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^{13}C isotopomer analysis of glutamate by heteronuclear multiple quantum coherence-total correlation spectroscopy (HMQC-TOCSY)

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Abstract **^{13}C has become an important tracer isotope for studies of intermediary metabolism. Information about relative flux through pathways is encoded by the distribution of ^{13}C isotopomers in an intermediate pool such as glutamate. This information is commonly decoded either by mass spectrometry or by measuring relative multiplet areas in a ^{13}C NMR spectrum. We demonstrate here that groups of glutamate ^{13}C isotopomers may be quantified by indirect detection of protons in a 2D HMQC-TOCSY NMR spectrum and that fitting of these data to a metabolic model provides an identical measure of the ^{13}C fractional enrichment of acetyl-CoA and relative anaplerotic flux to that given by direct ^{13}C NMR analysis. The sensitivity gain provided by HMQC-TOCSY spectroscopy will allow an extension of ^{13}C isotopomer analysis to tissue samples not amenable to direct ^{13}C detection (~10 mg soleus muscle) and to tissue metabolites other than glutamate that are typically present at lower concentrations.**

Key words: Indirect detection; ^{1}H Nuclear magnetic resonance; ^{13}C isotopomer analysis; Two-dimensional nuclear magnetic resonance; Heteronuclear multiple quantum coherence-total correlation spectroscopy

1. Introduction

^{13}C NMR isotopomer analysis is a powerful tool for evaluating relative fluxes through biochemical pathways [1–4]. The substantial advantages offered by the high information content in a ^{13}C NMR spectrum over other carbon tracer methods can be implemented whenever metabolite concentrations are high or the amount of tissue is not limited. Extension of these methods to human samples [5] or small tissue samples from mice [6], for example, will ultimately be limited by the NMR insensitivity of ^{13}C. One solution is to apply indirect methods whereby the heteronucleus is detected via its spin interactions with ^{1}H [7,8]. Although these methods do provide a gain in sensitivity, information about ^{13}C isotopomer populations that is normally derived from ^{13}C multiplet areas is typically lost. Numerous homonuclear [9–14] and heteronuclear [15–20] 2D NMR methods have been used to resolve, assign, or quantify tissue metabolite resonances, but this powerful type of experiment has not been applied to ^{13}C isotopomer analysis. We describe here a method for ^{13}C isotopomer analysis of tissue extracts using heteronuclear multiple quantum coherence-total correlation (HMQC-TOCSY) NMR spectroscopy [21–23]. The initial multiple quantum HMQC sequence filters out all protons not spin-coupled to a ^{13}C while addition of the TOCSY sequence distributes this information to all protons and thereby allows for the detection of long-range as well as direct ^{1}H-^{13}C correlations. We have found that this combined sequence provides different but complimentary positional ^{13}C isotopomer information which can be incorporated into existing mathematical models of intermediary metabolism. We show here that cross-peaks areas in a HMQC-TOCSY spectrum provides a readout of ^{13}C fractional enrichment of acetyl-CoA (F_{C2}) and relative anaplerotic flux (y) and demonstrate that the technique may be used to derive significant metabolic information from as little as 10 mg of muscle tissue.

2. Materials and methods

2.1. Heart perfusions

Rat hearts were rapidly excised from male Sprague-Dawley rats after general anesthesia. Isolated hearts were perfused using conventional Langendorff methods (retrograde) at 100 cm H_2O with Krebs-Henseleit bicarbonate buffer (KH) bubbled continuously with a 95/5 mixture of O_2/CO_2 at 37°C. The buffer (300 ml recirculating volume) contained 2 mM [2-^{13}C]acetate as the only oxidizable substrate. After 30 min, hearts were freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen. In a separate experiment, sodium [2,4,6,8-$^{13}C_4$]octanoate (30 mg dissolved in 200 μl of water) was infused into a live, anesthetized mouse for 30 min (1 mg/min). The animal was then killed and one soleus muscle was removed and freeze-clamped. All tissues were extracted with ice-cold perchloric acid, neutralized with KOH, freeze-dried, and dissolved in 600 μl of D_2O for NMR analyses. The pH of all extract samples was adjusted to 2.6–2.8 using HCl prior to 2D NMR analysis to simplify the ^{1}H NMR spectra of glutamate. By lowering the pH, the two H3 protons become more equivalent, and the chemical shift difference between H3 and H4 is increased, thus removing the overlap between ^{13}C satellites.

