METHOD ARTICLE

High-Speed Tracer Analysis of Metabolism (HS-TrAM) [version 1; referees: awaiting peer review]

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Abstract

Tracing the fate of stable isotopically-enriched nutrients is a sophisticated method of describing and quantifying the activity of metabolic pathways. Nuclear Magnetic Resonance (NMR) offers high resolution data, yet is under-utilised due to length of time required to collect the data, quantification requiring multiple samples and complicated analysis. Here we present two techniques, quantitative spectral filters and enhancement of the splitting due to J-coupling in ¹H,¹³C-HSQC NMR spectra, which allow the rapid collection of NMR data in a quantitative manner on a single sample. The reduced duration of HSQC spectra data acquisition opens up the possibility of real-time tracing of metabolism including the study of metabolic pathways in vivo. We show how these novel techniques can be used to trace the fate of labelled nutrients in a whole organ model of kidney preservation prior to transplantation using a porcine kidney as a model organ, and also show how the use of multiple nutrients, differentially labelled with ¹³C and ¹⁵N, can be used to provide additional information with which to profile metabolic pathways.

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Introduction

Investigations of metabolism in health and disease increasingly rely on tracing the use of stable isotope-enriched nutrients through the cell’s metabolic pathways. The most widely utilised technology platform to analyse the resulting complex patterns of labelling in multiple cellular metabolites is mass spectrometry (MS), due to its high sensitivity, short run times and a resulting low-cost operation\(^1\)–\(^12\). Conversely, NMR spectroscopy is relatively under-utilised, despite being able to provide higher resolution information on the conversion of synthetically produced stable isotopes of nutrients incorporated into cellular metabolites\(^12\)–\(^19\). This is because this platform has historically suffered from low sensitivity, long acquisition times and the need for complex analytical tools.

NMR spectroscopy is, however, ideally suited to answering some of the more pressing questions about metabolic control in health and disease. We currently have limited knowledge about the compartmentalisation of metabolic pathways in metabolically-active organelles, such as mitochondria, and therefore whether the same metabolite is selectively utilised for distinct purposes in different compartments. Given the recent drive to target metabolism in various diseases, understanding the control of metabolism by different tissues is critical to the ability to select specific therapies which target the desired pathways within appropriate cellular compartments.

Stable isotope-enriched metabolic precursors, such as glucose or glutamine, are employed as metabolic tracers. These synthetically produced nutrients are enriched in isotopes with a low natural abundance, such as \(^{13}\)C or \(^{15}\)N. Despite the fact that metabolites can arise from multiple sources, the contribution of the different metabolic pathways to the synthesis of this metabolite can be determined through the analysis of the \(^{13}\)C and/or \(^{15}\)N distribution within the metabolite (Figure 1).

Our recently published combined NMR and MS approach (CANMS) harnesses the strengths of both modalities to produce highly-resolved metabolism information in the form of metabolite isotopomers\(^19\). The detailed interpretation of MS isotopologue data, when using MS data in isolation, requires use of a pre-defined metabolic model. In contrast, the integrated analysis of NMR

