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### High-Speed Tracer Analysis of Metabolism (HS-TrAM)

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DOI:

[10.12688/wellcomeopenres.13387.1](https://doi.org/10.12688/wellcomeopenres.13387.1)

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#### *Document Version*

Publisher's PDF, also known as Version of record

#### *Citation for published version (Harvard):*

Smith, T, Patel, K, Munford, H, Peet, A, Tennant, D, Jeeves, M & Ludwig, C 2018, 'High-Speed Tracer Analysis of Metabolism (HS-TrAM)', Wellcome Open Research. <https://doi.org/10.12688/wellcomeopenres.13387.1>

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Checked for eligibility: 23/01/2018

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METHOD ARTICLE

# High-Speed Tracer Analysis of Metabolism (HS-TrAM) [version 1; referees: 2 approved with reservations]

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**v1** First published: 12 Jan 2018, 3:5 (doi: [10.12688/wellcomeopenres.13387.1](https://doi.org/10.12688/wellcomeopenres.13387.1))  
Latest published: 12 Jan 2018, 3:5 (doi: [10.12688/wellcomeopenres.13387.1](https://doi.org/10.12688/wellcomeopenres.13387.1))

## 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 <sup>1</sup>H, <sup>13</sup>C-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 <sup>13</sup>C and <sup>15</sup>N, can be used to provide additional information with which to profile metabolic pathways.

## Open Peer Review

Referee Status:  

	Invited Referees	
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<b>version 1</b> published 12 Jan 2018	 report	 report

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**Competing interests:** No competing interests were disclosed.

**How to cite this article:** Smith TB, Patel K, Munford H *et al.* **High-Speed Tracer Analysis of Metabolism (HS-TrAM) [version 1; referees: 2 approved with reservations]** Wellcome Open Research 2018, 3:5 (doi: [10.12688/wellcomeopenres.13387.1](https://doi.org/10.12688/wellcomeopenres.13387.1))

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**Grant information:** This work was supported by the Wellcome Trust [099185]; the National Institute for Health Research [13-0053]; Help Harry Help Others [HelpCU09]; UHB Charitable Funds [17-3-846] and the metabolic tracer analysis core (MTAC) at the University of Birmingham. *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

**First published:** 12 Jan 2018, 3:5 (doi: [10.12688/wellcomeopenres.13387.1](https://doi.org/10.12688/wellcomeopenres.13387.1))

## Introduction

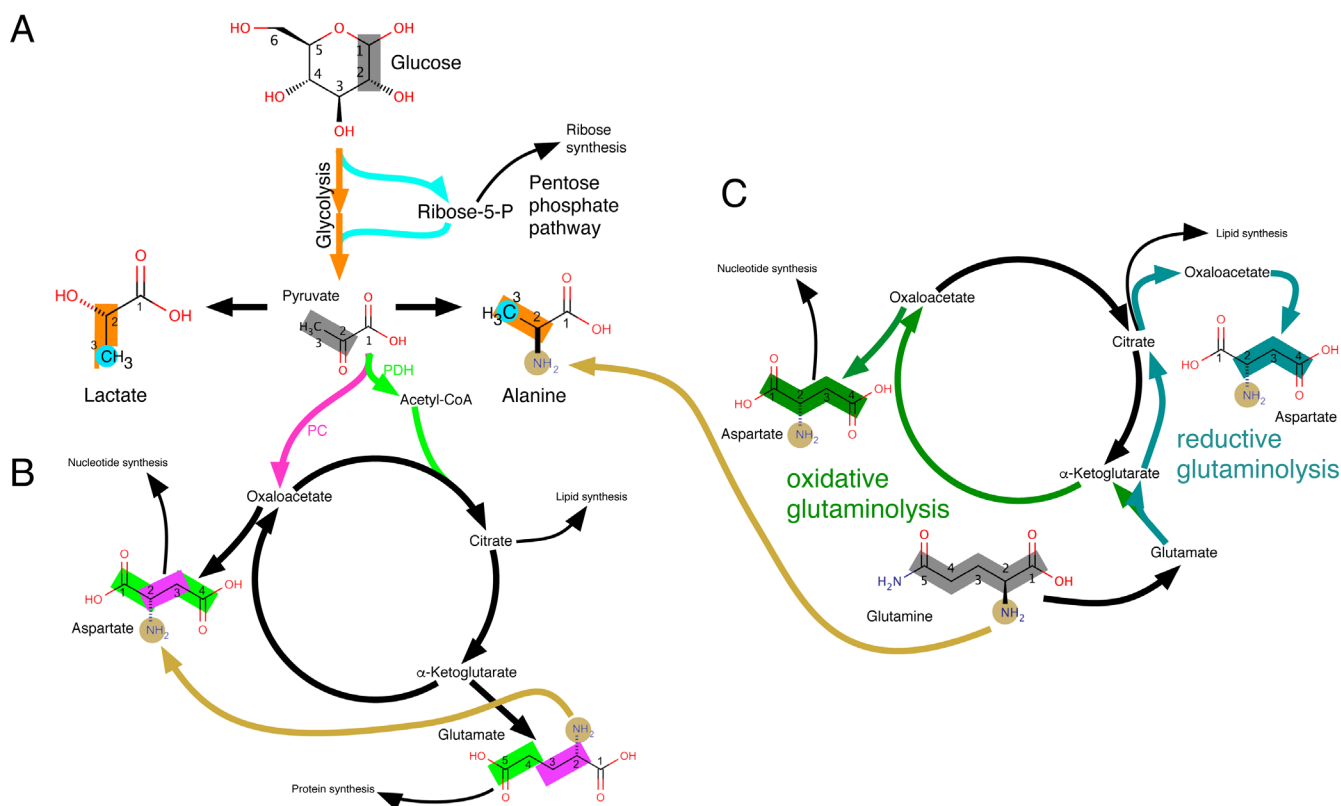
Investigations of metabolism in health and disease increasingly rely on tracing the use of stable isotope-enriched nutrients through the cells metabolic pathways. The most widely utilised technology platform to analyse the resulting complex patterns of labelling in multiple cellular metabolites is mass spectrometry (MS), due to its high sensitivity, short run times and a resulting low-cost operation<sup>1-12</sup>. 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 are incorporated into cellular metabolites<sup>12-19</sup>. 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 <sup>13</sup>C or <sup>15</sup>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 <sup>13</sup>C and/or <sup>15</sup>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<sup>19</sup>. 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