2.2. NMR spectroscopy

HMQC-TOCSY and ^{13}C NMR spectra were collected using 5 mm inverse and 5 mm broadband probes, respectively, on either an 11.75 T or 14.1 T Unity INOVA spectrometer (Varian Instruments, Palo Alto, CA). A TOCSY mixing time of 40 ms was used. The HMQC-TOCSY spectra were acquired with 2048 points in F2 and 128 increments ($n_i = 64$, phase arrayed) in F1. The spectral widths were chosen so that a range of 4 ppm would be covered in the ^{1}H dimension (1.5–5.5 ppm) and 40 ppm (22–62 ppm) in the ^{13}C dimension. The 2048×128 matrices were zero filled to 4096×512 and multiplied by gaussian functions in both dimensions before Fourier transformation.

Given that weighting functions applied to FIDs prior to transformation will affect measured cross-peak volumes in 2D spectra, all calibration standards and heart extracts were acquired and processed using identical parameters so that a direct comparison of cross-peak volumes could be made. Cross-peak volumes in the HMQC-TOCSY

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spectra were measured using Varian NMR software (Version 5.3b) after defining each cross-peak manually with a volume box that included approximately 25–30% baseline in each spectral dimension [24]. A comparison of volumes was made for different sizes of the volume boxes. Providing that adequate baseline was included in the contour map defining the cross-peak, no significant differences were observed in the volumes measured by the fitting program for a wide range of baseline percentages (20–100% relative to width of cross-peak).

^{13}C NMR spectra were acquired by using a 45° pulse, a 3 s repetition time, and WALTZ-16 for broadband ^{1}H decoupling. The FIDs were multiplied by an exponential weighting function corresponding to a LB = 0.5 Hz prior to transformation. ^{13}C NMR multiplets were deconvoluted using the Varian software and the line-fitting subroutine of the PC-based NMR software, NUTS (Acorn NMR, Fremont, CA).

2.3. Standard curves

Samples containing various, known amounts of [4-^{13}C]- and [U-$^{13}C_5$]glutamate were used to obtain normalization factors for all cross-peak volumes in the HMQC-TOCSY spectra. A stock solution containing ~10 mM [4-^{13}C]- and [U-$^{13}C_5$]glutamate, pH 2.75, was prepared in 99.9% D_2O also containing 300 mM NaCl (average ionic strength measured experimentally in heart extracts). This solution was diluted sequentially with a second 300 mM NaCl/D_2O solution to vary the isotopomer concentration over a range of concentrations similar to those found in tissue extracts.

2.4. Sensitivity enhancement

The experimental gain in sensitivity provided by the 2D HMQC-TOCSY experiment versus 1D direct ^{13}C observe was estimated using a mixture of glutamate ^{13}C isotopomers isolated from tissue. A standard 1D ^{13}C NMR spectrum was collected using the same acquisition parameters described above and a 2D HMQC-TOCSY spectrum was collected using the same total data collection time. Identical weighting functions as described above for tissue samples were applied prior to Fourier transformation. Average signal-to-noise was measured separately for the glutamate C2, C3 and C4 resonances in the 1D spectrum and for their respective ^{1}H traces in the HMQC-TOCSY spectrum.

3. Results

An 2D HMQC-TOCSY NMR spectrum of a solution containing an equimolar mixture of [4-^{13}C]- and [U-^{13}C]glutamate is shown in Fig. 1. Unlike a conventional 1D ^{1}H spectrum, this 2D spectrum contains ^{13}C isotopomer information because the cross-peaks correlate ^{13}C enrichment at every protonated carbon, not just the directly bonded carbon. This is illustrated by the three cross-peaks located at 31 ppm on the ^{13}C axis (C4 resonance) and 3.85 ppm on the ^{1}H axis (H2 resonance). This triad of cross-peaks consists of a singlet (C4H2S) and a doublet (C4H2D) component. The C4H2S cross-peak reflects all isotopomers having a ^{13}C at C4 but not at C2 (i.e. [4-^{13}C]glutamate), while C2H4D reflects all isotopomers having ^{13}C at both C2 and C4 (i.e. [U-$^{13}C_5$]glutamate). Thus, the ratio of these two cross-peak volumes should reflect the ratio of these two groups of isotopomers. As shown below, the cross-peak volumes in any S/D triad may be compared directly (no correction factors required), so for the equimolar mixture of [4-^{13}C]- and [U-$^{13}C_5$]glutamate used in this experiment, the C4H2S and C4H2D cross-peak volumes were equal.

