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METHOD ARTICLE

REVISED High-Speed Tracer Analysis of Metabolism (HS-TrAM)
[version 2; referees: 4 approved]

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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) spectroscopy offers high resolution data in terms of resolving metabolic pathway utilisation. Despite this, NMR spectroscopy 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 of ^{13}C signals due to homonuclear $^{13}C,^{13}C$ or heteronuclear $^{13}C,^{15}N$ J-coupling in $^{1}H,^{13}C$-HSQC NMR spectra. Together, these allow the rapid collection of NMR spectroscopy 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 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. In addition, we show how the use of multiple nutrients, differentially labelled with ^{13}C and ^{15}N, can be used to provide additional information with which to profile metabolic pathways.

Keywords

NMR, stable-isotope tracing, 13C, 15N, splitting enhancement, metabolism, tracer

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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 are incorporated into cellular metabolites[12–19]. This is because NMR spectroscopy 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[20]. 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. While sample analysis by high-resolution NMR spectroscopy is performed *ex vivo* or *in vitro*, the data obtained provide information on metabolic pathways *in vivo*.

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). The couplings

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 carboxylation (Panel **C**).

are visualised in the indirect dimension of an HSQC spectrum allowing the determination of the percentage incorporation of isotopic label into adjacent nuclei. While MS data does not need a reference sample to distinguish between labelled and unlabelled metabolites, it is not always possible to derive the exact distribution of labelled nuclei within a molecule. In contrast, NMR spectroscopy data can resolve label distribution at the atomic level, enabling a clearer picture of the label distribution in metabolites.

Our recently published combined NMR spectroscopy 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 spectroscopy 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 spectroscopy 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 spectroscopy 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. [1,2-^{13}C] 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 [2,3-^{13}C] lactate, the PPP derived isotopomers can be [3-^{13}C], [1-^{13}C] or [1,3-^{13}C] lactate. Although the first isotopomer can be assessed with NMR spectroscopy data, the other two isotopomers include labelling in C(1), which HSQC NMR spectroscopy is "blind" to. In these cases, MS data adds new information to the NMR spectroscopy data by contributing the isotopologues NMR spectroscopy is not able to detect, while NMR spectroscopy adds to the MS data by differentiating between [1,3-^{13}C] and [2,3-^{13}C] lactate.

A major drawback of utilising ^{13}C-^{13}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 2D-HSQC NMR spectrum with high-resolution in the ^{13}C dimension, even when using fast, state-of-the-art non-uniform sampling (NUS) techniques.

Here we describe two developments, quantitative spectral filters and signal splitting 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 spectroscopy as an investigative modality. Additionally, these developments facilitate fast detection of ^{15}N labelling, especially when combined with ^{13}C tracing, thus providing extra information allowing more accurate metabolic pathway profiling.

Methods and results

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

Experimental setup. A porcine kidney was procured from a slaughterhouse (FA Gill, Wolverhampton) following approximately 14 minutes warm ischaemic time (WIT) as per previous experimental methodology[18]. No animal was killed solely for experimental purposes; all were due for human consumption, 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 s interscan relaxation delay. A total of 32768 data points with a spectral width of 12 ppm was acquired for each FID using an adiabatic bi-level decoupling scheme to suppress ^{1}H, ^{13}C J-coupling during acquisition[21]. While decoupling for this long (2.25 s) was possible because of the cryogenic probe and may potentially work with a room temperature probe, care must be taken as there will be significant sample heating. The sample heating can be significantly reduced, with only very minor reduction in resolution, by acquiring for only 1.125 s. In order to estimate whether a specific spectrometer with a cryoprobe can tolerate the power dissipation originating from the decoupling sequence specific attention should be paid to the cryogas heater current, which should never fall below its system specific lower limit.

The spectra were processed within the MetaboLab software package (version 2018.07182055)[22]. A 0.5 Hz line broadening was applied with zero-filling of 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 highly sensitive tools with which 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 ^{1}H NMR spectra of protons bound to ^{13}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-^{1}H, ^{13}C-HSQC spectrum. However, signal intensities are not directly comparable with those in standard 1D-^{1}H NMR spectra. It is therefore not possible to directly derive ^{13}C percentages based on a comparison of those spectra with standard proton 1D spectra unless only a small subset of molecules is labelled with ^{13}C and one accompanying spectrum is scaled so that

the majority of signals within the two spectra are of same intensity[23]. Spectral filters such as BIRD, TANGO and POCE[24–28] 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 ^{13}C tracer.

Panels A-1 and B-1 in Figure 2 show the pulse sequences implementing the quantitative spectral filter. While the central
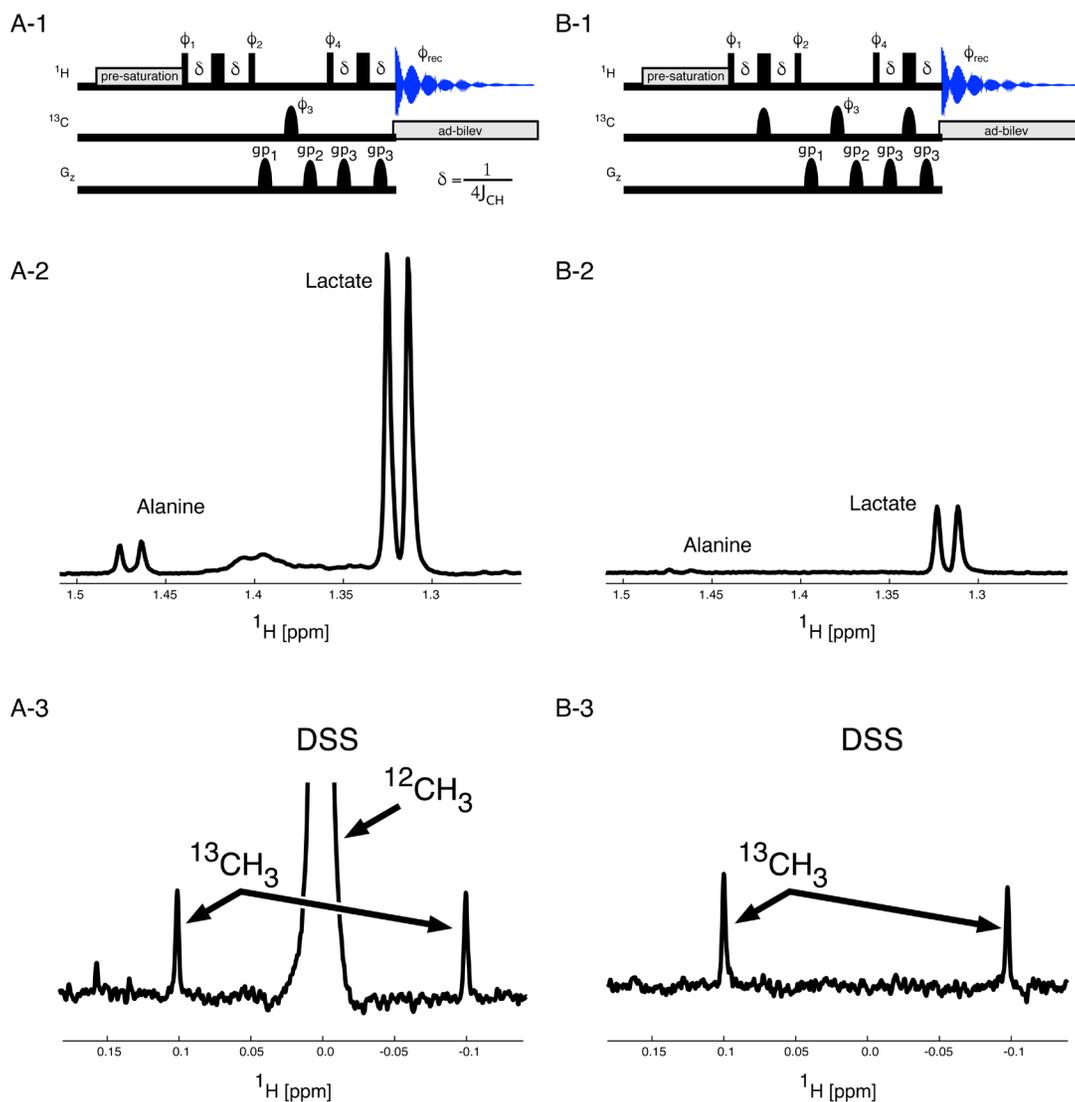
Figure 2. Spectral Filters in 1D spectroscopy. To determine percentage ^{13}C incorporation two spectra are acquired per sample. One spectrum (**A**) contains ^{1}H signals originating from all protons (all ^{1}H spectrum), while the second spectrum (**B**) only contains signals from protons attached to a ^{13}C nucleus. The central ^{13}C π–pulse with phase ϕ_3 is executed every second transient in both experiments. The proton magnetisation in the all ^{1}H pulse sequence is the same for ^{12}C and for ^{13}C bound protons and as a consequence, all ^{1}H magnetisation is longitudinal during the interval between ^{1}H pulses with the phases ϕ_2 and ϕ_4. Because of the additional ^{13}C π–pulses in the ^{13}C bound ^{1}H pulse sequence, the magnetisation for the two different kinds of protons develops differently. Here only the ^{13}C bound ^{1}H magnetisation is longitudinal in the interval between the ^{1}H pulses with the phases ϕ_2 and ϕ_4. Therefore, the ^{12}C bound ^{1}H magnetisation can be destroyed using the two pulse field gradients labelled gp_1 and gp_2. A recovery delay of 200 μs was used after each gradient. The central ^{13}C π–pulse with phase ϕ_4 improves magnetisation selection as it is accompanied by phase changes of the ^{1}H pulses and the receiver. All ^{13}C π-pulses are adiabatic Chirp pulses with γB_{1max} = 10 kHz. ^{1}H,^{13}C J-coupling is suppressed during acquisition using adiabatic bilevel decoupling (ad-bilev)[21]. The pulse phases are: ϕ_1 = x, x, -x, -x; ϕ_2 = x, x, -x, -x for the all ^{1}H spectrum (**A-1**) and y, y, y, y for the ^{13}C bound ^{1}H spectrum (**B-1**), ϕ_3 = x, y, -x, -y, ϕ_4 = x, -x, -x, x, y, -y, -y, y for the all ^{1}H spectrum (**A-1**) and y, -y, -y, y, -x, x, x, -x for the ^{13}C bound ^{1}H spectrum (**B-1**). Panels **A-2** and **B-2** show the peaks from Alanine and Lactate methyl protons in the all ^{1}H spectrum (**A-2**) and the ^{13}C bound ^{1}H spectrum (**B-2**). The scaling of the two spectra is identical allowing easy determination of the percentage incorporation of ^{13}C metabolites. Panels **A-3** and **B-3** demonstrate the complete removal of the ^{12}C bound proton signal from the ^{13}C edited spectrum (**B-3**) leaving only the natural abundance ^{13}C signals to be observed.

