCE) method retaining the high ^1H sensitivity, but forfeiting any ^{13}C -related information.

Figure 2.

(A) Repetition time variation as a function of evolution time t_1 calculated using Eqs. [1] and [2] with $TR_1/TR_2/T_1 = 3000/500/1000$ ms. The total number of t_1 increments, np_1 , and spectral width in the indirect dimension, sw_1 , were 256 and 2.0 kHz, respectively. *In vivo* 2D HSQC studies with $np_1 = 128$ would show an identical TR variation (over half the t_1 time base) thereby leading to an identical acceleration factor. (B) Simulated line broadening, measured as the full width at half maximum (FWHM) caused by the TR variation shown in (A) as a function of T_1 relaxation time for Lorentzian and Gaussian lines. *In vivo* 2D HSQC studies with $np_1 = 128$ would have exactly twice the line broadening for $T_1 = 1000$ ms, but more and less than twice for $T_1 < 1000$ ms and $T_1 > 1000$ ms, respectively.

Figure 3.

Correction of temporal B_0 modulations on a vegetable oil phantom. (A) Triglyceride methyl resonance at $\delta_H = 0.89$ ppm and $\delta_C = 14.16$ ppm obtained by 2D Fourier transformation of the experimental 2D HSQC data without apodization. (B) With a 2x increased vertical scale a highly asymmetrical doublet resonance surrounded by spurious signals (arrows) is apparent. (C) Magnitude FID data obtained by inverse 2D Fourier transformation of the methyl resonance region shown in (A/B). (D) Phase difference between the complex-valued FID signal in (C) and an ideal, complex-valued FID from a non-distorted methyl doublet resonance. (E/F) Complex multiplication of the experimental 2D HSQC time-domain data with the negative of the phase shown in (D) provides correction of temporal B_0 modulations and gives symmetrical signals upon 2D FFT. (F) is identical to (E) at a 2x increased vertical scale.

Figure 4.

(A) Structure of a triglyceride in which the β -glycerol position is esterified to linoleic acid. The α -glycerol positions are esterified to undefined R and R' fatty acid chains. The carbon position labels A and D through K are generally applicable, whereas labels B and C are unique in the case of linolenic and linoleic acid, respectively. In those cases the labels become B_{Ln} and C_L . (B/C) 2D HSQC data from a vegetable oil phantom acquired with (B) constant and (C) variable repetition times ($TR1/TR2/T_1 = 3000/500/1000$ ms). The indicated assignments correspond to the label defined in (A). Note that resonances B and C are split due to unique B_{Ln} and C_L moieties and resonance J is split due to the different chemical environments caused by the α and β -glycerol groups. Resonance D is composed of between 3 and 10 resonances. The chemical similarities of the various D groups make exact assignment difficult. (D/E) Summed 1D projections of the 2D HSQC spectra in (B/C) onto the carbon-13 axis demonstrate minor line increases in (E). The line width increase and associated peak height reduction is most noticeable for the methyl resonance at 14.16 ppm (see Table 1 for a quantitative line width evaluation).

Figure 5.

2D HSQC on human adipose tissue *in vivo*. (A) Anatomical MRI showing the placement of a $3 \times 1 \times 3$ cm voxel. (B) 2D HSQC spectra acquired from the voxel shown in (A) in 19 min with TR variation ($TR_1/TR_2/T_1 = 3000/500/1000$ ms). Besides the extended proton chemical shift axis, the 2D HSQC spectrum looks similar to those acquired *in vitro* (Fig. 4). Labels with an asterisk (*) indicate that the resonance is aliased along the carbon-13 dimension by an integer number of the carbon-13 spectral width. The inset shows the linolenic acid resonance B_{Ln} with a twenty-fold increased vertical scale, together with a 1H - ^{13}C resonance from correlation over multiple chemical bonds between the G position carbon-13 nucleus and the H position protons. (C-E) Summed 1D projections of the 2D HSQC spectra onto the carbon-13 axis. (C) 1D projection of the ^{13}C 'fingerprint' region summed over proton chemical shifts between 0.0 and 3.5 ppm. (D/E) 1D projection of the double-bond carbons (D) and glycerol carbons (E) summed over proton chemical shift ranges [4.5 ... 6.0] ppm and [3.5 ... 6.0] ppm, respectively. Since resonances K and L were aliased by different amounts, the 2D HSQC spectrum was frequency shifted to place K and L within the same spectral width before calculating the 1D projection.