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Figure 1. Tracing of metabolic pathways. The labelling patterns arising from [1,2-\(^{13}\)C] glucose (\(A\) & \(B\)) as well as from [U-\(^{13}\)C,\(^{15}\)N] glutamine (C for \(^{13}\)C labelling and A–C for \(^{15}\)N labelling) are shown. Metabolism of [1,2-\(^{13}\)C] glucose leads to distinctive labelling patterns in lactate and alanine ([2,3-\(^{13}\)C] lactate/alanine when using glycolysis and [3-\(^{13}\)C] lactate/alanine when using the pentose phosphate shunt, PPP) (Panel \(A\)). Similarly, glutamate and aspartate express different labelling patterns from [1,2-\(^{13}\)C] glucose, depending whether they were synthesised via pyruvate dehydrogenase (PDH; resulting in [4,5-\(^{13}\)C] glutamate) or via the pyruvate carboxylase (PC; resulting in [2,3-\(^{13}\)C] aspartate) route (Panel \(B\)). Metabolisation of labelled glutamine can reveal other anaplerotic pathway activities such as reductive glutaminolysis (Panel \(C\)).
and MS data makes fewer assumptions about the metabolic network, providing a more accurate insight into relative pathway contributions than is possible with current established methods or the independent analysis of MS or NMR data alone. For example, proton-less carbon atoms do not give rise to a signal in 2D-HSQC NMR spectra, although incomplete information on those carbons is available via splitting of adjacent carbon nuclear signals. The combination of NMR and MS analysis fills this gap as the MS data provides information on the amount of single carbon labelling into those carbon nuclei via “m+x” isotopologues. [13C-1,2] glucose is the optimal tracer to assess metabolic flux through glycolysis vs pentose phosphate pathway (PPP) shunting back into glycolysis. While the glycoolytically derived isotopomer of lactate is [13C-2,3] lactate, the PPP derived isotopomers can be [13C-3], [13C-1] or [13C-1,3] lactate. While the metabolic isotopomer can be assessed with NMR data, the other two isotopomers include labelling in C(1), which HSQC NMR is “blind” to. In these cases, MS data adds new information to the NMR data by contributing the isotopologues NMR is not able to detect, while NMR adds to the MS data by differentiating between [13C-1,3] and [13C-2,3] lactate.

A major drawback of utilising [13C-1,4C] scalar coupling information to derive isotopomer distributions is the time required to acquire spectra. For example, around four hours are required for the acquisition of a high-resolution 2D-HSQC NMR sample, even when using fast, state-of-the-art non-uniform sampling (NUS) techniques.

Here we describe two novel developments, quantitative spectral filters and J-coupling enhancement, to facilitate and speed-up the acquisition of NMR spectra for tracer based analysis of metabolism. Such techniques permit high throughput metabolic pathway profiling, increasing access, affordability and sensitivity when using NMR as an investigative modality. Additionally, these developments facilitate fast detection of 15N labelling, especially when combined with 13C tracing, thus providing the information required for more accurate metabolic pathway profiling.

Methods and results

Quantitative spectral filters for 13C tracer observation: 1D Spectral filters

Experimental setup. A porcine kidney (WIT-15 minutes) was procured from a slaughterhouse (FA Gill, Wolverhampton) following approximately 14 minutes warm ischaemia as per previous experimental methodology. No animal was killed solely for experimental purposes; all were due for human consumption and therefore no ethical approval was required. After 2 hours cold ischaemic time, kidneys were subject to 18 hours of hypothermic machine perfusion. The perfuse sample was collected after 6 hrs of perfusion and prepared for NMR analysis.

1D NMR spectra were acquired using a Bruker Avance III 600 MHz NMR spectrometer equipped with a 5mm z-PFG TCI Cryoprobe. 128 transients were acquired for each spectrum with a 5 seconds interscan relaxation delay. A total of 32768 data points with a spectral width of 12ppm was acquired for each FID using Bruker’s adiabatic bi-level decoupling scheme to suppress 1H,13C J-coupling during acquisition. Decoupling for this long (2.25s) was possible because of the cryogenic probe and will quite possibly lead to probe damage when using a NMR system equipped with a room temperature probe.

The spectra were processed within the MetaboLab software package (version 1.0.0.1). 0.5Hz line broadening was applied with zero-filling the data up to 131072 real data points prior to Fourier transformation. The resulting spectra were referenced using DSS and manually phase corrected. Subsequently the spectral baseline was corrected using MetaboLab’s spline baseline correction before the spectra were exported to Bruker format for metabolites to be quantified in the Chenomx software package (version 8.2, http://www.chenomx.com).