Before HMQC-TOCSY cross-peak volume data can be used in a metabolic isotopomer analysis, one must verify that the procedure is quantitative. Fig. 2 shows plots of HMQC-TOCSY cross-peak volumes as a function of glutamate isotopomer concentration, measured over a range typical of that found in heart tissue extracts. This titration was performed by diluting a sample initially containing 9.7 mM [U-$^{13}C_5$]glutamate and 8.5 mM [4-^{13}C]glutamate. Fig. 2a shows that the cross-peak S and D volumes within any C_xH_y group have identical slopes (i.e. slopes of C4H2D and C4H2S are equal and slopes of C4H3D and C4H3S are equal) and hence the C4H2S/C4H2D and C4H3S/C4H3D cross-peak volume ratios can be used in a metabolic analysis without further correction. This indicates that mixing of ^{1}H magnetization between H4 and either H2 or H3 is independent of ^{13}C enrichment at C2 or C3, respectively [25]. Although analogous titrations were not performed using mixtures of [2-^{13}C]-, [3-^{13}C]-, [2,4-$^{13}C_2$]-, [2,3-$^{13}C_2$]- or [3,4-$^{13}C_2$]glutamate (due to their commercial unavailability), we assume that no significant isotopic effect would be observed for any isotopomer since none was observed for [U-$^{13}C_5$]glutamate, the species that should have produced the largest isotope effect. Fig. 2b shows that volume comparisons *between* Ss or *between* Ds (C4H2S versus C4H3S or C4H2D versus C4H3D, for example) cannot be made without significant correction. This demonstrates, as expected, that transfer of ^{1}H magnetization between glutamate protons is not equivalent [26]. However, once calibration curves such as those shown have been determined for a particular set of NMR spectral acquisition conditions, these data can be used to correct all S/S and D/D cross-peak volume ratios in a HMQC-TOCSY spectrum of glutamate in any tissue extract. This allows one to directly quantify groups of ^{13}C isotopomers for use in a metabolic analysis.

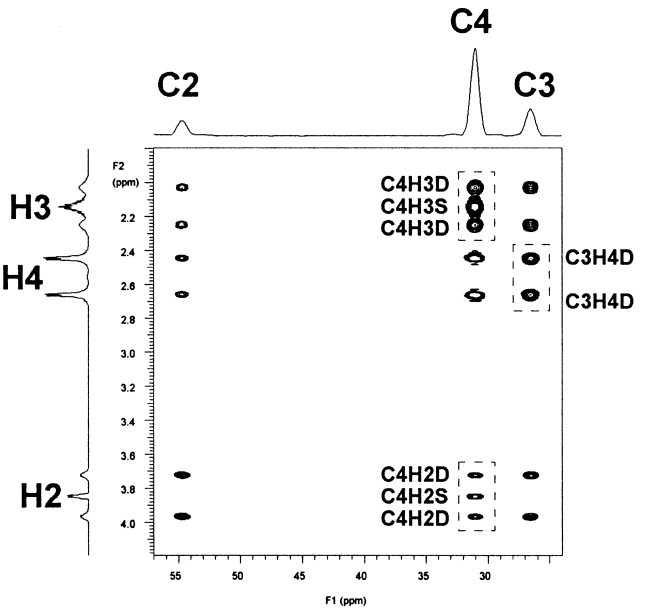

Fig. 1. A HMQC-TOCSY 2D NMR spectrum of an equimolar mixture (1 mM) of [4-^{13}C]- and [U-$^{13}C_5$]glutamate. The cross-peak doublet components, C_xH_yD, are due to the uniformly labeled isotopomer, and the singlet components, C_xH_yS, are due to the singly labeled isotopomer. The pH of the sample was 2.75.