**Figure 1. Tracing of metabolic pathways.** The labelling patterns arising from [1,2-<sup>13</sup>C] glucose (A & B) as well as from [U-<sup>13</sup>C,<sup>15</sup>N] glutamine (C for <sup>13</sup>C labelling and A-C for <sup>15</sup>N labelling) are shown. Metabolism of [1,2-<sup>13</sup>C] glucose leads to distinctive labelling patterns in lactate and alanine ([2,3-<sup>13</sup>C] lactate/alanine when using glycolysis and [3-<sup>13</sup>C] lactate/alanine when using the pentose phosphate shunt, PPP) (Panel A). Similarly, glutamate and aspartate express different labelling patterns from [1,2-<sup>13</sup>C] glucose, depending whether they were synthesised via pyruvate dehydrogenase (PDH; resulting in [4,5-<sup>13</sup>C] glutamate) or via the pyruvate carboxylase (PC; resulting in [2,3-<sup>13</sup>C] aspartate) route (Panel B). Metabolisation of labelled glutamine can reveal other anaplerotic pathway activities such as reductive carboxylation (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 nucleus 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. [<sup>13</sup>C-1,2] glucose is the optimal tracer to assess metabolic flux through glycolysis vs pentose phosphate pathway (PPP) shunting back into glycolysis. While the glycolytically derived isotopomer of lactate is [<sup>13</sup>C-2,3] lactate, the PPP derived isotopomers can be [<sup>13</sup>C-3], [<sup>13</sup>C-1] or [<sup>13</sup>C-1,3] lactate. While the first 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 [<sup>13</sup>C-1,3] and [<sup>13</sup>C-2,3] lactate.

A major drawback of utilising <sup>13</sup>C-<sup>13</sup>C 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 <sup>15</sup>N labelling, especially when combined with <sup>13</sup>C tracing, thus providing the information required for more accurate metabolic pathway profiling.

## Methods and results

### Quantitative spectral filters for <sup>13</sup>C 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<sup>18</sup>. 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 perfusate 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

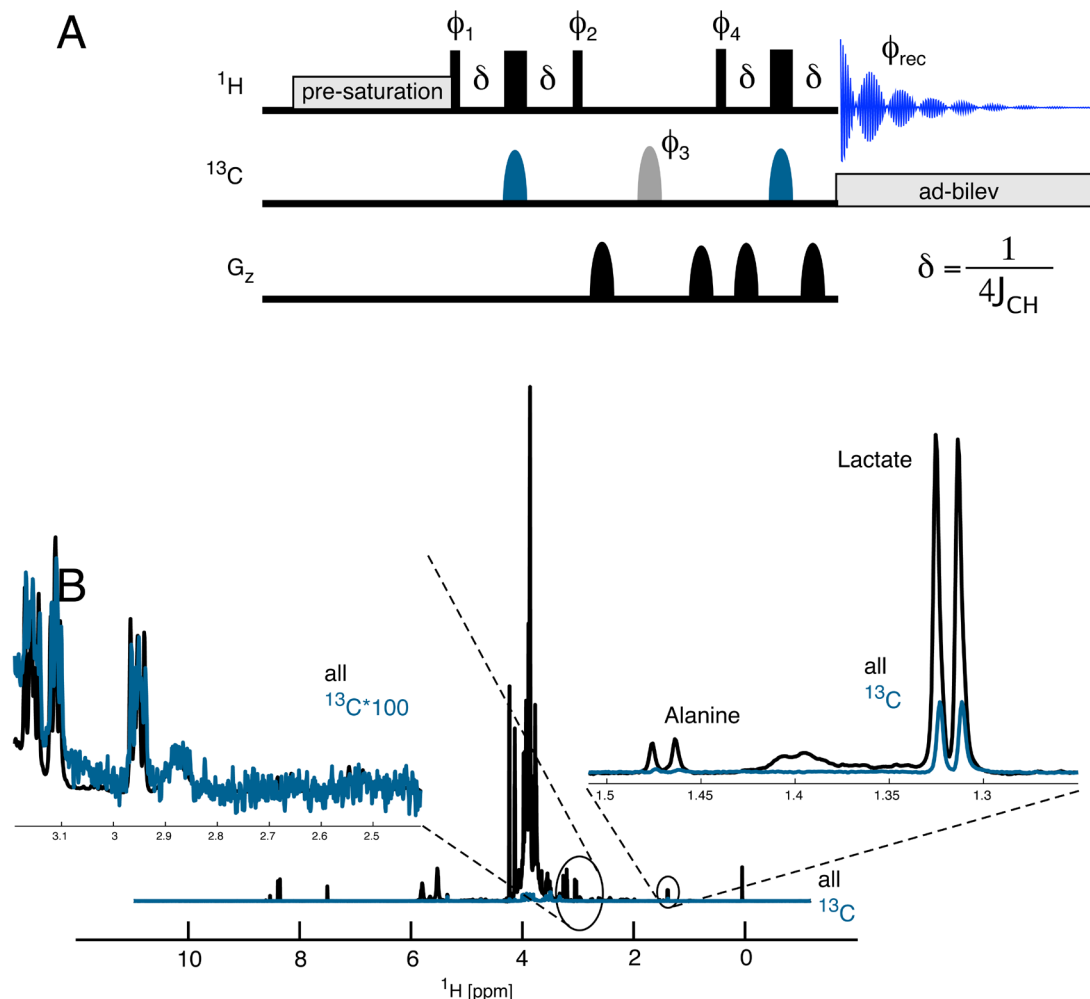
<sup>1</sup>H,<sup>13</sup>C J-coupling during acquisition. Decoupling for this long (2.25s) was possible because of the cryogenic probe and will quite possibly lead to probe damage when using a NMR system equipped with a room temperature probe.

The spectra were processed within the MetaboLab software package (version 1.0.0.1)<sup>20</sup>. 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 <sup>1</sup>H NMR spectra of protons bound to <sup>13</sup>C 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-<sup>1</sup>H,<sup>13</sup>C HSQC spectrum. However, signal intensities are not directly comparable with those in a standard 1D-<sup>1</sup>H NMR spectra. It is therefore not possible to directly derive <sup>13</sup>C percentages based on a comparison of those spectra with standard proton 1D spectra unless only a small subset of molecules is labelled with <sup>13</sup>C and one accompanying spectrum is scaled so that the majority of signals within the two spectra are of same intensity<sup>21</sup>. Spectral filters such as BIRD and TANGO<sup>22-25</sup> 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 <sup>13</sup>C tracer.

