NMR Sample Preparation

Volume of the NMR sample depends on NMR instrumentation available. For example, a 3 mm NMR tube has ~180 µL sample volume and 1.5 mm NMR tube has ~50 µL sample volume.

1. Mix 6 µL of "*DSS-standard*" (10% (v/v)) with 54 µL of "*sample buffer*" (90% (v/v)). Final sample will contain (nominal) 0.5 mM DSS and 0.02% sodium azide.
2. Dissolve the lyophilized pellet in 60 µL of the solution prepared in Step 1. Sonicate or vortex the sample for a few minutes and centrifuge the sample at 10000xg for five minutes.
3. Carefully remove 55 µL of supernatant from the sample and transfer to a new tube. This ensures that little to no salt is transferred to the final NMR sample.
4. Transfer the liquid from the new tube into NMR tube using an appropriate syringe.

NMR Spectroscopy

^{1}H setup

1. ^{1}H spectra with excellent lineshapes are a requirement for metabolomics and fluxomics. One robust approach to achieving good lineshapes is to shim the magnet using methyl resonance of DSS. Using a commercial NMR probe, full width at half-maximum values of 0.8 Hz or lesser yield spectra of high quality.
2. In some cases, DSS peak tend to be broad due to interference from other macromolecules (typically, lipids). In such cases, the lactate resonance (if present) is a good alternative for shimming the magnet.
3. Using water suppression will improve the quality of the spectra. Care must be taken to optimize water suppression such that nearby peaks are unaffected (e.g. glucose anomeric proton).

^{13}C setup

1. For isotopomer analysis, ^{1}H decoupled ^{13}C NMR spectra are straightforward to interpret. On a modern cryoprobe, ^{13}C spectra can be measured in about 20 hours.
2. Using Ernst angle optimized for spin lattice relaxation time (T_1) corresponding to the aliphatic region of the spectrum is advantageous. Alternatively, a 30° or 45° flip angle may be used.
3. ^{1}H decoupling should be employed for the entire duration of the pulse sequence to take advantage of NOE effects.

J-HSQC

An alternative to measure direct ^{13}C detection is to employ J-resolved heteronuclear single quantum coherence (j-HSQC) experiment. This experiment provides the same isotopomer information as the 1D ^{13}C experiment albeit at a much shorter experiment time. j-HSQC pulse sequence employing adiabatic pulses is described in detail elsewhere ***(1)***.

Data Processing

1. ^{13}C Spectra should be processed using standard NMR data processing approaches. Time domain data should be zero filled (as needed), Fourier transformed and line broadened. An exponential line broadening of 0.5 – 1 Hz is sufficient for most analysis.
2. ^{1}H spectra should be processed similar to ^{13}C spectra. Exponential line broadening of 0.5 Hz is sufficient for ^{1}H spectra.
3. If needed, baseline correction should be applied. (Also see below)

4. Reference the spectrum using an appropriate peak. For example the lactate resonance can be used as reference for ^{13}C (set to 21 ppm). ^{1}H spectra are referenced using DSS peak at 0 ppm.
5. In the ^{13}C spectra, identify glutamate resonances, fit the peaks to a mixed Gaussian-Lorentzian lineshape (such a fit for C-4 of glutamate is shown in Fig. 4), and tabulate the areas.
6. Calculate the peak ratios for each glutamate resonance (C1 – C5). These peak ratios will be used for metabolic modelling.

Depending on the NMR probe used, a rigorous baseline correction such as a spline may have to be applied to correct rolling baseline. Modern cryoprobes often suffer from spurious responses at the beginning of acquisition, which manifest as low frequency noise in the spectrum. Care must be taken to ensure that crowded regions of the spectra are not adversely affected during baseline subtraction. The peak ratios calculated are normalized to the total intensity for each carbon of glutamate, i.e., the singlets versus homonuclear coupled multiplets sum to 1. This is a fortunate circumstance, as within the relatively narrow frequency for each multiplet, the baseline correction is of secondary importance to the actual ratios of peak areas. The one exception is the input of the relative values of the areas defining the C3 and C4 resonances of glutamate. This important parameter gives direct insight into anaplerotic flux of carbons into the TCA cycle. Special care should be paid to baseline correction for the C3 at ~27 ppm and the C4 at ~34 ppm.

Refs

1. Merritt ME, Burgess SC, and Spitzer TD (2006) Adiabatic JHSQC for ^{13}C isotopomer analysis. Magn Reson Chem 44:463–466