[13]C π-pulse (phase ϕ_3) is used only in odd numbered transients and replaced with a delay of the same length during even numbered transients, the two other [13]C π-pulses are only used in the ^{12}C filtering experiment (B-1), where ^{1}H magnetisation to ^{12}C neighbours is filtered out, so that only ^{13}C bound ^{1}H magnetisation contributes to the signal intensities in the 1D spectrum. The phase cycle ϕ_1 changes as well between the 2 experiments, as indicated in the figure legend.

Panels A-2 and B-2 in Figure 2 depict 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 hypothermic machine perfusion. The standard unlabelled glucose constituent (10 mM) within classical UW MPS was replaced with 10 mM universally labelled glucose, for use as a metabolic tracer during the 18 hour perfusion.

While filters such as BIRD utilise relaxation to minimise the unwanted part of the magnetisation, methods such as TANGO or POCE generate magnetisation where ^{12}C bound ^{1}H atoms possess either the same or the opposite phase compared to the magnetisation of ^{13}C bound ^{1}H atoms, generating two different spectra. By subtraction of these two spectra, the magnetisation of ^{13}C bound ^{1}H atoms can then be calculated. In case of low ^{13}C incorporation, as with any difference technique, subtracting two very large signals in presence of a small signal can lead to substantial artefacts. The quantitative spectral filter works slightly differently compared to TANGO and POCE as the pulse sequence depicted in Figure 2, panel B-1 makes use of two gradient pulses (gp_1 and gp_2) to destroy unwanted magnetisation. As an example, panels A-3 and B3 in Figure 2 show a variant of the pulse sequences where the adiabatic bilevel decoupling (ad-bilev)[21] has been omitted, so that the ^{12}C and the ^{13}C bound ^{1}H signals appear separated in the spectrum. The [12]CH_3 signal in A-3 has been truncated to be able to visualise the ^{13}C satellites, which appear with 0.5% of the peak height of the [12]CH_3 signal. As can be seen in panel B-3, the [12]CH_3 signal is completely suppressed without leaving an artefact, so that even signals from naturally occurring ^{13}C alone are easily detectable in a ^{13}C decoupled spectrum.

The quantitative spectral filter is invariant with respect to differential ^{1}H relaxation rates or signal multiplicities. As with any J-coupling based filtering approach, the ^{12}C filtered spectrum will be scaled with a factor that is proportional to $\sin(2\pi\delta J_{CH})^2$, where δ is the delay during the first and last spin echo. δ is usually set to ¼J_{CH} with J_{CH} = 145 Hz. Assuming a minimum J_{CH} of 120 Hz and a maximum J_{CH} of 165 Hz, results in a maximum downscaling of 7.2%.

J-Coupling based splitting enhancement in 2D-NMR spectra

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 modified 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 0.7 L/min for the duration of the 24 hours perfusion period.

At the end of the perfusion period, 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, also under liquid nitrogen, and 0.5 g was placed in 7 ml 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 5.1 ml HPLC grade chloroform and vigorously agitated. Biphasic separation of polar and non-polar solvents was performed by centrifugation (1300 g, 15 minutes, 4°C), after which 4.5 ml of the polar layer was aspirated and dried overnight at 35°C.

The dried extracts were resuspended in 60 μl NMR buffer (0.1 M phosphate buffer, 0.5 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid, 2 mM imidazole and 10% D_2O). These suspensions were sonicated to dissolve micro particles and then 35 μl of this solution was added to 1.7 mm NMR tubes.

^{1}H,^{13}C-HSQC spectra were acquired using a Bruker Avance III 600 MHz NMR spectrometer equipped with a 1.7 mm 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 1.5 s interscan relaxation delay. The ^{1}H dimension was acquired with a spectral width of 13 ppm using 512 complex data points. The ^{13}C 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)[29] and processed using NMRPipe (version 9.2)[30]. 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[19]. 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 has previously been reported[31,32]. Here we apply this technique in order to negate the requirement for the collection of large number of increments in the ^{13}C dimension, which, together with methods such as non-uniform sampling[29,33] and variation of the repetition time[34] significantly reduces the time required to acquire 2D-HSQC spectra with sufficient resolution. It also means that at increasingly higher magnetic fields, the advantages of extra sensitivity and increased ^{1}H chemical shift resolution are not negated by the increased ^{13}C increments needed in in order to resolve J-couplings. Enhancement of the splitting due to J-coupling can be achieved by incrementing the spin echo delay after the period where chemical shift of ^{13}C evolves in parallel with the chemical shift evolution (Figure 3). This spin
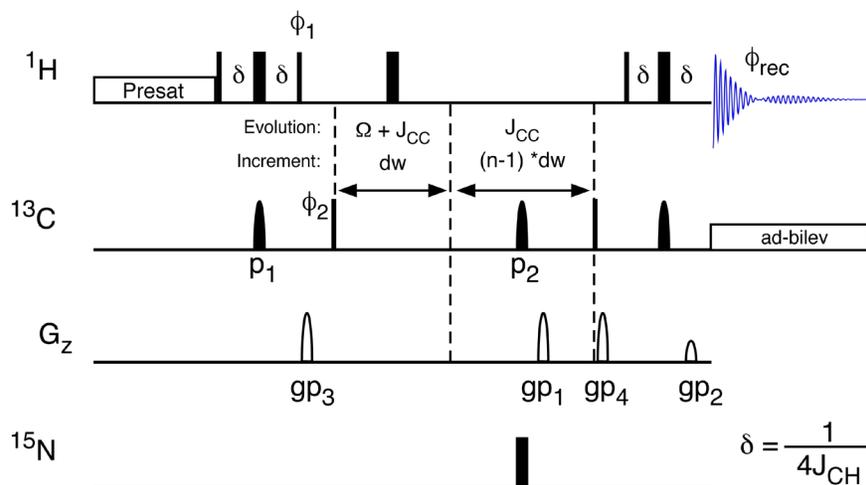
Figure 3. Splitting Enhanced HSQC Spectroscopy. The splitting enhancement due to J-coupling is achieved using an additional spin echo subsequent to the ^{13}C evolution period. The delays in the spin-echo for the J-coupling enhancement are multiples of the dwell time (dw). While the chemical shift evolves with dw, which is determined by setting the spectral width of the spectrum, splittings are enhanced depending on the increment of the ^{13}C gradient selection spin-echo. The HSQC spectrum is acquired using echo/anti-echo for quadrature detection to allow for efficient removal of artefacts in only two scans per increment. Optionally, the $^{13}C,^{15}N$-couplings can be scaled by the introduction of the ^{15}N π-pulse simultaneously with the ^{13}C π-pulse (labelled p2). $^{1}H,^{13}C$ J-coupling is suppressed during acquisition using adiabatic bilevel decoupling (ad-bilev)[21]. The pulse phases are: $\phi_1 = y$; $\phi_2 = x, -x$; $\phi_{rec} = x, -x$.

echo refocuses the ^{13}C chemical shift and the ^{1}H-^{13}C coupling, but allows the ^{13}C-^{13}C coupling to evolve further. The delays in the spin echo are proportional to those in the Ω+J_{CC} evolution period with the amount of extra coupling achieved being defined by the stretch of the J_{CC} increment compared to the Ω+J_{CC} increment. Thus, the ^{13}C-^{13}C J-couplings can be expanded as required (Figure 4). The ability to scale the signal splittings to varying extents means that the experiment can be tuned to the requirements of the sample and which metabolites are present, and of interest. Figure 4 demonstrates the effect of J-coupling splitting enhancement on 2D HSQC spectra, displaying C(6) of ^{13}C enriched glucose. The ^{13}C trace through the left-most signal (Figure 4D), demonstrates clearly that while the singlet in the middle of the multiplet does not change, the splitting due to the $^{1}J_{CC}$ coupling increases and in fact splits into multiple signals as the splittings of previously unresolved long-range couplings are amplified so that they are large enough to become resolved in the spectrum. These splittings can easily be simulated (Figure 4E), thereby providing additional information with which to model metabolic pathways.