Figure 2a shows the pulse sequences implementing the quantitative spectral filter. While the grey shaded <sup>13</sup>C π-pulse is used only in odd transients and replaced with a delay of the same length during even transients, the blue <sup>13</sup>C π-pulses are only used in the <sup>13</sup>C filtering experiment, where <sup>1</sup>H magnetisation to <sup>13</sup>C neighbours is filtered out, so that only <sup>13</sup>C bound <sup>1</sup>H magnetisation contributes to the signal intensities in the 1D spectrum. The phase cycle φ<sub>1</sub> changes as well between the 2 experiments, as indicated by the same colour scheme (black: all protons, blue, <sup>13</sup>C 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  $^{13}\text{C}$  incorporation two spectra are acquired per sample. One spectrum contains  $^1\text{H}$  signals originating from all protons (all  $^1\text{H}$  spectrum), while the second spectrum only contains signals from protons attached to a  $^{13}\text{C}$  nucleus. For the all  $^1\text{H}$  spectrum, the blue  $^{13}\text{C}$  180 degree pulses are omitted. The grey shaded  $^{13}\text{C}$  pulse is executed every second transient in both experiments. The proton magnetisation in the all  $^1\text{H}$  pulse sequence is the same for  $^{12}\text{C}$  and for  $^{13}\text{C}$  bound protons and as a consequence, all  $^1\text{H}$  magnetisation is longitudinal during the interval between  $^1\text{H}$  pulses with the phases  $\phi_2$  and  $\phi_4$ . Because of the additional  $^{13}\text{C}$  180 degree pulses in the  $^{13}\text{C}$  bound  $^1\text{H}$  pulse sequence, the magnetisation for the two different kinds of protons develops differently. Here only the  $^{13}\text{C}$  bound  $^1\text{H}$  magnetisation is longitudinal in the interval between the  $^1\text{H}$  pulses with the phases  $\phi_2$  and  $\phi_4$ . Therefore, the  $^{12}\text{C}$  bound  $^1\text{H}$  magnetisation can be destroyed using the two pulse field gradients labelled gp1 and gp2. The grey shaded 180 degree  $^{13}\text{C}$  pulse, which is only executed every second transient improves magnetisation selection as it is accompanied with phase changes of the  $^1\text{H}$  pulses and the receiver. All  $^{13}\text{C}$   $\pi$ -pulses are adiabatic Chirp pulses with  $\gamma B_{1\text{max}} = \text{Hz}$ . The pulse phases are:  $\phi_1 = x, x, -x, -x$ ;  $\phi_2 = x, x, -x, -x / y, y, -y, -y$ ;  $\phi_3 = x, y, -x, -y$ ;  $\phi_4 = x, -x, -x, x, y, -y, y / y, -y, -y, y, -x, x, x, -x$ . The blue phases refer to the  $^{13}\text{C}$  bound  $^1\text{H}$  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 ( $-80^\circ\text{C}$ ) 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

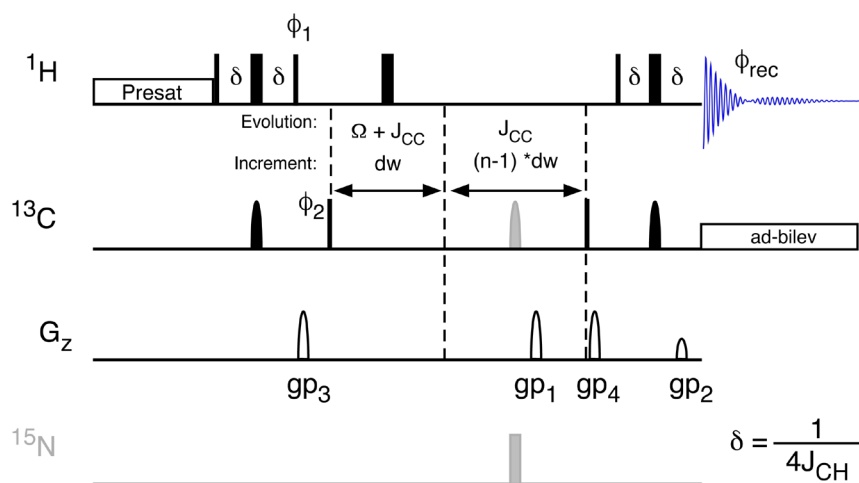
vigorously agitated. Biphasic separation of polar and non-polar solvents was performed by centrifugation (1300g, 15 minutes, 4°C), after which 4.5ml of the polar layer was aspirated and dried overnight at 35°C.

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

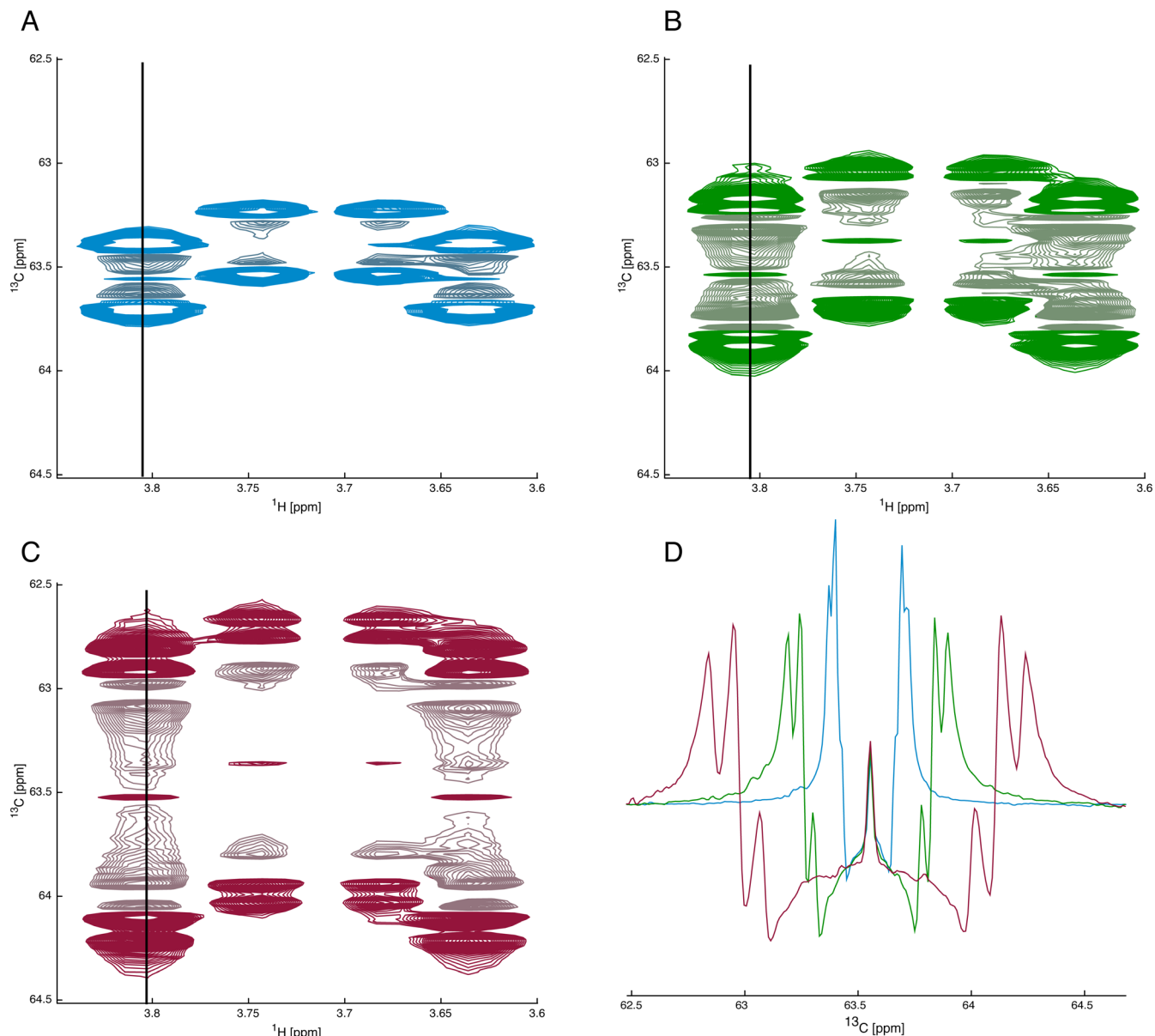
2D <sup>1</sup>H/<sup>13</sup>C-HSQC NMR spectra were acquired using a Bruker Avance III 600 MHz NMR spectrometer equipped with a 1.7mm z-PFG TCI Cryoprobe. The HSQC spectra were acquired using 2 transients per increment with echo/anti-echo gradient coherence selection and an additional pre-saturation 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)<sup>26</sup> and processed using NMRPipe (version 9.2)<sup>27</sup>. 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 patterns<sup>19</sup>. 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