NMR methodology. Despite their relative simplicity and limited resolution, 1D-NMR spectra are a highly sensitive tool to identify and quantify metabolites. Spectral filters enable the acquisition of spectra which filter out certain signals, thereby reducing ambiguity in 1D spectra associated with attributing peaks to nuclei within metabolites. For example, one can acquire 1D 1H NMR spectra of protons bound to 13C only, simplifying signal assignment and analysis of the acquired spectra. The simplest approach to collect such spectra would be to acquire the first increment of a 2D-1H,13C HSQC spectrum. However, signal intensities are not directly comparable with those in a standard 1D-1H NMR spectra. It is therefore not possible to directly derive 13C percentages based on a comparison of those spectra with standard proton 1D spectra unless only a small subset of molecules is labelled with 13C and one accompanying spectrum is scaled so that the majority of signals within the two spectra are of same intensity. Spectral filters such as BIRD and TANGO originated in protein NMR spectroscopy to filter out certain parts of the magnetisation and therefore quantitative data cannot be gained from resultant output spectra. Quantitative comparisons between unfiltered and filtered spectra are usually unnecessary, except for tracer based analysis. Here we present a novel spectral filter which enables quantitative analysis of resultant spectra from single samples, enriched with 13C tracer.

Figure 2a shows the pulse sequences implementing the quantitative spectral filter. While the grey shaded 13C π-pulse is used only in odd transients and replaced with a delay of the same length during even transients, the blue 13C π-pulses are only used in the 12C filtering experiment, where 1H magnetisation to 13C neighbours is filtered out, so that only 13C bound 1H magnetisation contributes to the signal intensities in the 1D spectrum. The phase cycle φi changes as well between the 2 experiments, as indicated by the same colour scheme (black: all protons, blue, 13C bound protons only).

Figure 2b depicts two sample spectra from a perfusate sample where a cadaveric porcine kidney was perfused with modified University of Wisconsin machine perfusion solution (UW MPS) during a period of commonly used hypothermic machine perfusion. The standard unlabelled glucose constituent (10mM) within classical UW MPS was replaced with universally labelled glucose, at the same concentration, for use as a metabolic tracer.
Figure 2. Spectral Filters in 1D spectroscopy. To determine percentage 13C incorporation two spectra are acquired per sample. One spectrum contains 1H signals originating from all protons (all 1H spectrum), while the second spectrum only contains signals from protons attached to a 13C nucleus. For the all 1H spectrum, the blue 13C 180 degree pulses are omitted. The grey shaded 13C pulse is executed every second transient in both experiments. The proton magnetisation in the all 1H pulse sequence is the same for 12C and for 13C bound protons and as a consequence, all 1H magnetisation is longitudinal during the interval between 1H pulses with the phases φ2 and φ4. Because of the additional 13C 180 degree pulses in the 13C bound 1H pulse sequence, the magnetisation for the two different kinds of protons develops differently. Here only the 13C bound 1H magnetisation is longitudinal in the interval between the 1H pulses with the phases φ2 and φ4. Therefore, the 13C bound 1H magnetisation can be destroyed using the two pulse field gradients labelled gp1 and gp2. The grey shaded 180 degree 13C pulse, which is only executed every second transient improves magnetisation selection as it is accompanied with phase changes of the 1H pulses and the receiver. All 13C π-pulses are adiabatic Chirp pulses with γB1max = Hz. The pulse phases are: φ1 = x, x, -x, -x; φ2 = x, x, -x, -x; φ3 = x, y, -x, -y; φ4 = x, -x, -x, x. The blue phases refer to the 13C bound 1H spectrum where applicable.

J-Coupling enhancement

Experimental setup. Slaughterhouse porcine kidneys (WIT-15 minutes) were cannulated and flushed with chilled Soltran solution (Baxter) as performed in clinical practice. Kidneys were placed in static cold storage en route to our laboratory, where they were immediately perfused with KPS-1 using the Lifeport Kidney Transporter 1.0 (Organ Recovery Systems), which has been modified to include a paediatric oxygenator. Oxygen was supplied at a flow rate of 0.7L/min and perfusion time was 24 hours.