Large expansion of J-coupling also allows for rapid collection of data, as the resolution required to resolve them becomes diminished (Figure 5). However, this should be tempered by the need to avoid unnecessary overlap of signals. To date, the authors have acquired 2D spectra with up to eight fold enhanced ^{13}C J-couplings, combined with shortening the acquisition by using variable pulse sequence repetition times[34], leading to an overall decrease in acquisition time by a factor larger than 10 (Figure 5). Panels A to D show the spectral region of the methyl groups of lactate and alanine. Panel E shows a cross section, as marked in the 2D spectra, from alanine, whereas panel F shows the corresponding simulations of those multiplets. The acquisition times for the different spectra were 233, 110, 51 and 24 minutes (Table 1). Whilst the lines in

the spectrum become broader due to the shorter acquisition times, this is negated by the increase in splittings, allowing the analysis of the multiplets with similar precision. Shorter acquisition times may be achieved by including spectral folding in the acquisition protocol or by incorporating new fast acquisition schemes such as ASAP- or ALSOFAST-HSQC[35].

^{15}N tracing

Experimental setup. *2D ^{14}N spectral filter* - Sample preparation is described elsewhere[16]. 2D ^{1}H ^{13}C-HSQC NMR spectra with and without ^{14}N filtering were acquired using a Bruker Avance III 600 MHz NMR spectrometer equipped with a 1.7 mm 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 during the 1.5 s interscan relaxation delay to suppress the water resonance. The ^{1}H dimension was acquired with a spectral width of 13 ppm 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 splitting enhancement. The human Renal Proximal tubule cell line (RPTEC/TERT1, supplied by Evercyte GmBH, Austria) was used to investigate the metabolic fates of both carbon from glucose, and carbon and nitrogen from glutamine. Cells were expanded as described elsewhere[36], with population doubling level (PDL) routinely tracked using in-house software (PDL calculator, EcoCyto). Cells with PDL between 43 and 45 were collated and seeded at a density of $4\times10^4/cm^2$ in 75 cm^2 flasks containing 240 μl/cm^2 flux media (Zenbio, cat DMEMf12-NGG002), supplemented as above with the addition of 17.5 mM [1,2-^{13}C] D-Glucose (sigma 453188) and 2 mM [U-^{13}C,U-^{15}N] L-Glutamine (sigma, 607983). Cell
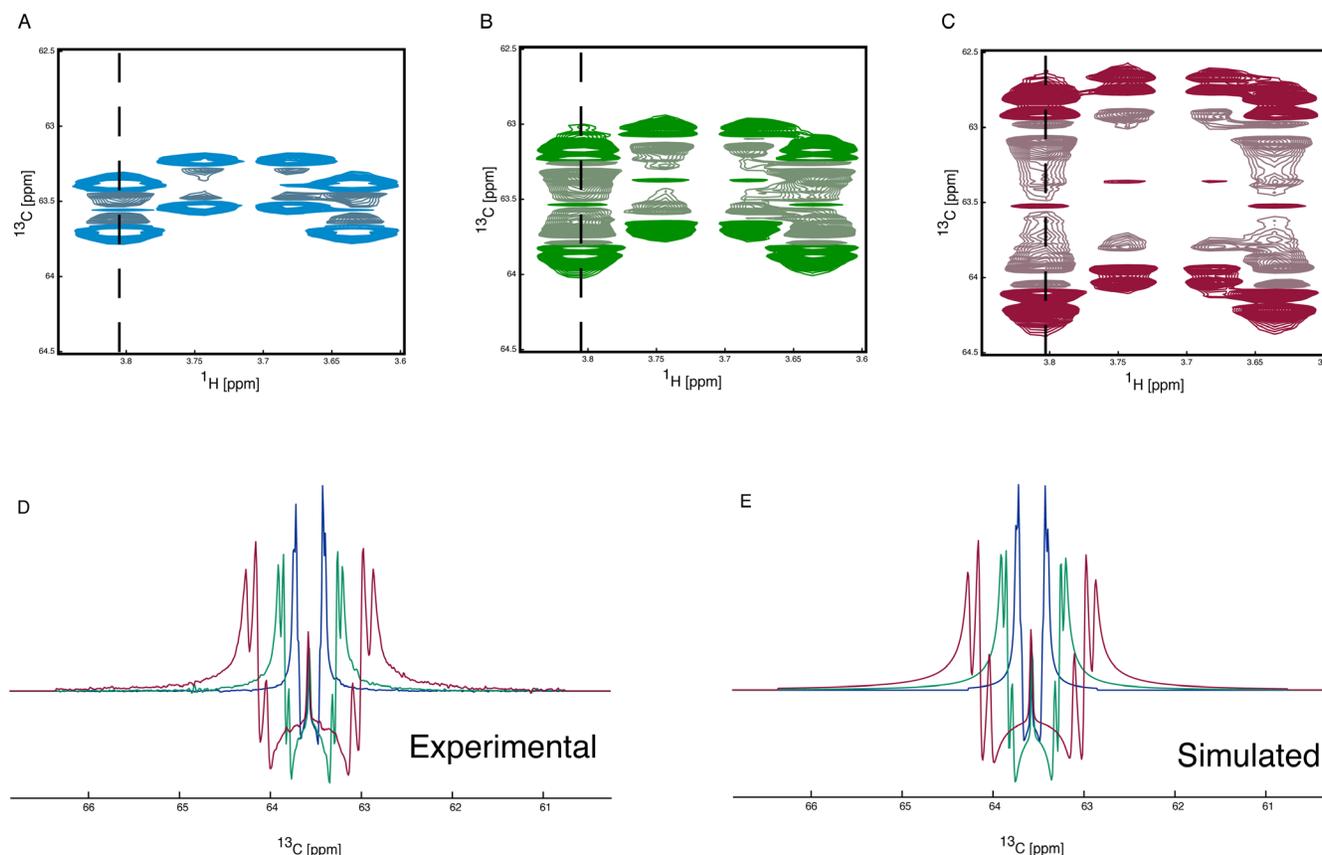
Figure 4. J-coupling splitting enhancement HSQC spectroscopy. ^{1}H,^{13}C HSQC spectra showing the C(6) of glucose are shown (**A**, **B** and **C**). The spectrum with no J-coupling splitting enhancement is shown in blue (**A**), with an enhancement of two in green (**B**) and with an enhancement of four in red (**C**). The ^{13}C trace of the HSQC spectra (**D**), taken from the ^{1}H frequency as depicted by the dashed line, clearly shows the increase in observed splitting. The J-coupling splitting enhancement is achieved using an additional spin echo subsequent to the ^{13}C evolution period. The delays to achieve the scaling of the splittings are multiples of dw such the use of a delay of 3*dw will result in a J-coupling splitting enhancement of 4 (one from the t_1 evolution and three from the J-coupling splitting enhancement spin echo). The observed splitting can be simulated (**E**) giving the following incorporation percentages. From the no J-coupling splitting enhancement spectrum 6.8% / 41.2% / 52 % for [6-^{13}C] / [5,6-^{13}C] / [U-^{13}C], from the two-fold J-coupling splitting enhancement 6.3% / 41.4% / 52.3 % for [6-^{13}C] / [5,6-^{13}C] / [U-^{13}C] and from the four-fold J-coupling splitting enhancement 5.9% / 41.6% / 52.5 % for [6-^{13}C] / [5,6-^{13}C] / [U-^{13}C].

culture was continued for 48 hours to allow isotopic labelling, after which cells were washed with ice-cold saline solution (0.9%) and collected by scraping into 2 ml pre-chilled methanol (-20°C), 2 ml water (4°C) and 2 ml chloroform (-20°C). The solution was vigorously mixed for 10 minutes, after which lysates were centrifuged at 15,000 *g* for 15 min at 4°C. 1 ml of the sample was aspirated for NMR analysis. Samples were dried using a Savant (SPD1010) speedvac concentrator and then resuspended in 60 μL of 100 mM sodium phosphate buffer, containing 0.5 mM DSS, 2 mM Imidazole in D$_2$O, pH 7.0. The samples were vortexed and subsequently sonicated for 10 min and then centrifuged at 15000 *g* for 30 seconds to collate the fluid. Finally 35 μl of the samples were transferred to 1.7 mm NMR tubes.