<sup>13</sup>C dimension. It also means that at increasingly higher magnetic fields, the advantages of extra sensitivity and increased <sup>1</sup>H chemical shift resolution are not negated by the increased <sup>13</sup>C 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 <sup>13</sup>C evolves in parallel with the chemical shift evolution (Figure 3). This spin echo refocuses the <sup>13</sup>C chemical shift and the <sup>1</sup>H-<sup>13</sup>C coupling, but allows the <sup>13</sup>C-<sup>13</sup>C coupling to evolve further. The delays in the spin echo are proportional to those in the  $\Omega + J_{CC}$  evolution period with the amount of extra coupling achieved being defined by the stretch of the  $J_{CC}$  increment compared to the  $\Omega + J_{CC}$  increment. Thus, the <sup>13</sup>C-<sup>13</sup>C 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 <sup>13</sup>C J-couplings, combined with shortening the acquisition by using variable pulse sequence repetition times<sup>28</sup>, 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 <sup>13</sup>C 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  $J_{CC}$  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 <sup>13</sup>C 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 <sup>13</sup>C 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 <sup>13</sup>C,<sup>15</sup>N-couplings can be scaled by the introduction of the <sup>15</sup>N  $\pi$ -pulse parallel to the grey <sup>13</sup>C  $\pi$ -pulse, also shown in grey. The pulse phases are:  $\phi_1 = y$ ;  $\phi_2 = x, -x$ ;  $\phi_{rec} = x, -x$ .



**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  $^{13}\text{C}$  trace of the HSQC clearly shows the increase in splitting. The J-coupling enhancement is achieved using an additional spin echo subsequent to the  $^{13}\text{C}$  evolution period. The delays in the J-scaling are multiples of  $d_w$  such the use of a delay of  $3 \cdot d_w$  will result in a J-scaling of 4 (one from the  $t_1$  evolution and three from the J-scaling spin echo).

#### $^{15}\text{N}$ tracing

**Biological methods.** 2D  $^{14}\text{N}$  spectral filter - Sample preparation is described in 16. 2D  $^1\text{H}/^{13}\text{C}$ -HSQC NMR with and without  $^{14}\text{N}$  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  $^1\text{H}$  dimension was acquired with a spectral width of 13ppm using 512

complex data points. The  $^{13}\text{C}$  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.

**$^{13}\text{C}, ^{15}\text{N}$  J-coupling enhancement.** The human Medulloblastoma cell line DAOY from ATCC (HTB-186<sup>TM</sup>) 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.

$3 \times 10^6$  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- $^{13}\text{C}_5$ ,  $^{15}\text{N}_2$  (sigma, 607983) and 10mM 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 ( $-20^\circ\text{C}$ ), 600 $\mu\text{L}$  water ( $4^\circ\text{C}$ ) and 1.2 mL chloroform ( $-20^\circ\text{C}$ ). Cell lysates were vortexed for 15 min at  $4^\circ\text{C}$  and instantly centrifuged at 15,000 g for 15 min at  $4^\circ\text{C}$ .

Samples were dried using a Savant (SPD1010) speedvac concentrator and then resuspended in 60  $\mu\text{L}$  of 100 mM sodium phosphate buffer containing 500  $\mu\text{M}$  DSS and 2 mM Imidazole, 10%  $\text{D}_2\text{O}$ , pH 7.0. Samples were vortexed, sonicated for 10min and centrifuged momentarily (these steps were repeated twice), prior to being manually transferred to 1.5ml NMR tubes.