Table 1Evaluation of spectral differences due to t_1 -dependent TR variation

Carbon position ¹	label ²	experimental line widths (Hz) ³		proton T ₁ (ms)	theoretical line width differences (Hz) ⁴	integral (%) ⁵
		proton	carbon-13		carbon-13 (Lorentzian/Gaussian)	
$\text{CH}_3\text{-CH}_2\text{-}$	A	13.8/-0.2	7.4/+1.0	898	2.0/1.1	98
$\text{-CH}_2\text{-CH}_2\text{-COOR}$	I	23.2/+0.2	9.3/+0.3	332	0.6/0.3	101
$\text{=CH-CH}_2\text{-CH=}$	H	14.1/-0.3	7.7/+0.5	469	1.0/0.5	99
$\text{=CH-CH}_2\text{-CH}_2\text{-}$	E	20.5/+0.1	8.9/+0.4	433	0.9/0.5	101
$\text{-CH}_2\text{-CH}_2\text{-COOR (}\alpha\text{)}$	J	17.4/0.0	8.3/+0.3	366	0.7/0.4	103

¹ C indicates the specific carbon position along the lipid carbon chain.² Carbon position label is defined in Figure 5A.³ Line widths are defined as 'FWHM without TR variation/FWHM with TR variation minus FWHM without TR variation'.⁴ Theoretical line width increase defined as 'FWHM with TR variation minus FWHM without TR variation' for Lorentzian and Gaussian lines.⁵ Integral ratio defined as ' $100 \times \text{integral without TR variation} / \text{integral with TR variation}$ '.

Table 2
Proton and carbon-13 chemical shifts and heteronuclear scalar couplings for triglycerides *in vivo*²

Carbon position	I	label	chemical shift (ppm)		scalar coupling (Hz)	remarks
			proton	carbon-13	$^1J_{HC}$	
$\underline{CH}_3\text{-CH}_2\text{-}$		A	0.895 ± 0.004	14.163 ± 0.010	126.6 ± 0.7	
$CH_3\text{-}\underline{CH}_2\text{-CH=}$		B _{Ln}	2.040 ± 0.018	20.737 ± 0.072	132.8 ± 4.6	linolenic 'ω3' acid
$CH_3\text{-}\underline{CH}_2\text{-CH}_2\text{-}$		B	1.310 ± 0.005	22.941 ± 0.009	130.0 ± 0.6	
$\text{-}\underline{CH}_2\text{-CH}_2\text{-COOR}$		I	1.580 ± 0.003	25.043 ± 0.006	136.1 ± 1.4	
$=\underline{CH}\text{-}\underline{CH}_2\text{-CH=}$		H	2.764 ± 0.003	25.821 ± 0.009	127.4 ± 2.2	
$=\underline{CH}\text{-}\underline{CH}_2\text{-CH}_2\text{-}$		E	2.030 ± 0.005	27.406 ± 0.005	129.4 ± 1.1	cis isomer
$\text{-(}\underline{CH}_2\text{)}_n\text{-}$		D	1.329 ± 0.004	29.313 ± 0.029	133.8 ± 2.5	
$\text{-(}\underline{CH}_2\text{)}_n\text{-}$		D	1.313 ± 0.017	29.628 ± 0.026	128.6 ± 2.8	
$\text{-(}\underline{CH}_2\text{)}_n\text{-}$		D	1.324 ± 0.013	30.088 ± 0.010	132.9 ± 1.9	
$CH_3\text{-CH}_2\text{-}\underline{CH}_2\text{-CH}_2\text{-}$		C _L	1.295 ± 0.007	31.883 ± 0.030	127.8 ± 2.1	linoleic 'ω6' acid
$CH_3\text{-CH}_2\text{-}\underline{CH}_2\text{-CH}_2\text{-}$		C	1.275 ± 0.006	32.238 ± 0.013	125.8 ± 1.0	
$\text{-CH}_2\text{-}\underline{CH}_2\text{-COOR (}\alpha\text{)}$		J(α)	2.250	33.850	131.7 ± 1.3	chemical shift reference
$\text{-CH}_2\text{-}\underline{CH}_2\text{-COOR (}\beta\text{)}$		J(β)	2.251 ± 0.007	34.120 ± 0.024	132.9 ± 2.1	
$H_2\text{-}\underline{COOR (}\alpha\text{)}$		L	4.302 ± 0.010	62.053 ± 0.010	161.3 ± 0.9	glycerol H1
$H_2\text{-}\underline{COOR (}\alpha\text{)}$		L	4.074 ± 0.006	62.060 ± 0.012	159.3 ± 1.8	glycerol H1'
$H\text{-}\underline{COOR (}\beta\text{)}$		K	5.220 ± 0.004	69.210 ± 0.014	155.4 ± 2.0	glycerol H2
$\text{-CH=}\underline{CH}\text{-CH}_2\text{-}\underline{CH=CH-}$		G	5.307 ± 0.005	128.164 ± 0.008	159.8 ± 2.8	
$\text{-CH=CH-CH}_2\text{-CH=}\underline{CH-}$		F	{	5.331 ± 0.003	129.683 ± 0.015	157.5 ± 2.3
$\text{-CH}_2\text{-}\underline{CH=CH-CH}_2\text{-}$				5.333 ± 0.003	129.905 ± 0.017	156.4 ± 0.9

I_C indicates the specific carbon position along the lipid carbon chain. Carbon position label is defined in Fig. 5A.

² All values represent the mean ± standard deviation over six subjects.