2D-^{1}H,^{13}C-HSQC and 2D-^{1}H,^{15}N-HSQC NMR spectra were acquired using a Bruker Avance III 600 MHz NMR spectrometer equipped with a 1.7 mm z-PFG TCI Cryoprobe. The HSQC

spectra were acquired using 2 transients per increment with echo/anti-echo gradient coherence selection with an additional pre-saturation to suppress the water resonance during the 1.5 s interscan relaxation delay. The ^{1}H dimension of the ^{1}H, ^{13}C-HSQC spectra 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 160 ppm using 25% (2048) of 8192 data points using a non-uniform sampling scheme. The ^{1}H dimension of the ^{1}H,^{15}N-HSQC spectra 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 256 data. All non-uniformly sampled spectra were reconstructed via the compressed sensing algorithm within MDDNMR (version 2.5)[29] and processed using NMRpipe (version 9.2)[30]. All spectra were processed without baseline correction to avoid complications in the multiplet analysis procedure especially with regards to the negative peaks caused by the echo/anti-echo coherence selection with gradients.
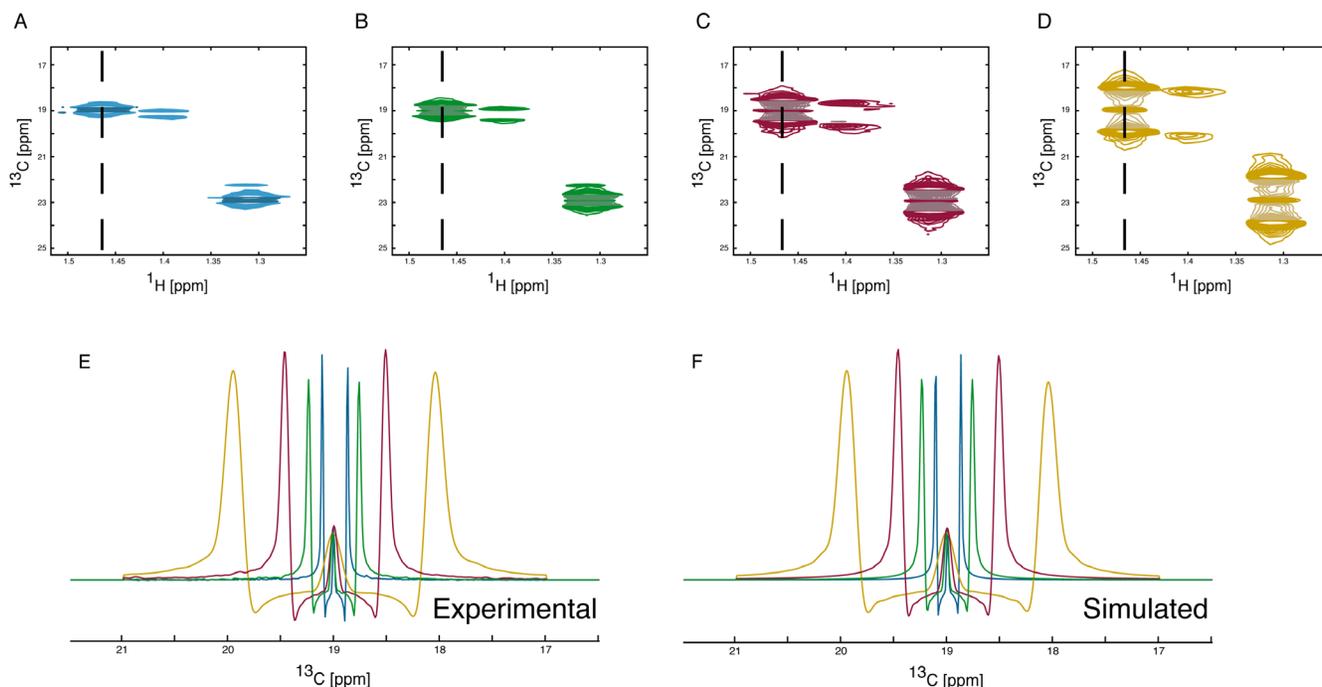
Figure 5. Simulation of J-coupling splitting enhanced spectra. $^1H,^{13}C$ HSQC spectra showing the C(3) of alanine are shown (**A**, **B**, **C** and **D**). The enhancement of J-coupling in the $^1H,^{13}C$-HSQC spectra leads to increased separation of coupled peaks and allows the collection of data with reduced numbers of increments in the ^{13}C dimension resulting in shorter acquisition times. The number of points collected in the ^{13}C dimension can be reduced to match the increase in J-coupling enhancement as the loss of resolution will be counteracted by the increase in separation of the coupled peaks. Spectra with no enhancement (**A**), two-fold enhancement (**B**), four-fold enhancement (**C**) and eight-fold enhancement (**D**) were collected. The overlay of the differently enhanced spectra clearly shows the effect of the enhancement (**E**). The enhancement can be tailored to meet the need in order to maximise separation without significantly increasing signal overlap whilst achieving the maximum reduction in acquisition time possible. The splitting enhancement can easily be included in the simulation parameters (**F**) resulting in no adverse effects on the simulated spectra. The simulation gives the following incorporation percentages. From the no J-coupling splitting enhancement spectrum 12.1% / 87.9% for [3-^{13}C] / [2,3-^{13}C], from the two-fold J-coupling splitting enhancement 12.0% / 88.0% for [3-^{13}C] / [2,3-^{13}C], from the four-fold J-coupling splitting enhancement 12.0% / 88.0% for [3-^{13}C] / [2,3-^{13}C] and from the eight-fold J-coupling splitting enhancement 12.1% / 87.9% for [3-^{13}C] / [2,3-^{13}C].

Table 1. Comparison of spectroscopic techniques. The acquisition time and signal to noise ratios of various experiments used in this study. A good signal to noise ratio can be achieved using the spectral filtering allowing rapid measurement of quantitative spectra. The signal to noise benefit of the HSQC over the 1D ^{13}C acquisition is clearly seen. The effect of increasing the J-coupling splitting enhancement whilst simultaneously reducing the number of increments on the acquisition time of $^1H,^{13}C$-HSQC spectra is also shown.

Experiment	Acquisition time [mins]	Signal to noise ratio (CH$_3$ of lactate)	Transients	TD	SW [ppm]	Splitting enhancement
^1H 1D (all ^1H)	2	327.31	8	16384	12	1
^1H 1D (^{13}C bound ^1H)	2	66.13	8	16384	12	1
$^1H,^{13}C$-HSQC	233	823.86	2	1024/8192	12/160	1
$^1H,^{13}C$-HSQC	110	770.98	2	1024/4096	12/160	2
$^1H,^{13}C$-HSQC	51	426.41	2	1024/2048	12/160	4
$^1H,^{13}C$-HSQC	24	283.38	2	1024/1024	12/160	8
^{13}C 1D (30° excitation)	1414	59.95	16384	65538	239	1

All NMR spectra in this article were processed within the MetaboLab software package (version 2018. 07182055; http://metabolab.uk)[22].