At experimental endpoint, the kidney was removed from the perfusion circuit and laterally bisected. Sections of cortex and medulla were isolated and snap frozen in liquid nitrogen. These tissues were powdered under liquid nitrogen, and 0.5g was placed in 7ml homogenisation tube (Precellys, CK28), containing 5.1 ml of HPLC grade methanol (−80°C) to quench metabolism. These were homogenised using the Precellys 24 dual homogeniser (8x 5000 rpm for 15 s). The samples were mixed with 4.65 ml deionised water and 5.1ml HPLC grade chloroform and...
vigorously agitated. Biphasic separation of polar and non-polar solvents was performed by centrifugation (1300g, 15 minutes, 4°C), after which 4.5ml of the polar layer was aspirated and dried overnight at 35°C.

The dried extracts were re-suspended in 60 µl NMR buffer (0.1M phosphate buffer, 0.5mM 4,4-dimethyl-4-silapentane-1-sulfonic acid, 2mM imidazole and 10% D2O). These suspensions were sonicated to dissolve micro particles and then 35 µl of this solution was added to 1.7mm NMR tubes.

2D 1H/13C-HSQC NMR spectra were acquired using a Bruker Avance III 600 MHz NMR spectrometer equipped with a 1.7mm z-PFG TCI Cryoprobe. The HSQC spectra were acquired using 2 transients per increment with echo/anti-echo gradient coherence selection and an additional pre-saturation for suppressing the water resonance during the 1.5s interscan relaxation delay. The 1H dimension was acquired with a spectral width of 13ppm using 512 complex data points. The 13C dimension was acquired with a spectral width of 160 ppm using 25% (2048) of 8192 data points using a non-uniform sampling scheme. The non-uniformly sampled spectra were reconstructed via the compressed sensing algorithm within the MDDNMR (version 2.5)26 and processed using NMRPipe (version 9.2)27. All spectra were processed without baseline correction to avoid complications in the multiplet analysis procedure.

NMR methodology. The relatively long acquisition times of 2D-HSQC spectra are necessary to generate the spectral resolution required to resolve complex multiplet patterns28. Here we present a technique to manipulate the appearance of NMR multiplets in the indirect dimension of 2D-HSQC spectra. The ability to expand the splitting caused by J-coupling negates the requirement for the collection of large number of increments in the 13C dimension. It also means that at increasingly higher magnetic fields, the advantages of extra sensitivity and increased 1H chemical shift resolution are not negated by the increased 13C increments needed in order to resolve J-couplings. Increased J-coupling splitting can be achieved by incrementing the spin echo delay after the period where chemical shift of 13C evolves in parallel with the chemical shift evolution (Figure 3). This spin echo refocuses the 13C chemical shift and the 1H-13C coupling, but allows the 13C-13C coupling to evolve further. The delays in the spin echo are proportional to those in the Ω+Jcc evolution period with the amount of extra coupling achieved being defined by the stretch of the Jcc increment compared to the Ω+Jcc increment. Thus, the 13C-13C J-couplings can be expanded as required (Figure 4). The ability to scale the J-couplings to varying extents means that the experiment can be tuned to the requirements of the sample and which metabolites are present, and of interest. Large expansion of J-coupling allows for rapid collection of data, as the resolution required to resolve them becomes diminished. However, this should be tempered by the need to avoid unnecessary overlap of signals. To date, the authors have acquired 2D spectra with 8-fold enhanced 13C J-couplings, combined with shortening the acquisition by using variable pulse sequence repetition times28, leading to an overall decrease in acquisition time by a factor larger than 10 (i.e. an acquisition time of 22 minutes compared to 4 hours for normal NUS acquisition). This can be stretched to even shorter acquisition times by including spectral folding in the acquisition protocol. Figure 4 demonstrates the effect of J-scaling on 2D HSQC spectra, displaying C(6) of 13C enriched glucose. The trace through the left-most signal, displayed in panel D, demonstrates clearly that while the singlet in the middle of the multiplet does not change, the apparent Jcc constant increases and in fact splits into multiple signals as previously unresolved long-range couplings are amplified so that they are large enough to become resolved in the spectrum.