1D ^{13}C NMR and 2D HMQC-TOCSY spectra of an extract of a rat heart perfused to steady state with [2-^{13}C]acetate are compared in Fig. 3. Each spectrum contains different but complimentary information about the population of ^{13}C isotopomers in the sample. In the ^{13}C spectrum, relative ^{13}C enrichment at each carbon is given by the resonance areas (after appropriate correction for T_1 and nOe differences) while enrichment at nearest-neighbor carbons is reported by the one-bond ^{13}C-^{13}C multiplets within each resonance. We

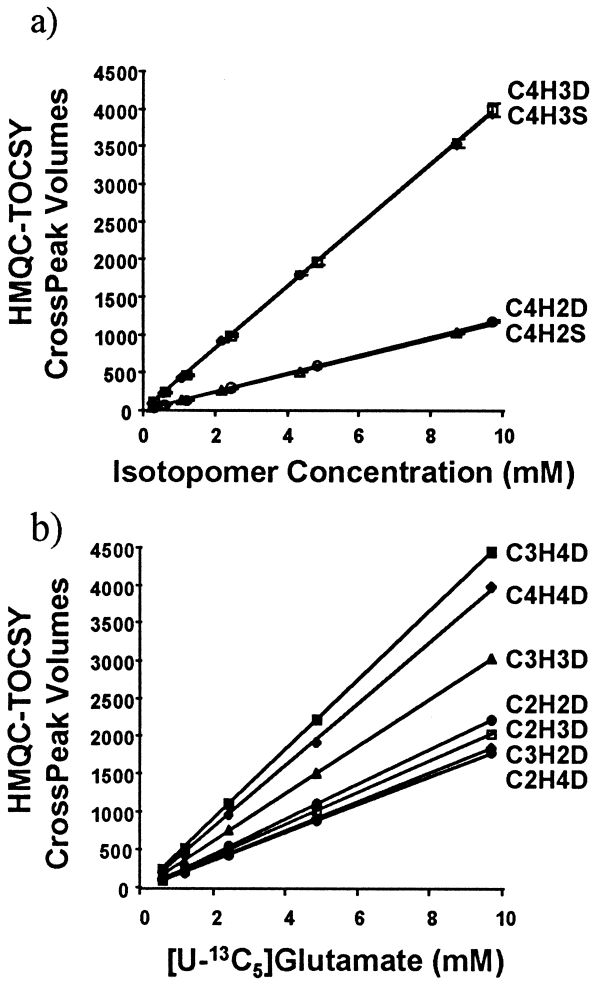

Fig. 2. Plots of measured HMQC-TOCSY cross-peak volumes as a function of glutamate isotopomer concentration. a: The plots demonstrate equal slopes for C4H3D and C4H3S (410.3 ± 3.5 and 408.4 ± 2.8) and equal but different slopes for C4H2D and C4H2S (121.5 ± 2.9 and 117.9 ± 1.8). b: The slopes of the plots refer to the other doublet cross-peak components due to [U-$^{13}C_5$]glutamate: C3H4D (462.3 ± 4.2); C4H4D (409.8 ± 3.1); C3H3D (315.6 ± 6.5); C2H2D (234.4 ± 2.2); C3H2D (210.2 ± 3.0); C2H3D (190.2 ± 2.1); C2H4D (186.7 ± 2.5). These may be used as correction factors for HMQC-TOCSY spectra of extracts collected at 14.1 T, pH 2.6–2.8, 25°C, and using the spectral acquisition conditions outlined in Section 2. Four HMQC-TOCSY spectra were acquired for each mixture in order to estimate changes in cross-peak volumes.

have previously shown [1] that the ^{13}C fractional enrichment of the methyl carbon (C2) of acetyl-CoA entering the citric acid cycle (F_{C2}) and anaplerosis (y) are reported in the ^{13}C spectrum as:

$$F_{C2} = \text{C4D34 (C4/C3)} \quad (1)$$

$$y = [(\text{C4/C3}) - 1]/2 \quad (2)$$

where C4D34 is the area of the J_{34} doublet in C4 relative to the total area of the C4 resonance while C4/C3 is the ratio of total C4 to C3 resonance areas (the latter ratio may require a correction factor, depending upon how the spectrum was collected). For a group of five hearts perfused with [2-^{13}C]acetate, the multiplet and resonance area ratios reported in the ^{13}C NMR spectra indicate that $F_{C2} = 0.93 \pm 0.03$ and $y = 0.04 \pm 0.01$, identical to previously published values [1,3].