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 two 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[16]. The pulse sequence (Figure 6) is a gradient selected $^{1}H,^{13}C$-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 ^{14}N spectral filter (Panel C-2,
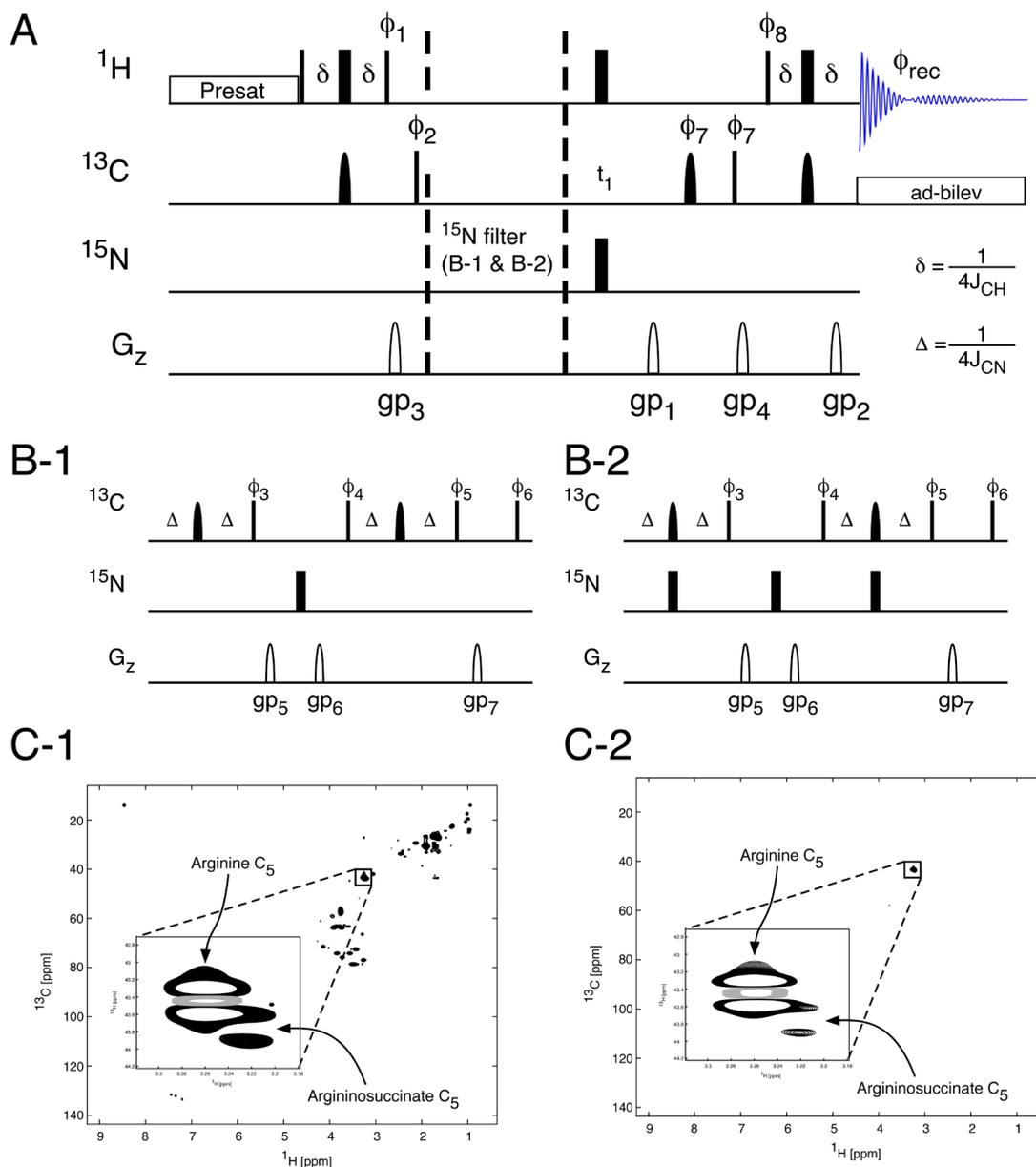
Figure 6. Filtered HSQC spectroscopy. The application of a ^{15}N filtering block in the $^{1}H,^{13}C$-HSQC pulse sequence (**A**) allows the observation of $^{1}H,^{13}C$ groups directly coupled to ^{15}N nuclei. In the sequence in panel **B-1** no filtering will be observed and the resulting spectrum (**C-1**) will contain all ^{1}H, ^{13}C groups adjacent to either ^{14}N or ^{15}N nuclei. The use of a ^{15}N filter (**B-2**) will result in only those ^{1}H, ^{13}C groups adjacent to a ^{15}N nuclei being observed in the resulting ^{1}H, ^{13}C HSQC spectrum (**C2**). $^{1}H,^{13}C$ J-coupling is suppressed during acquisition using adiabatic bilevel decoupling (ad-bilev)[21]. The pulse phases are: ϕ_1 = y; ϕ_2 = x, -x; ϕ_3 = x for the no filter sequence (panel **B-1**) and y for the ^{15}N filtered sequence (panel **B-2**); ϕ_4 = y, –y for the no filter sequence and y, y for the ^{15}N filtered sequence; ϕ_5 = y, -y for the no filter sequence x, -x for the ^{15}N filtered sequence; ϕ_6 = x, x, -x, -x; ϕ_7 = x, x, x, x, -x, -x, -x, -x; ϕ_8 = x, x, x, x, y, y, y, y; ϕ_{rec} = x, -x, -x, x, y, -y, -y, y.

Figure 6) 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 $^1J_{CN}$ constant. J-coupling splitting enhancement on the other hand can be easily extended to include ^{13}C-^{15}N J- coupling splitting enhancement. Indeed, the addition of a single ^{15}N π–pulse simultaneous with the central ^{13}C π–pulse (Figure 3) is sufficient to enhance the apparent

^{13}C-^{15}N J-coupling splitting in ^{1}H,^{13}C-HSQC spectra, an example of which is given in Figure 7. The 2D signals for the J_{CN} splitting scaled spectrum are shown in panel A. Panels B and C show traces of the ^{13}C multiplets for carbon atoms 2 and 3 of alanine. While the J_{CC} splittings are enhanced by a factor of 4 in both spectra, the apparent J_{CN} splittings are unchanged in the spectrum in panel B, whereas they are enhanced by a factor of 4 in the spectra in panel C. Because the $^2J_{CN}$ coupling between C(3) and N is negligible, both traces for C(3) overlap perfectly. C(2) on the other hand experiences a $^1J_{CN}$ coupling,
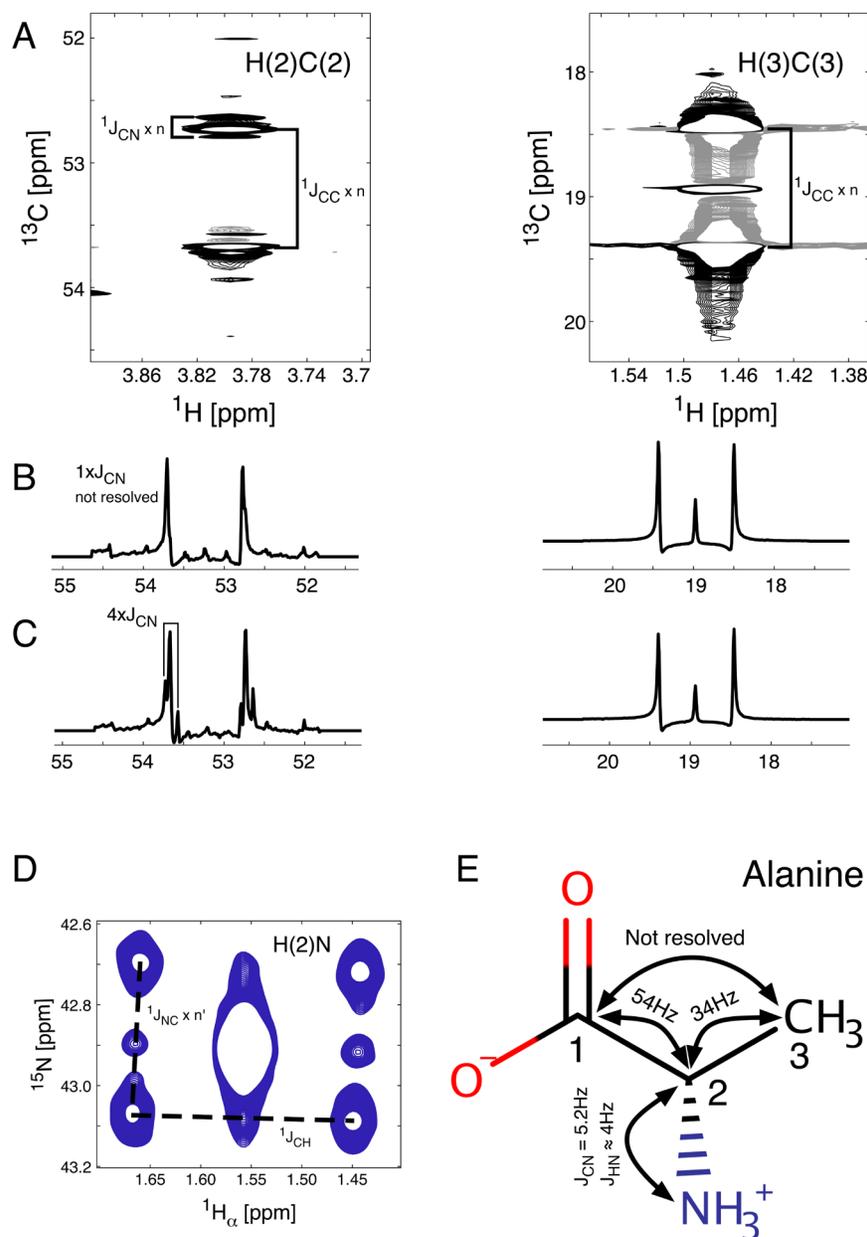
Figure 7. Splitting due to ^{15}N and ^{13}C incorporation. Regions of the ^{1}H,^{13}C-HSQC spectrum containing signals from alanine (panels **A** & **B**). The ^{13}C traces of the alanine signals are shown with either no J_{CN}-coupling splitting enhancement (**B**) or four-fold J_{CN}-coupling splitting enhancement (**C**). The signals are split by either $^1J_{CC}$ or $^1J_{CN}$ coupling contributions. The long range ^{1}H,^{15}N-HSQC spectrum (**D**) is composed of unlabelled alanine (central peak) and ^{13}C/^{15}N labelled alanine (6 outer signals, 4 split by $^2J_{HC}$ and $^1J_{NC}$ couplings and 2 only split by $^1J_{HC}$ (values for the coupling constants are shown in panel **E**)).

which is too small to be resolved when the J_{CN} splitting is not enhanced and is only detectable in panel C.