**Figure 3. J-Coupling Enhanced HSQC.** The J-coupling enhancement is achieved using an additional spin echo subsequent to the 13C evolution period. The delays in the spin-echo for the J-coupling enhancement are multiples of the dwell time (dw). While the chemical shift evolves with dw, which is determined by setting the spectral width of the spectrum, J-coupling is enhanced depending on the increment of the 13C gradient selection spin-echo. The HSQC is acquired using echo-antiecho for quadrature detection to allow for efficient removal of artefacts in only two scans per increment. Optionally the 13C, 15N-couplings can be scaled by the introduction of the 15N π-pulse parallel to the grey 13C π-pulse, also shown in grey. The pulse phases are: φ1 = y; φ2 = x, -x; φrec = x, -x.
Figure 4. J-coupling enhancement HSQC. The spectrum with no J-coupling enhancement is shown in blue, with an enhancement of two in green and with an enhancement of four in red. The $^{13}$C trace of the HSQC clearly shows the increase in splitting. The J-coupling enhancement is achieved using an additional spin echo subsequent to the $^{13}$C evolution period. The delays in the J-scaling are multiples of dw such the use of a delay of 3*$dw$ will result in a J-scaling of 4 (one from the $t_1$ evolution and three from the J-scaling spin echo).

$^{15}$N tracing

Biological methods. 2D $^{14}$N spectral filter - Sample preparation is described in 16. 2D $^{1}$H/$^{13}$C-HSQC NMR with and without $^{14}$N filtering spectra were acquired using a Bruker Avance III 600 MHz NMR spectrometer equipped with a 1.7mm z-PFG TCI Cryoprobe. The HSQC spectra were acquired using 2 transients per increment with echo/anti-echo gradient coherence selection and an additional pre-saturation for suppressing the water resonance during the 1.5s interscan relaxation delay. The $^1$H dimension was acquired with a spectral width of 13ppm using 512 complex data points. The $^{13}$C dimension was acquired with a spectral width of 160 ppm using 2048 data points. The spectra were processed with quadratic cosine window functions and without baseline correction to avoid complications in the multiplet analysis procedure.

$^{13}$C/$^{15}$N J-coupling enhancement. The human Medulloblastoma cell line DAOY from ATCC (HTB-186™) was used to investigate the fate of both carbon and nitrogen from glutamine. The importance of glutamine in relation to cancer biology has been
exhausted in the literature, with numerous cancers being addicted to glutamine despite it being a non-essential amino acid. Glutamine serves as a vital metabolite to deliver both carbon and nitrogen for several cellular processes including growth and redox control. It is therefore important to track the flux of carbon and nitrogen from glutamine together, providing a more detailed account of this metabolite in tumour biology. One such metabolite that may require both carbon and nitrogen from glutamine is alanine. Alanine is typically synthesised from pyruvate by reductive amination, where an aminotransferase enzyme facilitates the addition of the amino group from glutamate to pyruvate.

3 × 10^6 cells were seeded onto 15 cm dishes and cultured in standard medium overnight. Basic formulation DMEM media (Sigma, D5030) was supplemented with 2 mM L-Glutamine, ^13^C_p, ^15^N (sigma, 607983) and 10 mM of 13C6 glucose (Sigma, 389374) and subsequently added to cells for 24 h. At the conclusion of tracer experiments, cells were washed with ice-cold saline solution (0.9%) and collected by scraping in 1.2 mL pre-chilled methanol (-20°C), 600 µL water (4°C) and 1.2 mL chloroform (-20°C). Cell lysates were vortexed for 15 min at 4°C and instantly centrifuged at 15,000 g for 15 min at 4°C.

Samples were dried using a Savant (SPD1010) speedvac concentrator and then resuspended in 60 µL of 100 mM sodium phosphate buffer containing 500 µM DSS and 2 mM Imidazole, 10% D_2O, pH 7.0. Samples were vortexed, sonicated for 10 min and centrifuged momentarily (these steps were repeated twice), prior to being manually transferred to 1.5 mL NMR tubes.