This same metabolic information is also encoded in the HMQC-TOCSY spectrum. As in the ^{13}C spectrum, we define the S and D cross-peak volumes as a fraction of total volume within each C_xH_y group, i.e. C4H3S+C4H3D = 1. Such fractional volumes may be used to evaluate the same metabolic data as that described above for the ^{13}C spectrum. The chance that a molecule of oxaloacetate with ^{13}C at C2 (the carbonyl carbon) will condense with [2-^{13}C]acetyl-CoA in the citric acid cycle is simply F_{C2}. Since oxaloacetate C2 translates into glutamate C3 on each turn of the cycle, F_{C2} is determined by the ratio of all glutamate isotopomers having ^{13}C at both C3 and

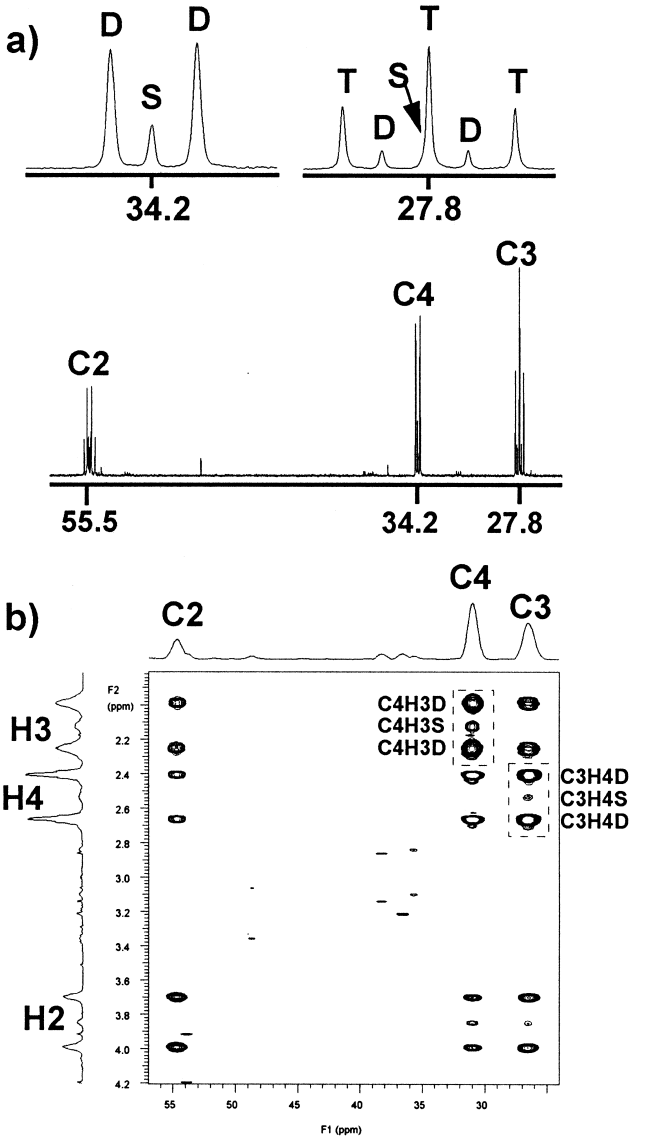

Fig. 3. ^{13}C NMR (a) and HMQC-TOCSY (b) spectra of an extract of a rat heart perfused to steady state with [2-^{13}C]acetate. The spectra were collected at 11.75 T using the same 600 μl sample but at different pH values (pH = 7.20 for ^{13}C and 2.75 for HMQC-TOCSY). The 1D ^{13}C spectrum (a) was collected over 2.4 h while the 2D HMQC-TOCSY spectrum (b) was collected over 24 min. The insets in the ^{13}C spectrum show expanded C3 and C4 resonances containing the multiplet information used in Eqs. 1 and 2 for a ^{13}C isotopomer metabolic analysis. The highlighted triad of cross-peaks in the HMQC-TOCSY spectrum (C4H3S+C4H3D = 1 and C3H4S+C3H4D = 1) represent the volume elements used in Eqs. 3 and 4 to perform a similar metabolic analysis.