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 spectrum can be acquired. In such a spectrum proton magnetisation is transferred from H_α (the proton bound to C(2)) via the $^2J_{HN}$ coupling. The splitting due to the J_{NC} 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 $^1J_{CH}$ coupling constant. The result in this case is therefore a signal split into 7 2D components (Figure 7, panel D), demonstrating that alanine was either recycled from unlabelled alanine which was incorporated into proteins, synthesised *de-novo* from [U-^{13}C] glucose and ^{15}N labelled glutamate which originated from [U-^{13}C, U-^{15}N] glutamine that was added to the growth medium in addition to the [U-^{13}C] glucose or just synthesised *de novo* from [U-^{13}C] glucose with an unlabelled amino group transferred to form alanine. In conjunction with MS data, this complementarity between the 2D-^1H,^{13}C- and the 2D- ^1H,^{15}N-HSQC spectra enables a model-free metabolism analysis using multiple nutrients as tracer sources in a single sample.

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 spectroscopy. Parallel advancement in the methods used to acquire and analyse data from samples will increase the amount of information we can gain from 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 spectroscopy 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 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 spectrum is a powerful tool in the study of metabolism as it takes advantage of the increased sensitivity of the ^1H 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 HSQC spectra a much more attractive method in the study of tracer-based metabolism. The use of echo/anti-echo for quadrature detection ensures efficient elimination of unwanted artefacts, whilst using only two scans per increment in the indirect dimension. The changes observed in line shape due to the quadrature detection are predictable and can be easily incorporated into line fitting analysis. As described elsewhere[19], the simulation procedure assumes weak coupling between the different carbon nuclei. The simulation is implemented as a spin echo before the acquisition of a ^{13}C-FID to allow the evolution of ^{13}C,^{13}C spin coupling prior to the first increment.

The ability to scale the visualised splittings due to J-coupling allows HSQC spectra to be acquired in time equivalent to that of a 1D ^1H spectrum, but the HSQC spectrum contains significantly more information. The reduced time required to acquire HSQC spectra means that it is feasible to apply 2D 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 (Table 1). Expansion of the splitting due to J-coupling can also bring out smaller long-range couplings that were not apparent in a normal HSQC spectrum. Thus, the scaling of splittings can either be used to decrease acquisition imes by allowing data collection at lower resolution or to bring out smaller couplings not previously visible. These small couplings include the $^1J_{CN}$ 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 6), the cells used for this experiment were deficient in the expression of fumarate hydratase[16] and therefore contained high fumarate levels. One hypothesis was that argininosuccinate is synthesised 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 produced in the cells containing the knock out, but not in wild-type cells[16]. 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 signal splittings due to J-coupling 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, the use of 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*[37].

Data availability
All experimental data for this article is available at: http://doi.org/10.17605/OSF.IO/EQHN3[38].

Experimental NMR datasets for HS-TrAM: ^{12}C filtered (subdirectory 2) ^{1}H spectrum, both of which are ^{13}C decoupled during acquisition. Subdirectories 3 and 4 contain an unfiltered (3) and a ^{12}C filtered (4) spectrum, both without ^{13}C decoupling during acquisition. Subdirectories 5 and 6 contain POCE spectra, either with ^{12}C- and ^{13}C-bound ^{1}H with same phase (subdirectory 5), or with opposite phase (subdirectory 6), both without ^{13}C decoupling during acquisition. Subdirectory 7 contains a 30 degree excitation 1D ^{13}C spectrum, acquired using a TXO Cryoprobe. The file jEnhanced_13C_HSQC.zip contains the ^{1}H, ^{13}C-HSQC spectra with different J-coupling splitting enhancements.

The file 13C_HSQC_14N_filter_and_15N_HSQC.zip contains an unfiltered (subdirectory 1) and a ^{14}N filtered (subdirectory 2)

^{1}H,^{13}C-HSQC spectrum. The file jEnhanced_13C_15N_HSQC.zip contains the following spectra: 4 x ^{13}C,^{13}C splitting enhancement ^{1}H,^{13}C-HSQC in subdirectory 1, 4 x ^{13}C,^{13}C splitting enhancement and 4 x ^{13}C,^{15}N splitting enhancement ^{1}H,^{13}C-HSQC in subdirectory 2 and 4 x ^{13}C,^{15}N splitting enhancement ^{1}H,^{15}N-HSQC in subdirectory 3.

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Open Peer Review

Current Referee Status: ✓ ✓ ✓ ✓

Version 2

Referee Report 19 September 2018

✓   **Paul C. Driscoll**   iD

Francis Crick Institute, London, UK

The revised manuscript has been significantly improved compared to the original version, and give an enhanced prospect that other researchers can attempt to employ the new approach to measurement of stable isotope enrichment in biological samples.

I think it worth pointing out the following:

It is not made clear in the figure legend for Figure 2 (though it is mentioned in the main text) that the data in panels A-3 and B-3 were obtained in the absence of 13C-decoupling.

Page 6: The authors refer to the invariance of the spectral filter with respect to differential relaxation 1H rates. This comment appears to refer to chemically distinct 1H atoms. It seems entirely probable that the relaxation rate of chemically equivalent 12C- and 13C-bound 1H nuclei would be non-identical. Such differences would introduce a systematic difference in the signal response for the different (X-enriched/X-nonenriched) sets of molecules and therefore lead to a (perhaps small) error in the derived level of X-enrichment - particularly when the X-enrichment is low.

The performance of 13C-pulses and 13C-decoupling may be 13-C offset -dependent. The paper contains no demonstration that the choice of offset leads to any systematic error in the derived 13C-enrichments.

Figure 6 legend: presumably the spectrum in C-1 also contained 1H/!3C-signals from CH group unattached to any nitrogen atom.

Figure 7D: The 1H chemical shift scale does not appear to be consistent with the 1Halpha NMR resonances.

Overall, the paper would be improved by further copyediting. On panel C-2 of Figure 6 is directly referred to in the main text. I note numerous non-sequiturs, inappropriate letter cases (e.g. page 7: D-Glucose); inappropriate use of numerals instead of number words (e.g. Figure 7 legend (better: six outer signals, four split...)

Competing Interests: No competing interests were disclosed.

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.

Hector C. Keun   iD

Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London, UK

The revised version submitted is certainly much improved with greater clarity in the presentation. I'm not sure that all suggestions have been addressed, particularly with respect to comparison and justification versus other approaches. There is still no validation with known levels of enrichment. However I think there is sufficient detail in the manuscript to understand and repeat the experimental set up and thus for the sequences proposed to be evaluated in more complete way. Hence the work should be indexed pending the minor corrections below.

Minor corrections:
In figure 2 panels A-3/B-3: these should be labelled on figure and it should be stated in legend that the 13C decoupling during acquisition has been switched off. The reader only gets this from the main text and it is confusing.
Given that figure 2 suggests that the authors have the same samples with/without decoupling, then they can report the estimation of 13C enrichment from the DSS peak presented using their method and the differences in 13C enrichment estimates of lactate from the decoupled and coupled spectra.

Competing Interests: No competing interests were disclosed.

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.

Sebastien Serres   iD

University of Nottingham, Nottingham, UK

This is a much improved version that fully covers all the points I mentioned in my previous report. Simplifying the biological model has helped to get the message across. The utility of the method is now evident and mislabeling in figure and legends have been corrected.
I am happy with these corrections and approve indexing of this paper.

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.

Version 1

? **Hector C. Keun** (iD)

Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London, UK

In this manuscript the authors present a set of novel experimental schemes designed to reduce the time and experimental complexity of measuring isotope enrichment in biological samples.   They present a filter designed to allow quantitative analysis of enrichment without an unlabelled reference sample, and then expand to include J-scaling to reduce experiment time and present a 15N filtered 1H-13C HSQC to monitor labile 15N via JNC. The methods could be very useful for many isotope-labelling experiments, increasing the confidence and ease with which various features can be extracted from such experiments.

Main points:

- The authors state that the new filter presented will negate the requirement for a matched unlabelled sample to calculate isotopic enrichment. It would be really helpful to see that explicitly demonstrated (could be with simulated mixtures of 15N/13C/unlabelled substrates perhaps spiked into a relevant background, not necessarily a real labelling expt in a biological system) and the performance compared to more conventional experimental approaches e.g. 1D 13C with proton decoupling, or indeed mass spec? Would be particularly useful to know the minimum amounts of fractional enrichment that can be identified.

- J-(up)scaling has been reported before, implemented in different ways (e.g. Furihata & Tashiro MRC 2013[1], Willker et al. JMR 1997[2] and refs within, several by Zangger et al). It would be good to cite these instances and highlight what is different about the authors' scheme.

- While the J-scaling approach clearly ought to help resolution and quantitative analysis of overlapping multiplets from different isotopomers, again it would be good to see some complete analysis to that effect and a comparison to the standard 2D experiments or other J-scaling alternatives.

- As much of the utility of the method for its stated purpose (isotopomer analysis) is contingent on the peak fitting algorithms used, some further details on this should be provided.

- In general there are lot of apparent typos with respect to subscript/superscript fonts, mislabelling in figures/legends – I presume something went wrong with formatting into final form, the authors should check through thoroughly.

- Some better annotation / commenting of the pulse sequence in the main text would be useful (gradient pulses used, necessary additional delays etc).

- The blending of methods and results leads to some confusion when going through figures. In general these are not annotated such that the reader understands what the sample in question is, nor why the analysis is presented thus. It can be inferred from the main text but is not always made explicit. For example in Figure 2 one presumes that we are shown resonances where there is believed to be enrichment e.g. lactate, but others where there is not (and are scaled by 100 to demonstrate this). I think the Figure legends and text should be more explicit.