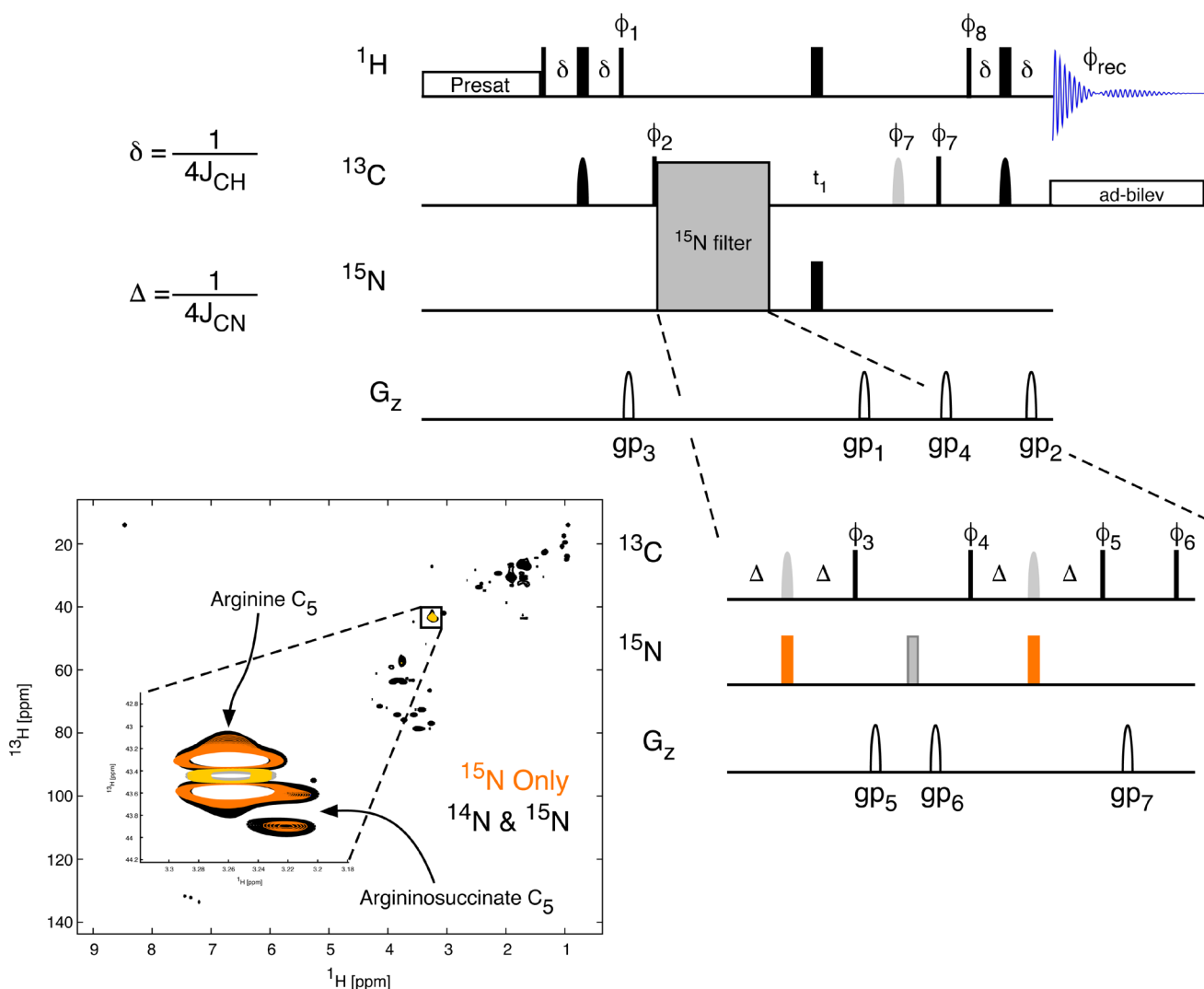
2D  $^1\text{H}^{13}\text{C}$ -HSQC and 2D  $^1\text{H}^{15}\text{N}$ -HSQC NMR spectra were acquired using a Bruker Avance III 900 MHz NMR spectrometer equipped with a 5mm z-PFG TCI Cryoprobe. The HSQC spectra were acquired using 2 transients per increment with echo/anti-echo gradient coherence selection and with an additional pre-saturation for suppressing the water resonance during the 1.5s interscan relaxation delay. The  $^1\text{H}$  dimension of the  $^{13}\text{C}$ -HSQC was acquired with a spectral width of 12ppm using 512 complex data points. The  $^{13}\text{C}$  dimension was acquired with a spectral width of 165 ppm using 30% (2458) of 8192 data points using a non-uniform sampling scheme. The  $^1\text{H}$  dimension of the  $^{15}\text{N}$ -HSQC was acquired with a spectral width of 12 ppm using 1024 complex data points. The  $^{15}\text{N}$  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)<sup>26</sup> and processed using NMRpipe (version 9.2)<sup>27</sup>. 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  $^{15}\text{N}$  labelling in metabolites, which alongside  $^{13}\text{C}$  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  $^{13}\text{C}$  nuclei that are adjacent to  $^{15}\text{N}$  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  $^{15}\text{N}$  labelling in the presence of  $^{13}\text{C}$  labelling within the metabolite. If spectral simplification is the goal, a single spectrum is sufficient<sup>16</sup>. The pulse sequence (Figures 5) is a gradient selected 2D- $^1\text{H},^{13}\text{C}$  HSQC spectrum with the spectral filter added once the  $^1\text{H}$  magnetisation has been transferred to the  $^{13}\text{C}$  nucleus. The spectrum collected with the  $^{15}\text{N}$  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  $^1\text{J}_{\text{CN}}$  constant. J-scaling on the other hand can be easily extended to include  $^{13}\text{C}$ - $^{15}\text{N}$  J-scaling. Indeed, the addition of a single  $^{15}\text{N}$   $\pi$ -pulse parallel to the grey shaded  $^{13}\text{C}$   $\pi$ -pulse in Figure 3 is sufficient to upscale the apparent  $^{13}\text{C}$ - $^{15}\text{N}$  J-coupling constant in 2D- $^1\text{H},^{13}\text{C}$  HSQC spectra. An example is given in Figure 6. Panels C and D show traces of the  $^{13}\text{C}$  multiplets for carbon atoms 2 and 3 of alanine. While the  $\text{J}_{\text{CC}}$  couplings are enhanced by a factor of 4 in both spectra, the apparent  $\text{J}_{\text{CN}}$  couplings are unchanged in the amber spectrum, whereas they are enhanced by a factor of 2 in the blue spectra. Because the  $^2\text{J}_{\text{CN}}$  coupling between C(3) and N is negligible, both traces for C(3) overlap perfectly. C(2) on the other hand experiences a  $^1\text{J}_{\text{CN}}$  coupling, which leads to the much more complex blue spectrum. The 2D signals for the  $\text{J}_{\text{CN}}$  scaled spectrum are shown in Figures 6A and B.

$\text{J}_{\text{CN}}$  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  $\text{H}_\alpha$  (the proton bound to C(2)) via the  $^2\text{J}_{\text{HN}}$  coupling. The  $\text{J}_{\text{NC}}$ -coupling then can be enhanced to show the appearance of  $^{13}\text{C}$  labelling in molecules which contain  $^{15}\text{N}$  next to those labelled carbon nuclei. As in this case, where the  $^{13}\text{C}$  nucleus is also bound to the proton determining the chemical shift on the horizontal axis,