2D $^1$H$^1$C-HSQC and 2D $^1$H$^{15}$N-HSQC NMR spectra were acquired using a Bruker Avance III 900 MHz NMR spectrometer equipped with a 5 mm z-PFG TCI Cryoprobe. The HSQC spectra were acquired using 2 transients per increment with echo/anti-echo gradient coherence selection and with an additional pre-saturation for suppressing the water resonance during the 1.5 s interscan relaxation delay. The $^1$H dimension of the $^{13}$C-HSQC was acquired with a spectral width of 12 ppm using 512 complex data points. The $^{13}$C dimension was acquired with a spectral width of 165 ppm using 30% (2458) of 8192 data points using a non-uniform sampling scheme. The $^1$H dimension of the $^{15}$N-HSQC was acquired with a spectral width of 12 ppm using 1024 complex data points. The $^{15}$N dimension was acquired with a spectral width of 40 ppm using 47.5% (122) of 256 data points using a non-uniform sampling scheme. All non-uniformly sampled spectra were reconstructed via the compressed sensing algorithm within the MDDNMR (version 2.5) and processed using NMRpipe (version 9.2). All spectra were processed without baseline correction to avoid complications in the multiplet analysis procedure.

**NMR methodology.** Both aforementioned methods can be used to detect $^{15}$N labelling in metabolites, which alongside $^{13}$C isotope incorporation can provide additional much-needed information on the overlapping activity of multiple metabolic pathways. 2D spectroscopic filters are an extension of the 1D concept and as such can be used to simplify increasingly complex 2D spectra by selectively observing a subset of metabolites in which nuclei of interest have been incorporated. For example, the analysis of the $^{13}$C nuclei that are adjacent to $^{15}$N nuclei using 2D spectra permits a simplified unequivocal description of the nature in which two metabolic pathways converge.

Similar to the 1D method, the acquisition of 2 spectra is required in order to enable a quantitative analysis of the amount of $^{15}$N labelling in the presence of $^{13}$C labelling within the metabolite. If spectral simplification is the goal, a single spectrum is sufficient. The pulse sequence (Figures 5) is a gradient selected 2D-$^1$H-$^{13}$C HSQC spectrum with the spectral filter added once the $^1$H magnetisation has been transferred to the $^{13}$C nucleus. The spectrum collected with the $^{15}$N spectral filter (Figure 5) contains only two visible NMR signals, corresponding to arginine and arginosuccinate, clearly showing how the filter can simplify complex spectra for easier analysis.

While 2D spectral filters serve a purpose, their quantitative usage is limited by the variability of the $J_{CN}$ constant. J-scaling on the other hand can be easily extended to include $^{13}$C-$^{15}$N J-scaling. Indeed, the addition of a single $^{15}$N π-pulse parallel to the grey shaded $^{13}$C π-pulse in Figure 3 is sufficient to upscale the apparent $^{13}$C-$^{15}$N J-coupling constant in 2D-$^1$H-$^{13}$C HSQC spectra. An example is given in Figure 6. Panels C and D show traces of the $^{13}$C multiplets for carbon atoms 2 and 3 of alanine. While the $J_{OC}$ couplings are enhanced by a factor of 4 in both spectra, the apparent $J_{CN}$ couplings are unchanged in the amber spectrum, whereas they are enhanced by a factor of 2 in the blue spectra. Because the $^{13}$C-$^{15}$N coupling between C(3) and N is negligible, both traces for C(3) overlap perfectly. C(2) on the other hand experiences a $J_{CN}$ coupling, which leads to the much more complex blue spectrum. The 2D signals for the $J_{CN}$ scaled spectrum are shown in Figures 6A and B.