Minor points:

Figure 2. "gp1 and gp2"? Not labelled. "Chirp pulses with yB1max = ?? Hz"?
Figure 5: "13H ppm"? Also does yellow indicate negative intensity?
P8. "D20"? "1.
Figure 6. Need to label couplings on the molecule for clarity

References
1. Furihata K, Tashiro M: BASHD-J-resolved-HMBC, an efficient method for measuring proton-proton and heteronuclear long-range coupling constants.*Magn Reson Chem*. **52** (1-2): 27-31 PubMed Abstract | Publisher Full Text
2. Willker W, Flögel U, Leibfritz D: Ultra-high-resolved HSQC spectra of multiple- 13C-labeled biofluids.*J Magn Reson*. 1997; **125** (1): 216-9 PubMed Abstract | Publisher Full Text

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?
Yes

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: metabolomics in oncology and toxicology

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.

Author Response 17 Aug 2018

Christian Ludwig, University of Birmingham, UK

We thank Hector Keun for his comments and suggestions. These have been very helpful to improve the quality of the manuscript.

Reviewer: The authors state that the new filter presented will negate the requirement for a matched unlabelled sample to calculate isotopic enrichment. It would be really helpful to see that explicitly demonstrated (could be with simulated mixtures of 15N/13C/unlabelled substrates perhaps spiked into a relevant background, not necessarily a real labelling expt in a biological system) and the performance compared to more conventional experimental approaches e.g. 1D 13C with proton decoupling, or indeed mass spec? Would be particularly useful to know the minimum amounts of fractional enrichment that can be identified.

Response: We included spectra without $^{13}C,^{1}H$ decoupling demonstrating not only the quality of the $^{12}CH_3$ signal removal from the spectrum, but also the linearity of the intensities between the spectra, as the satellites in both spectra appear at 0.5% intensity of the $^{12}CH_3$ signal. This can also serve as an indication for the minimum of fractional enrichment possible to identify. If possible, we prefer to acquire mass spec data. However, this may not always be possible, e.g. when analysing samples of intact tissue.

Reviewer: *J-(up)scaling has been reported before, implemented in different ways (e.g. Furihata & Tashiro MRC 20131, Willker et al. JMR 19972 and refs within, several by Zangger et al). It would be good to cite these instances and highlight what is different about the authors' scheme.*

Response: We included those references in the main text.

Reviewer: While the J-scaling approach clearly ought to help resolution and quantitative analysis of overlapping multiplets from different isotopomers, again it would be good to see some complete analysis to that effect and a comparison to the standard 2D experiments or other J-scaling alternatives.
As much of the utility of the method for its stated purpose (isotopomer analysis) is contingent on the peak fitting algorithms used, some further details on this should be provided.

Response: We included complete multiplet analyses for splitting scaling with enhanced resolution for small couplings (figure 4) as well as a series of 2D spectra demonstrating the potential to accelerate acquisition times (figure 5).

Reviewer: In general there are lot of apparent typos with respect to subscript/superscript fonts, mislabelling in figures/legends – I presume something went wrong with formatting into final form, the authors should check through thoroughly.

Response: We have checked thoroughly.

Reviewer: Some better annotation / commenting of the pulse sequence in the main text would be useful (gradient pulses used, necessary additional delays etc).

Response: We redesigned most of the figures and enhanced the annotation of the pulse sequences.

Reviewer: The blending of methods and results leads to some confusion when going through figures. In general these are not annotated such that the reader understands what the sample in question is, nor why the analysis is presented thus. It can be inferred from the main text but is not always made explicit. For example in Figure 2 one presumes that we are shown resonances where there is believed to be enrichment e.g. lactate, but others where there is not (and are scaled by 100 to demonstrate this). I think the Figure legends and text should be more explicit.

Response: We redesigned most figures (including figure 2) and their legends and made references to the figures more explicit in the main text.

Reviewer: Figure 2. "gp1 and gp2"? Not labelled. "Chirp pulses with yB1max = ?? Hz"? Figure 5: "13H ppm"?

Response: All pulses are now properly labelled. We added the missing value for gB_{1max} and changed ^{13}H to ^{13}C in figure 5.

Reviewer: Also does yellow indicate negative intensity?

Response: We redesigned this figure and removed colour from the spectra plots, this should be clearer now.

Reviewer: P8. "D20"? "1.

Response: All D20 occurences have been changed to D2O

Reviewer: Figure 6. Need to label couplings on the molecule for clarity

Response: We included all detectable couplings in the molecule plot.

Competing Interests: No competing interests were disclosed.

Referee Report 12 February 2018

✓   **Julian L. Griffin**
Department of Biochemistry, Cambridge Systems Biology Centre, University of Cambridge, Cambridge, UK

Smith and colleagues describe a new approach for acquiring 13C NMR labelling data as part of flux based analyses of metabolism. There has been much recent interest in metabolomics and metabolism research in the application of stable isotope approaches to probe metabolite fluxes. While mass spectrometry is increasingly being used for these labelling experiments, this approach cannot readily determine the position of labelling, limiting the information that can be obtained – for example, different pathways can result in different labelling patterns of certain metabolites. While NMR spectroscopy can determine where labelling is in a given molecule, the approach has poor sensitivity and can take significant time to acquire the datasets. Smith and colleagues set out to improve one type of 2-dimensional NMR technique – a heteronuclear single quantum coherence (HSQC) spectroscopy – to reduce the time and increase the sensitivity of the approach, thus aiding those who use NMR spectroscopy for flux analysis. The manuscript is well written and the data and tools used available to the reader which should aid the dissemination of the products of this research. I particularly liked the application using 13C and 15N dual labelling to follow amino acid metabolism – both in terms of the carbon backbone and the ammine group of amino acids. I do have some specific questions though that I would like the authors to address.

1. Abstract: Strictly speaking it should be NMR spectroscopy rather than simply NMR.
2. Abstract: What splitting are they referring to? Could they be more precise in the abstract?
3. Introduction: "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." A good reference (or references) for the use of 13C NMR spectroscopy to examine metabolic compartmentation would be the work of E.D. Lewandowski.
4. 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." This should be expanded on - with NMR spectroscopy we can observe exactly where the label is in a metabolite - this is exceptionally hard using mass spectrometry and probably open to a lot of confusion.
5. NMR methodology. It would be useful to have the approximate times for the various pulse sequences discussed so the reader can judge how long the spectra required to acquire.
6. NMR methodology. "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 $^{1}J_{CC}$ 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." Does this create problems for quantification if the signals are beginning to be split?
7. In Figure 2 why can no 13C satellites be seen around the 1H NMR doublet of lactate? Presumably I'm missing something as I note the 13C spectrum is uncoupled from 1H-13C interactions and we only "see" the doublet from 1H-1H couplings. Note the figure legend needs to discuss A and B in terms of labels.
8. Figure 4D. How easy is it to quantify from such complex line shapes as shown in Figure 4D?

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?
Yes

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?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: The application of metabolomics to type 2 diabetes and metabolic diseases.

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.

Author Response 17 Aug 2018
Christian Ludwig, University of Birmingham, UK

We thank Julian Griffin for his comments and suggestions. These have been very helpful to improve the quality of the manuscript.
Reviewer: Smith and colleagues describe a new approach for acquiring 13C NMR labelling data as part of flux based analyses of metabolism. There has been much recent interest in metabolomics and metabolism research in the application of stable isotope approaches to probe metabolite fluxes. While mass spectrometry is increasingly being used for these labelling experiments, this approach cannot readily determine the position of labelling, limiting the information that can be obtained – for example, different pathways can result in different labelling patterns of certain metabolites. While NMR spectroscopy can determine where labelling is in a given molecule, the approach has poor sensitivity and can take significant time to acquire the datasets. Smith and colleagues set out to improve one type of 2-dimensional NMR technique – a heteronuclear single quantum coherence (HSQC) spectroscopy – to reduce the time and increase the sensitivity of the approach, thus aiding those who use NMR spectroscopy for flux analysis. The manuscript is well written and the data and tools used available to the reader which should aid the dissemination of the products of this research. I particularly liked the application using 13C and 15N dual labelling to follow amino acid metabolism – both in terms of the carbon backbone and the ammine group of amino acids. I do have some specific questions though that I would like the authors to address.
  1. Abstract: Strictly speaking it should be NMR spectroscopy rather than simply NMR.
Response: We changed NMR to NMR spectroscopy

  1. Reviewer: Abstract: What splitting are they referring to? Could they be more precise in the abstract?
Response: We changed the description to be explicit about the homonuclear 13C,13C and heteronuclear 13C,15N J-couplings referred to in the abstract.

  1. Reviewer: Introduction: "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." A good reference (or references) for the use of 13C NMR spectroscopy to examine metabolic compartmentation would be the work of E.D. Lewandowski.