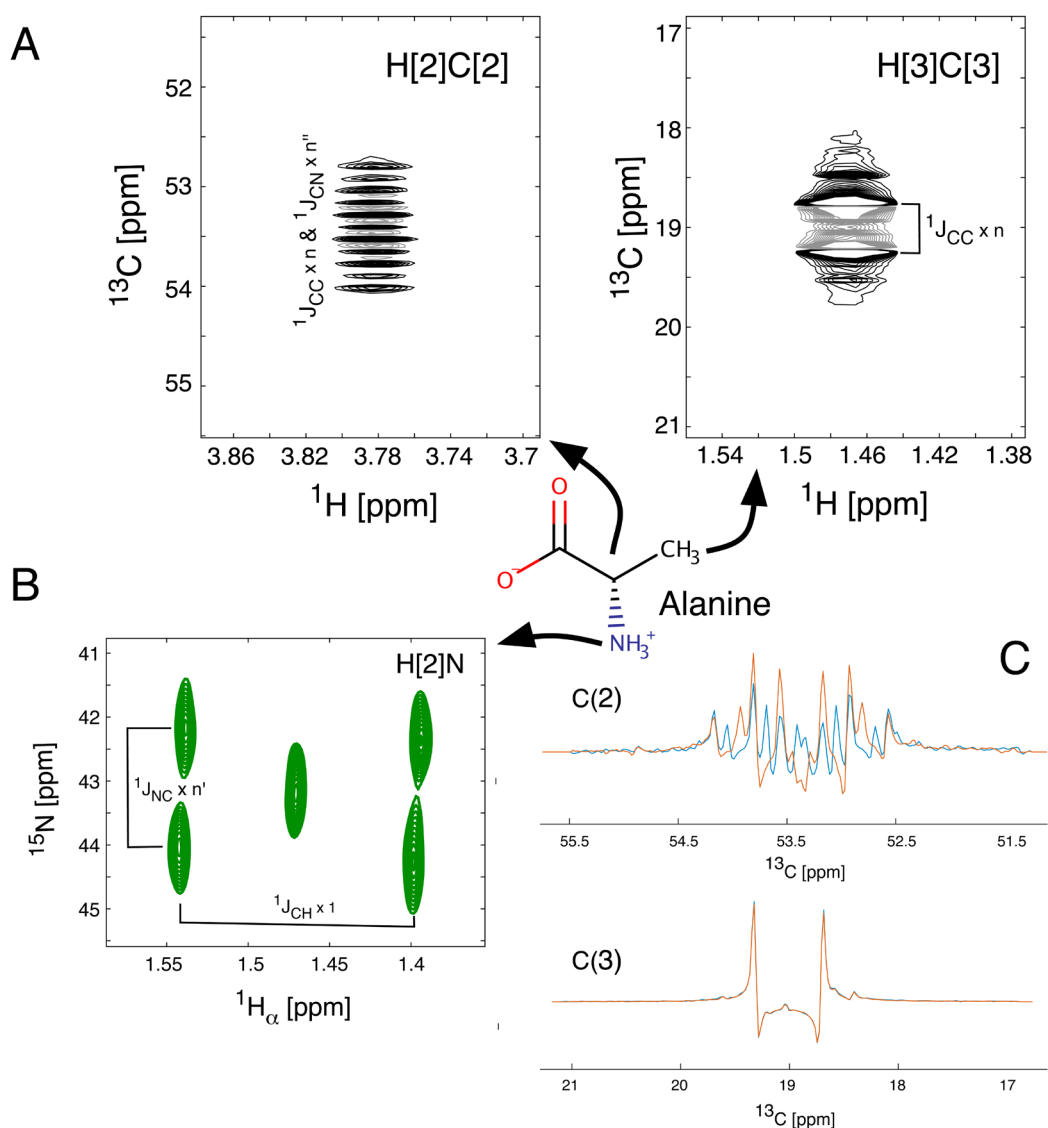


**Figure 5. Filtered HSQC.** Unfiltered (black) and filtered (amber) 2D- $^1\text{H}$ , $^{13}\text{H}$  HSQC spectra. In the filtered spectrum only carbon nuclei adjacent to  $^{15}\text{N}$  are visible. Similar to the filtered pulse sequence in Figure 1, the amber pulses are only executed in the  $^{14}\text{N}$  filtered pulse sequence and the grey  $^{15}\text{N}$   $\pi$ -pulse is executed every second transient. The pulse phases are:  $\phi_1 = y$ ;  $\phi_2 = x, -x$ ;  $\phi_3 = x / y$ ;  $\phi_4 = y, -y / y, y$ ;  $\phi_5 = y, -y / x, -x$ ;  $\phi_6 = x, x, -x, -x$ ;  $\phi_7 = x, x, x, x, -x, -x, -x, -x$ ;  $\phi_8 = x, x, x, x, y, y, y, y$ ;  $\phi_{\text{rec}} = x, -x, -x, x, y, -y, y$ .

that same proton signal will be split by the  $^1\text{J}_{\text{CH}}$ -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  $[\text{U-}^{13}\text{C}]$  glucose and  $^{15}\text{N}$  labelled glutamate which originated from  $[\text{U-}^{13}\text{C}, \text{U-}^{15}\text{N}]$  glutamine which was added to the growth medium in addition to the  $[\text{U-}^{13}\text{C}]$  glucose. In conjunction with MS data, this complementarity

between the 2D- $^1\text{H}$ , $^{13}\text{C}$  and the 2D- $^1\text{H}$ , $^{15}\text{N}$  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>)<sup>20</sup>. Details on sample preparation, data acquisition, processing and analysis are available in supporting information.



**Figure 6. Splitting due to  $^{15}\text{N}$  and  $^{13}\text{C}$  incorporation.** Alanine regions of the 2D- $^1\text{H}$ ,  $^{13}\text{C}$  HSQC spectrum (**A & B**). The signals are split by either  $^1J_{\text{CC}}$  or  $^1J_{\text{CN}}$  coupling contributions. The long range 2D- $^1\text{H}$ ,  $^{15}\text{N}$  HSQC (**C**) is composed of unlabelled alanine (middle peak) and  $^{13}\text{C}/^{15}\text{N}$  labelled alanine (4 outer signals, split by  $^2J_{\text{HN}}$  and  $^1J_{\text{NC}}$  couplings).

## 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 micro-probes, 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  $^{13}\text{C}$  incorporation percentages. However, samples cannot be assumed to be biologically identical, thus making analyses problematic due to the inability to determine accurate  $^{13}\text{C}$  isotope incorporation

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  $^{13}\text{C}$  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  $^1\text{H}$  nucleus over  $^{13}\text{C}$  and using the splitting due to J-coupling in the  $^{13}\text{C}$  dimension allows the indirect visualisation of the  $^{13}\text{C}$  incorporation into quaternary carbons. However, long acquisition 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 much 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  $^1J_{\text{CN}}$  couplings that are found in many metabolites after the addition of metabolites labelled  $^{15}\text{N}$  in conjugation with  $^{13}\text{C}$ . 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<sup>16</sup> 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- $^{13}\text{C}$ , U- $^{15}\text{N}$ ] arginine and were able to show that  $^{15}\text{N}$  labelled argininosuccinate was being formed in the cells containing the knock out, while wild-type cells did not produce  $^{15}\text{N}$  labelled argininosuccinate<sup>16</sup>. 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  $^{13}\text{C}$  and  $^{15}\text{N}$ . 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*<sup>29</sup>.