$J_{CN}$ coupling, as any J-coupling, works in two directions, therefore a similar approach can be followed from the opposite direction. While amine groups of small molecules are notoriously difficult to observe due to chemical exchange of amine protons with solvent molecules, a long-range HSQC can be acquired. In such a spectrum proton magnetisation is transferred from $^2$H (the proton bound to C(2)) via the $J_{CN}$ coupling. The JOC-coupling then can be enhanced to show the appearance of $^{13}$C labelling in molecules which contain $^{15}$N next to those labelled carbon nuclei. As in this case, where the $^{13}$C nucleus is also bound to the proton determining the chemical shift on the horizontal axis,
that same proton signal will be split by the $^{1}J_{CH}$-coupling constant. The result in this case is therefore a signal split into 4 2D components, demonstrating that alanine was either recycled from unlabelled alanine which was incorporated into proteins, or synthesised de-novo from [U-$^{13}$C] glucose and $^{15}$N labelled glutamate which originated from [U-$^{13}$C, U-$^{15}$N] glutamine which was added to the growth medium in addition to the [U-$^{13}$C] glucose. In conjunction with MS data, this complementarity between the 2D-$^{1}$H,$^{13}$C and the 2D-$^{1}$H,$^{15}$N HSQC spectra enables a model-free metabolism analysis using multiple nutrients as tracer source in a single sample.

All NMR spectra in this article were processed within the MetaboLab software package (version 1.0.0.1; http://metabolab.uk)

Figure 5. Filtered HSQC. Unfiltered (black) and filtered (amber) 2D-$^{1}$H,$^{13}$H HSQC spectra. In the filtered spectrum only carbon nuclei adjacent to $^{15}$N are visible. Similar to the filtered pulse sequence in Figure 1, the amber pulses are only executed in the $^{15}$N filtered pulse sequence and the grey $^{15}$N $\pi$-pulse is executed every second transient. The pulse phases are: $\phi_{1} = y$; $\phi_{2} = x$, $-x$; $\phi_{3} = x$, $x$, $-x$, $-x$; $\phi_{4} = x$, $x$, $x$, $y$, $y$, $y$; $\phi_{5} = y$, $-y$, $x$, $-x$; $\phi_{6} = x$, $x$, $-x$, $-x$; $\phi_{7} = x$, $x$, $x$, $y$, $-y$, $-y$; $\phi_{8} = y$, $-y$, $y$; $\phi_{rec} = x$, $-x$, $-x$, $x$, $y$, $-y$, $-y$.

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\delta = \frac{1}{4J_{CH}}
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\[
\Delta = \frac{1}{4J_{CN}}
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Discussion

Changes in metabolism are increasingly being recognised as central to the pathogenesis of a number of different diseases. Although metabolomic studies have helped determine aspects of disease phenotype, tracing the changing use of specific metabolic pathways using stable isotope-enriched nutrients provides higher resolution information on altered metabolic pathway activity that may lead to the identification of specific novel therapeutic targets. Over the last few years, development of magnet and probe technology, including innovative ultra-sensitive microprobes, has enabled the study of systems that were not previously amenable to NMR. Parallel advancement in the methods used to acquire and analyse data from samples will increase the amount of information we can gain from the such samples.

In this paper, we describe how spectral filters and J-splitting enhancement can be used in tracer based metabolism studies. These techniques overcome some of the major hurdles in the use of NMR spectroscopy. A challenge in the analysis of NMR HSQC data has been the need for an additional “unlabelled” sample in order to determine absolute per carbon $^{13}$C incorporation percentages. However, samples cannot be assumed to be biologically identical, thus making analyses problematic due to the inability to determine accurate $^{13}$C isotope incorporation.

Figure 6. Splitting due to $^{15}$N and $^{13}$C incorporation. Alanine regions of the 2D-$^1$H,$^{13}$C HSQC spectrum (A & B). The signals are split by either $^1J_{CC}$ or $^1J_{CN}$ coupling contributions. The long range 2D-$^1$H,$^{15}$N HSQC (C) is composed of unlabelled alanine (middle peak) and $^{13}$C/$^{15}$N labelled alanine (4 outer signals, split by $^2J_{CN}$ and $^1J_{NC}$ couplings).
values. Systems that demonstrate greater inter-sample variation, such as in vivo tracer studies, are even more prone to these analytical issues. The use of spectral filters negates the requirement for two samples and instead a single sample can be used to determine absolute percentage $^{13}$C incorporation and thus allow the scaling of multiplets.