Response: We added this reference

1. Reviewer: 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 13C and/or 15N distribution within the metabolite." This should be expanded on - with NMR spectroscopy we can observe exactly where the label is in a metabolite - this is exceptionally hard using mass spectrometry and probably open to a lot of confusion.

Response: We added a statement about the potential of NMR data.

1. Reviewer: NMR methodology. It would be useful to have the approximate times for the various pulse sequences discussed so the reader can judge how long the spectra required to acquire.

Response: We added a table with acquisition times as well as pros and cons of the approaches.

1. Reviewer: NMR methodology. "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 1JCC 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." Does this create problems for quantification if the signals are beginning to be split?

Response: We included a quantitative analysis with multiplet simulations, demonstrating that the multiplet compositions do not change with different amounts of scaling.

1. Reviewer: In Figure 2 why can no 13C satellites be seen around the 1H NMR doublet of lactate? Presumably I'm missing something as I note the 13C spectrum is uncoupled from 1H-13C interactions and we only "see" the doublet from 1H-1H couplings. Note the figure legend needs to discuss A and B in terms of labels.

Response: We redesigned the figure and included a spectrum without $^{13}C,^{1}H$ decoupling. This should make it easier to understand why there are no satellites around lactate.

1. Reviewer: Figure 4D. How easy is it to quantify from such complex line shapes as shown in Figure 4D?

Response: We included a quantitative analysis including a line shape fit.

Competing Interests: No competing interests were disclosed.

Paul C. Driscoll iD
Francis Crick Institute, London, UK

In this manuscript the authors have built on their own previous work wherein high resolution 2D ^{1}H^{13}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}C-^{15}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}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}H relaxation rates, signal multiplicities and ^{1}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}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}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}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}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}H ^{15}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 $^{2}J_{NH}$ coupling, but should properly be C(3) (Cbeta) and $^{2}J_{NCbeta}$. 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}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}H-decoupled ^{13}C spectrum; (ii) a non-aliased 2D ^{1}H^{13}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 D_2O, not D_20; 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.

Author Response 17 Aug 2018
Christian Ludwig, University of Birmingham, UK

We thank Paul Driscoll for his comments and suggestions. These have been very helpful to improve the quality of the manuscript.
Reviewer: In this manuscript the authors have built on their own previous work wherein high resolution 2D 1H13C 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 13C-15N 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.

Response: We separated the biological methods from the NMR methods.

Reviewer: 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 13C 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.

Response: We changed the text to reflect that it is only the splittings that are enhanced in the spectra. We added more details on the line shape simulation and explicitly stated the weak coupling assumption behind the data analysis procedure.

Reviewer: 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.

Response: We added relevant citations.

Reviewer: 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 1H relaxation rates, signal multiplicities and 1JCH 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?

Response: We added more caveats and explanations addressing all those points.

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

Response: Although we didn't try zeroth order baseline corrections, we don't really need this as the baseline is centred around zero. Higher order baseline corrections will interefere with the negative aspects of the multiplets.

Reviewer: 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.

Response: We redesigned most figures and have taken care to ensure that labels are referred to appropriately.

Reviewer: 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.

Response: Figure 2 has been redesigned and all colour references have been removed. The explanation has been improved.

Reviewer: 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.

Response: Figure 2 has been redesigned and all colour references have been removed. The explanation has been improved. ad-bilev is now explained and the reference has been added.

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

Response: This was done to demonstrate the ability to detect natural abundance of ^{13}C. We understand that this could be confusing and have now removed the scaled spectrum and replaced it with a non-decoupled spectrum of DSS with its ^{13}C satellites. We hope that the new figure is clearer.

Reviewer: 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 13C 'J-scaling' pulse shown in gray? Caption: 'simultaneously with' would be better than 'parallel'.

Response: We removed all occurrences of J-scaling and replaced them with a more suitable term. We replaced parallel with simultaneously.

Reviewer: 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 13C 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).

Response: This figure has been redesigned, addressing all points above.

Reviewer: Figure 5: A and B labels for the different figure elements are missing. Why are some of the 13C 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 15N pulse is employed along with the amber pulses. I am not keen on the use of colour in the exposition of the phase cycles.

Response: This figure (now figure 6) has been redesigned, removing colour from the spectrum plots.

Reviewer: 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 1H 15N 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 2JNH coupling, but should properly be C(3) (Cbeta) and 2JNCbeta. 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.

Response: We addressed all these points.

Reviewer: When 1D spectra are mentioned (page 11) is this meant to refer to 13C 1D spectra?

Response: This referred to 1D ^{1}H spectra. This has been made explicit in the text now.

Reviewer: 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 1H-decoupled 13C spectrum; (ii) a non-aliased 2D 1H13C-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?

Response: We added spectra using accelerated acquisition. We acquired a 1D 13C spectrum, however we could not reach a sufficient signal to noise ratio given the amount of sample we had available. As we can reach acquisition times comparable to those for a 1D 1H spectrum and given that the analysis software would need substantial changes to be able to deal with aliased NMR spectra, we chose not to acquire aliased NMR spectra. The samples we acquired we believe are of sufficient complexity and variability (e.g. perfusion fluids as well as cell extracts).

Reviewer: 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 D_2O, not $D20$; 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.

Response: We checked all these carefully and changed the text accordingly.

Reviewer: 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.

Competing Interests: No competing interests were disclosed.

? **Sebastien Serres** (iD)

University of Nottingham, Nottingham, UK

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 ^{13}C and ^{15}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 ^{13}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 13C enriched glucose.
5. Why using a different NMR spectrometer for glutamine experiment (900 vs 600MHz)?

 "2D 1 H13C-HSQC and 2D 1 H15N-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.

Author Response 17 Aug 2018
Christian Ludwig, University of Birmingham, UK

We thank Sebastien Serres for his comments and suggestions. These have been very helpful to improve the quality of the manuscript.
Reviewer: 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.

Response: We acquired new data from a renal epithelial cell line. Unfortunately, whole organ perfusion experiments with ^{13}C,^{15}N glutamine would be prohibitively expensive due to the amount of glutamine needed in the perfusion fluid. In addition, glutamine is not part of the formulation of KPS-1, so that the results obtained with such a modified perfusion solution would not lead to data directly comparable with clinical data.
We have simplified the biological side by concentrating more on kidney metabolism and removing the cancer cell line.

Reviewer: 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 13C and 15N 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?

Response: As this is a proof of concept paper, we did not run biological replicates. We expect that the reproducibility will be similar to our recently published paper in Angewandte Chemie, where we demonstrate benefits of combining NMR and MS data. The error margins obtained there were very small, resulting in correlation coefficients very close to 100% when comparing experimental and simulated signals.
We acquired a 1D ^{13}C NMR spectrum from one of the samples. In order to obtain the best signal to noise ratio, we diluted the sample from 30 ul to 600 ul and acquired the carbon spectrum using a 5 mm TXO cryoprobe, which is optimised for ^{13}C detection. As expected the signal to noise ratio was much poorer in the 1D 13C spectrum compared to the HSQC spectrum, despite a very long acquisition time for the ^{13}C 1D spectrum. We added the ^{13}C 1D spectrum to the deposited data.
We also acquired POCE spectra, which require the subtraction of two very large signals to obtain the amount of ^{13}C incorporation. This can result in significant subtraction artefacts, which are not seen in our approach because of the use of pulsed field gradients. We included a brief discussion in the main text and added the spectra to the deposited data.

Reviewer: Specific comments:
1. Reviewer: 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.

Response: We added a statement to this effect to the main text.

1. Reviewer: 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 13C data collection using mass spectrometry?

Response: We were referring to the ability to resolve metabolic pathway utilisation. We added a statement to this effect to the abstract.

1. Reviewer: Authors claim that" such techniques permit high throughput metabolic pathway profiling, increasing access, affordability and sensitivity when using NMR as an investigative modality"

Response: We added a new figure that demonstrates the acceleration of acquisition.

Reviewer: Are these sequences freely available and easy to implement on Bruker NMR spectrometers?

Response: In accordance with the Wellcome openresearch journal's rules, all sequences are freely available. They have been designed to comply with Bruker's pulse sequence

nomenclature so that a standard setup is easily possible.

Reviewer: However not every sites in the world have access to cryoprobe.

Response: It may be possible to run all pulse sequences on room temperature probes. However, as we have not tested the sequences on RT probes, we can't give recommendations. We included a short discussion mentioning RT probes in the text.

1. Reviewer: "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?

Response: We added this information

1. Reviewer: To correct: "needed in in order to resolve J-couplings "

Response: Corrected

1. Reviewer: 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.

Response: We added such a table to the main text.

1. Reviewer: Figure 4 legend did not mention that the spectrum displays C(6) of 13C enriched glucose.

Response: This has been corrected.

1. Reviewer: Why using a different NMR spectrometer for glutamine experiment (900 vs 600MHz)? "2D 1 H13C-HSQC and 2D 1 H15N-HSQC NMR spectra were acquired using a Bruker Avance III 900 MHz NMR spectrometer equipped with a 5mm z-PFG TCI Cryoprobe"

Response: We simplified the biology of the samples and now acquired all spectra used for this paper at 600 MHz.

Competing Interests: No competing interests were disclosed.