### Data availability

All experimental data for this article is available at: <http://doi.org/10.17605/OSF.IO/EQHN3><sup>30</sup>.

Experimental NMR datasets for HS-TrAM: The file filtered\_1D\_spectra.zip contains one unfiltered (subdirectory 1) and one  $^{13}\text{C}$  filtered (subdirectory 2) 1D- $^1\text{H}$  spectrum. The file jEnhanced\_13C\_HSQC.zip contains the 2D- $^1\text{H}$ , $^{13}\text{C}$  HSQC spectra with different J-coupling enhancement. The file 13C\_HSQC\_14N\_filter\_and\_15N\_HSQC.zip contains an unfiltered (subdirectory 1) and a  $^{14}\text{N}$  filtered (subdirectory 2) 2D- $^1\text{H}$ , $^{13}\text{C}$  HSQC spectrum. The file jEnhanced\_13C\_15N\_HSQC.zip contains the following spectra:

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

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### Competing interests

No competing interests were disclosed.

### Grant information

This work was supported by the Wellcome Trust [099185]; the National Institute for Health Research [13-0053]; Help Harry Help Others [HelpCU09]; UHB Charitable Funds [17-3-846] and the metabolic tracer analysis core (MTAC) at the University of Birmingham.

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

### Acknowledgements

We thank HWB-NMR at the University of Birmingham for providing open access to their Wellcome Trust-funded 900 MHz spectrometer. Organ Recovery Systems donated perfusion equipment.

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# Open Peer Review

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## Version 1

Referee Report 23 January 2018

doi:[10.21956/wellcomeopenres.14535.r29774](https://doi.org/10.21956/wellcomeopenres.14535.r29774)



**Paul C. Driscoll** 

Francis Crick Institute, London, UK

In this manuscript the authors have built on their own previous work wherein high resolution 2D  $^1\text{H}^{13}\text{C}$  HSQC NMR spectra were used together with sophisticated data-fitting tools to provide quantitative measures of isotope incorporation at single positions within metabolites. The specific procedures developed here significantly expand the scope of the method and in principle constitute a new paradigm in isotope tracer analysis. One aspect (and it is only one of several utilities that arise) is that the approach should enable real time measurements in isotopomer-based NMR metabolic profiling with a vastly higher time resolution than would otherwise be possible. A particularly advantageous aspect is the demonstration of the detection of adjacent  $^{13}\text{C}$ - $^{15}\text{N}$  dual isotope labelling with enhanced sensitivity and precision, opening up the potential to simultaneously assess the metabolic flux of both carbon and nitrogen atoms.

I have abundant enthusiasm for the ingenuity and potential of this work, and find very little that is technically questionable with the approach. However I did find several aspects where the manuscript should be enhanced or, indeed, corrected to make it more useful to the more- or less-informed reader.

I found a number of aspects of the structure of the document require attention. Namely: Aspects of Methods are unnecessarily (and confusingly) mixed up with the Results, for example on pages 6-7 Biological and NMR Methods are completely jumbled.

I do not think it is pedantry to say that J-couplings cannot be 'enhanced'. The resonance splittings due to [magnetization evolution due to] J-coupling can be enhanced, but J-coupling constants are exactly that: constant. More care should be taken with the language used to describe the 'J-scaling' (itself strictly a misnomer in my opinion) concept employed. I note that the treatment assumes weak coupling between  $^{13}\text{C}$  atoms (well, in that the line shape is not described anywhere – or shown fitted here, I expect that assumption is behind the Metabolab treatment). It is not a bad assumption but this condition should be stated.

Is 'J-scaling' truly 'novel'? I found reference to downscaling of J-couplings from the relatively early NMR literature, and wonder whether upscaling of J-splittings has been employed in the more recent field of residual dipolar coupling measurements.

I would feel more comfortable for the less expert reader if the authors would comment on the extent to which, if at all, their two approaches might be sensitive to differential  $^1\text{H}$  relaxation rates, signal

multiplicities and  $^1\text{JCH}$  coupling constants. I think the section on page 3 describing the adiabatic decoupling during the acquisition of the FID requires greater caveating than is provided. For example, one should check the manufacturer's specification for the particular probe employed, and the authors here could indicate the average power dissipation and whether the cryogas heater current is above its lower limit during such a long period of RF irradiation. When 0.5 Hz line broadening apodization is being employed, do you need 2.25 s of acquisition?

I do not understand why baseline correction (e.g. zeroth order to remove offsets) would interfere with data fitting, as mentioned more than once.

The figure captions are mostly inadequate. Despite the figure panels being labelled A, B, C etc. the captions themselves do not refer to these labels. More detail is required here.

I struggled with the description of the spectral filter experiment sketched in Figure 2 and described on page 3. To assist the less NMR-savvy reader, I would prefer to see this cast in terms of two different NMR experiments with separate depictions of the pulse sequence(s).

Throughout the main text I would prefer not to see references to 'the blue spectrum', 'the grey pulse', but rather more explicit terms used.

Figure 2A: see comment above. Labels for gp1 and gp2 are missing. 'ad-bilev' is not explained. Reference should be made to time gaps constituted by the gradient recovery delays.

Figure 2B: it is not explicit what is the utility of scaling the blue spectrum by 100?

Figure 3: The J-coupling is not enhanced (see above). The Evolution and Increment lines in the figure are (confusingly) not horizontally aligned. 'n' is not explained. Why is the  $^{13}\text{C}$  'J-scaling' pulse shown in gray? Caption: 'simultaneously with' would be better than 'parallel'.

Figure 4: The cross-sectional vertical lines would be better in dashed format. Positive and negative contour colour differences are not explained. It might be worthwhile to comment on the asymmetric structure of the cross peak in the  $^{13}\text{C}$  dimension. Caption: there is no reference to A,B,C,D panel labels. The description of the pulse sequence scaling evolution delays needs to be improved ('the J-scaling' is shorthand; 'the J-only evolution' period might be better).

Figure 5: A and B labels for the different figure elements are missing. Why are some of the  $^{13}\text{C}$  pulses shown in grey? I would prefer it if the two NMR spectra were not superposed, but rather plotted side by side. Caption: It is ambiguous whether the grey  $^{15}\text{N}$  pulse is employed along with the amber pulses. I am not keen on the use of colour in the exposition of the phase cycles.