The 2D HSQC is a powerful tool in the study of metabolism as it takes advantage of the increased sensitivity of the $^1$H nucleus over $^{13}$C and using the splitting due to J-coupling in the $^{13}$C dimension allows the indirect visualisation of the $^{13}$C incorporation into quaternary carbons. However, long acquisitions times, even when using the latest NUS techniques, limits the number of samples that can be acquired. Reducing the experimental time makes the use of HSQCs a more attractive method in the study of tracer based metabolism. The use of echo-antiecho for quadrature detection ensures efficient elimination of unwanted artefacts whilst using only 2 scans per increment in the indirect dimension. The changes observed in lineshape due to the quadrature detection are predictable and can be easily incorporated into line fitting analysis. The spin echo required for the echo-antiecho can further be utilised to enhance the observed splitting due to J-coupling.

The ability to scale the visualised J-couplings allows HSQC spectra to be acquired in time equivalent to that by 1D spectra but the HSQC contains significantly more information. The reduced time required to acquire spectra mean that it is feasible to apply 2D HSQC methods to in vivo tracer based metabolism studies, as well as allowing the use of greater sensitivity of higher field spectrometers while avoiding longer experiment times. Expanding the splitting due to J-coupling can also bring out smaller long-range couplings that were not apparent in a normal HSQC. Thus, the J-scale can either be used to decrease acquisition times by allowing data collection at lower resolution or to bring out smaller couplings not previously visible. These small couplings include the $J_{	ext{CN}}$ couplings that are found in many metabolites after the addition of metabolites labelled $^{15}$N in conjugation with $^{13}$C. This increases the information available and allows more in-depth analysis of complex metabolic pathways. In the example shown (Figure 5), the cells used for this experiment lacked the expression of fumarate hydratase and therefore contained high fumarate levels. One hypothesis was that the cells form argininosuccinate from fumarate and arginine to minimise intracellular fumarate. In order to ascertain the signal assignment, we used [U-$^{13}$C, U-$^{15}$N] arginine and were able to show that $^{15}$N labelled argininosuccinate was being formed in the cells containing the knock out, while wild-type cells did not produce $^{15}$N labelled argininosuccinate. This shows the utility of using multiple labelled nutrients to answer fundamental questions in metabolism.

In summary, the spectroscopic tools presented here open up new avenues for tracer based metabolism studies. Scaling of J-coupling constants leads to faster data collection of samples supplemented with nutrients enriched in stable isotopes, such as $^{13}$C and $^{15}$N. This enables profiling of metabolic pathways and can also be used to enhance sensitivity beyond current technical developments whilst maintaining reasonable data acquisition times. Ultimately, use of the 1D spectral filters as well as the fast acquisition of HSQC spectra leads to the possibility of tracing metabolism in real-time. In addition, simultaneous tracing with multiple nutrients will lead to unprecedented insight into the interplay of converging and intersecting metabolic pathways, both in vitro and in vivo.

Data availability
All experimental data for this article is available at: http://doi.org/10.17605/OSF.IO/EQHN3.

Experimental NMR datasets for HS-TrAM: The file filtered_1D_spectra.zip contains one unfiltered (subdirectory 1) and one $^{13}$C filtered (subdirectory 2) 1D-$^1$H spectrum. The file jEnhanced_13C_HSQC.zip contains the 2D-$^1$H,$^{13}$C HSQC spectra with different J-coupling enhancement. The file 13C_HSQC_14N_filter_and_15N_HSQC.zip contains an unfiltered (subdirectory 1) and a $^{14}$N filtered (subdirectory 2) 2D-$^1$H,$^{13}$C HSQC spectrum. The file jEnhanced_13C_15N_HSQC.zip contains the following spectra:

1. 4x $^{13}$C, $^{15}$N J-Coupling enhancement $^1$H,$^{13}$C-HSQC in subdirectory 1
2. 4x $^{13}$C, $^{15}$N J-Coupling enhancement and 2x $^{13}$C,$^{14}$N J-Coupling enhancement $^1$H,$^{13}$C-HSQC in subdirectory 2
3. 32x $^{13}$C,$^{14}$N J-Coupling enhancement $^1$H,$^{13}$C-HSQC in subdirectory 3

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Competing interests
No competing interests were disclosed.

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