C4 versus those having ^{13}C at C3 but not C4. This is directly reported in the HMQC-TOCSY spectrum because all glutamate isotopomers having ^{13}C at both C3 and C4 contribute to the C3H4D cross-peak volume while all isotopomers having a ^{13}C at C3 but *not* at C4 contribute to the C3H4S cross-peak volume. It follows that:

$$F_{C2} = \text{C3H4D} \quad (3)$$

The fraction of unenriched acetyl-CoA entering the cycle, F_{C0}, is obtained by the difference $1-F_{C2}$ or simply C3H4S. This is a non-steady-state measurement of F_{C2} because isotopic steady state is not required for the relationship to be valid; the only requirement is that enough ^{13}C has entered the C3 position of glutamate to allow detection by HMQC-TOCSY with reasonable signal-to-noise. The advantages of HMQC-TOCSY versus ^{13}C NMR for the determination of F_{C2} are twofold; first, only one experimental measurement is necessary (compare Eqs. 1 and 3) and, second, the data do not require correction for spectral acquisition conditions. A third, less obvious advantage, is that F_{C2} can also be evaluated in the same spectrum by the volume element, C2H4D, assuming complete randomization of the C2 and C3 carbons of symmetrical four-carbon cycle intermediates. This may find use as an alternative measure of F_{C2} in situations where C3H4D is obscured by other metabolite resonances (C2H4D is in a more open region of the 2D spectrum).

Anaplerosis is defined as flux of metabolites other than acetyl-CoA through citric acid cycle pools relative to citric acid cycle flux ($y = a/c$). Since acetate can only be oxidized by mammalian tissue and cannot enter the cycle as an anaplerotic substrate, no labeling of anaplerotic substrate is possible in hearts perfused with [2-^{13}C]acetate. Given this metabolic condition, anaplerosis is proportional to total ^{13}C enrichment of C4/total ^{13}C enrichment of C3 (see Eq. 2). In the 2D HMQC-TOCSY spectrum, this ratio is reported by the total volume of the C4H3 triad/total volume of the C3H4 triad,

$$y = [(\text{C4H3triad}/\text{C3H4triad}) - 1]/2 \quad (4)$$

where C4H3 triad = C4H3S+C4H3D and C3H4 triad = C3H4S+C3H4D. Note that this volume ratio required correction (C3H4 = 1.13 × C4H3) because data were obtained from two different cross-peak volume elements. As usual, measurement of y also requires that the tissue be at metabolic and isotopic steady state. Using volume data from HMQC-TOCSY spectra collected on five different hearts and Eqs. 3 and 4, $F_{C2} = 0.94 \pm 0.03$ and $y = 0.05 \pm 0.02$, both in good agreement with the respective values determined by direct ^{13}C analysis.

The comparison presented above shows that identical metabolic information can be derived from glutamate multiplet areas in a 1D ^{13}C NMR spectrum and from glutamate cross-peak volumes in a 2D HMQC-TOCSY spectrum. For typical perfused heart studies where tissue glutamate is not limiting, the 2D method offers no clear advantage over direct ^{13}C observe. However, in situations where either ^{13}C fractional enrichment is low or total tissue glutamate (or other metabolite of interest) is limited, the sensitivity gain offered by HMQC-TOCSY could become critical. By comparing signal-to-noise ratios in 1D and 2D spectra collected over identical total collection periods, we found that our experimental gain in sensitivity was 5.8, 2.5, and 5.1 for glutamate C2, C3 and C4, respectively. Presumably, C3 shows a lower gain in sensitivity due to the chemical shift non-equivalence of the H3 protons. Nevertheless, a 5–6-fold increase in sensitivity should allow an extension of ^{13}C isotopomer methods to smaller samples (a few mg of tissue) or to metabolites normally not detected by direct ^{13}C observe. To illustrate this point, the HMQC-TOCSY spectrum of an extract of a single soleus muscle (wet weight ~10 mg) removed from a mouse after infusion of [2,4,6,8-^{13}C]octanoate is presented in Fig. 4. Glutamate was not detected in the ^{13}C NMR spectrum of this sample after an overnight acquisition yet the glutamate cross-peaks are easily distinguished in a HMQC-TOCSY spectrum. Substitution of the cross-peak volume element, C3H4D, into Eq. 3 gave a F_{C2} value of 83%, showing that a majority of the acetyl-CoA oxidized by this muscle was derived from octanoate.