Figure 6: Panel C seems to have more to do with A than B, so order is confusing. 'n', 'n"', 'n"' 'H[2]N', 'H[2]C[2]' and 'H[3]C[3]' are not clearly explained (molecular structure needs numbering). The caption is in error when referencing B and C. Panel C switches from C[2] to C(2), etc. What do the different colour traces in panel C indicate? The caption does not explain. Note 1: on page 7 of the text there is reference to a panel D, and this strangle out of order as reference to panels A and B comes later. Note 2: Later the text describes the long range  $^1\text{H}$   $^{15}\text{N}$  HSQC spectrum shown in panel C but without reference to it. It appears to describe the spectrum in terms of carbon C(2) and a two bond  $^2\text{J}_{\text{NH}}$  coupling, but should properly be C(3) (Cbeta) and  $^2\text{J}_{\text{NCbeta}}$ . Note 3: The text goes on (page 8) to discuss the metabolic

pathway in a section that would more properly be in the Discussion, and then there is a short paragraph about Metabolab that should be in the Methods section.

Discussion:

When 1D spectra are mentioned (page 11) is this meant to refer to  $^{13}\text{C}$  1D spectra?

There is a claim for 'unprecedented' detail. The utility of J-scaling to reveal small couplings is not very well exemplified by the results, since no fitted cross sections are shown. Also whilst real-time measurements of metabolism using this approach can be anticipated, no (even synthetic) example is shown. The 'High Speed' moniker in the title for the manuscript is not strongly justified by the data shown, and not strongly emphasized by the account given. Would it not be preferable to provide side-by-side demonstrations of the effective information content, for a given complex sample\* in (i) a 1D  $^1\text{H}$ -decoupled  $^{13}\text{C}$  spectrum; (ii) a non-aliased 2D  $^1\text{H}^{13}\text{C}$ -HSQC spectrum with minimum phased cycle; (iii) an aliased version of (ii); and (iv) the 'J-scaled' versions of (ii) and (iii)? \* of arbitrary complexity and concentration?

Throughout the manuscript, greater care is required for the use of numerals instead of words (where appropriate, e.g. two scans, no 2 scans); spaces between numbers and units; avoidance of unexplained abbreviations (e.g. WIT-15); appropriate use of spectra/spectrum; avoidance of ambiguous orphan pronouns (e.g. 'this/it' [verb]); anthropomorphization (e.g. 'cells form...' page 10); it is  $\text{D}_2\text{O}$ , not  $\text{D}_2\text{O}$ ; almost invariably 'spectrum' should follow HSQC. I would be happy to proof read any revised version of the manuscript to help iron out any similar issues remaining.

In summary, whilst I see great value in the ideas presented here, and hope to employ them in our own future work, I believe that the authors could substantially upgrade the description of the new approach in a revised version of the manuscript.

**Is the rationale for developing the new method (or application) clearly explained?**

Yes

**Is the description of the method technically sound?**

Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**

Partly

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**

Partly

**Competing Interests:** I am a member of the External Advisory Board for the Henry Wellcome Biomolecular NMR Centre at Birmingham University. One of the authors of the current manuscript (CL) is the referee of a 2017 submission to Wellcome Open Research of which I am a co-author. Another of the authors (MJ) was formerly a postdoctoral fellow in my own research group.



**Referee Expertise:** Biological NMR spectroscopy

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Referee Report 23 January 2018

doi:[10.21956/wellcomeopenres.14535.r29772](https://doi.org/10.21956/wellcomeopenres.14535.r29772)



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I did answer partly for 2 questions which do not alter the quality of this paper. My comments are listed below:

#### **General comments:**

In this manuscript, Smith *et al.* present two techniques - quantitative spectral filters and enhanced J-coupling- that allow rapid collection of heteronuclear NMR data using HSQC NMR spectroscopy. The main advantage of these methods is that 2D- HSQC NMR spectra contain information about 1H-13C, 13C-13C and 13-15N coupling in a fraction of time it takes 1D spectra to acquire the same sample. Combining this NMR approach with metabolic modelling could be very powerful for quantify metabolic fluxes in cells or tissues.

This paper is interesting and novel in terms of pulse-sequence but suffers lack of consistency and rationale regarding experimental approaches (i.e. biological models). Although the NMR approach using spectral filtering and enhanced J-coupling is clever, I don't really understand why 3 biological models have been used in this paper. I understand that this work is a proof-of-concept but for consistency only the porcine kidney perfusion model should be used. In addition, this model is relevant to the primary aim of this study- the real-time tracing of metabolic pathways in whole organ prior to transplantation. Additional data with porcine kidney perfusion are needed to improve this paper or at least a good rationale for why authors have used tumour cells for 15N tracing. I believe that 13C-15N glutamine metabolism can be detected in their porcine kidney model. This will add strength and rigour to the paper.

What is the variability in quantitative spectral filters and enhanced J-coupling between experimental repeats? The authors don't state the number of repetitions per experiment, so the reproducibility cannot be assessed. I believe that the percentage incorporation of <sup>13</sup>C and <sup>15</sup>N into metabolites could be assessed in this study and thus should be presented. Did authors run their samples using conventional 1D 13C NMR (Rodrigues et al 2013 Front Neuroenergetics) or proton-observed carbon-edited (POCE) strategy (Rothman et al. 1985 PNAS) for comparison?

#### **Specific comments:**

1. To avoid any confusion, authors should mention that NMR analysis is done *ex vivo* or *in vitro* but provides information on metabolic pathways *in vivo*.
2. Not sure about what authors mean by high-resolution data. Is it related to sensitive measurements of metabolites (~microM) or a better signal splitting with ultra-high field NMR? Or is it in comparison with <sup>13</sup>C data collection using mass spectrometry?
3. Authors claim that" such techniques permit high throughput metabolic pathway profiling, increasing access, affordability and sensitivity when using NMR as an investigative modality"

Are these sequences freely available and easy to implement on Bruker NMR spectrometers? However not every sites in the world have access to cryoprobe.

1. "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". How long was the labelling time for?
2. To correct: "needed in in order to resolve J-couplings "
3. Please add a table that summarizes the pros and cons of their new techniques (e.g. acquisition time, SNR, J coupling enhancement etc...). This need to be compared with conventional 1D NMR spectra or POCE.
4. Figure 4 legend did not mention that the spectrum displays C(6) of  $^{13}\text{C}$  enriched glucose.
5. Why using a different NMR spectrometer for glutamine experiment (900 vs 600MHz)?

"2D  $^1\text{H}$ - $^{13}\text{C}$ -HSQC and 2D  $^1\text{H}$ - $^{15}\text{N}$ -HSQC NMR spectra were acquired using a Bruker Avance III 900 MHz NMR spectrometer equipped with a 5mm z-PFG TCI Cryoprobe"

**Is the rationale for developing the new method (or application) clearly explained?**

Yes

**Is the description of the method technically sound?**

Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**

Partly

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Metabolic biochemistry

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

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