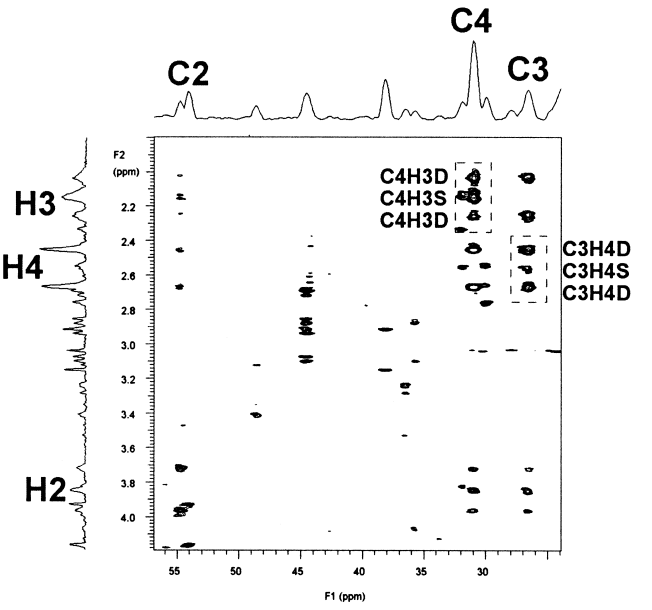

Fig. 4. HMQC-TOCSY spectrum collected at 14.1 T of an extract of a single mouse soleus muscle (~10 mg wet weight) after an infusion of sodium [2,4,6,8-^{13}C]octanoate.

4. Discussion

Previous applications of ^{1}H detect 2D NMR methods to follow metabolism of a ^{13}C-enriched substrate have most often involved linear pathways where a single ^{13}C isotopomeric metabolic product is formed [16,20]. To our knowledge, this report describes the first application of HMQC-TOCSY to quantify groups of ^{13}C isotopomers in a metabolic intermediate (glutamate) and to relate that data to metabolic parameters of interest using simple analytical relationships. The fraction of acetyl-CoA derived from [2-^{13}C]acetate (F_{C2}) and relative anaplerotic flux (y) determined by HMQC-TOCSY were shown to be identical to those determined from ^{13}C multiplet areas in a 1D high resolution ^{13}C NMR spectrum.

We have estimated that the sensitivity gain offered by indirect detection of glutamate isotopomers on our 600 MHz system (using different probes, inverse versus broad-band, for the two experiments) ranged from 2.5 to 5.8. The theoret-

ical maximum gain in sensitivity for this experiment has been reported to be 32-fold [27], yet much lower enhancements have been observed in practice. Novotny et al. [28], using a 1D spin-echo ^{1}H observe ^{13}C editing method, measured a 14-fold increase in sensitivity for ^{1}H observe over ^{13}C observe for detection of the methyl resonance of [2-^{13}C]ethanol in rabbit brain. Most recently, Willker and coworkers [29] compared 2D HMQC versus 1D ^{13}C observe and reported an average signal-to-noise enhancement factor of 2.5 for several metabolites in cell extracts (values ranged from 0.5 to 14). The 5–6-fold gain in sensitivity observed here for glutamate has allowed us to extend ^{13}C isotopomer methods to sample sizes typically used in tissue biopsies. It also offers the possibility of performing a ^{13}C isotopomer analysis of other intermediates that are less abundant in most tissues (i.e. malate, succinate, citrate, or α-ketoglutarate), thereby allowing incorporation of isotopomer data from several citric acid cycle intermediates in existing models of metabolic flux [3–5].

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