Biomarker discovery using NMR based metabolomics of tissue

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Abstract

NMR-based metabolomics has shown promise in the diagnosis of diseases as it enables identification and quantification of metabolic biomarkers. Using high-resolution magic-angle-spinning (HR-MAS) NMR spectroscopy, metabolic profiles from intact tissue specimens can be obtained with high spectral resolution. In addition, HR-MAS NMR requires minimal sample preparation and the sample is kept intact for subsequent analyses. In this chapter we describe a typical protocol for NMR based metabolomics of tissue samples. We cover all major steps ranging from tissue sample collection to determination of biomarkers, including experimental precautions taken to ensure reproducible and reliable reporting of data in the area of clinical application.

Key Words: High-resolution magic angle spinning MR spectroscopy, tissue analysis, biopsies, metabolism, metabolomics, biomarkers, protocols

1 Introduction

Endogenous metabolites serve as diagnostic biomarkers for a variety of medical conditions, since disease frequently is associated with altered concentrations of one or more metabolites. A universally known example is phenylketonuria, an inherited genetic disorder where dysfunctional metabolic degradation causes build-up of phenylalanine to potentially toxic levels [1]. Elevated phenylalanine levels in blood is the diagnostic biomarker used when newborns are screened for this disease. Thus, blood and other biofluids serve as matrices for metabolic phenotyping and biomarker detection in many diseases. However, in the search for novel biomarkers, direct analysis of the affected tissue has been suggested as a potential method for identification of metabolites or metabolic patterns that hold prognostic or predictive information. This approach has been widely applied to cancer, but analysis of biopsy material is also reported in various inflammatory diseases, pre-eclampsia, neurological disorders and more [2-5].

High resolution magic angle spinning (HR-MAS) NMR spectroscopy has emerged as a technique that is highly suitable for metabolic analyses of intact tissue specimens *ex vivo.* The technique, based on the principles of nuclear magnetic resonance, was first described by Andrew in 1958 [6], and differs from the solution state NMR spectroscopy in that it involves spinning the sample of solid or semi-solid specimen at magic angle, 54.7° to the main magnetic field. This causes averaging of orientation-dependent interactions between the nuclear spins and their surroundings, including chemical shift anisotropy, dipole-dipole and quadrupolar couplings. This causes narrowing of the signals in the NMR spectra and results in high spectral resolution similar to the solution state spectra. Generally, the rotor containing tissue specimen is spun at high speed (typically 3 to 5 kHz) activated by flow of pressurized air through the NMR probe, leaving the sample intact for further analyses. From the resulting NMR spectra,

individual metabolites can be identified and quantified. Alternatively, metabolic patterns or "fingerprints" representing various disease states can be identified.

For discovery and validation of novel biomarkers for potential use in clinical practice, it is of outmost importance that analytical methods can be trusted to provide accurate and reproducible data. To achieve this, attention must be paid to rigorous quality assurance and validation of the methods, and standardized protocols optimized for generation of reliable data must be used. Obtaining metabolic information using HR-MAS NMR for clinical use depends on built-in systems for quality assurance and validation ensuring that the laboratory meets relevant criteria for robustness and analytical accuracy. The performance of the NMR spectrometer must be monitored regularly. Sample handling protocols must ensure that the metabolic state of the sample is kept stable from collection to analysis. Importantly, the NMR spectra reflect the composition of a complex biological samples, and care must be taken to process and analyze the raw NMR data in an objective and standardized way, without introducing systematic bias or error. Therefore, a reliable and robust pipeline for data management must be defined. In this chapter, we provide detailed guidance on how to perform HR-MAS NMR analysis of tissue specimens for the purpose of identification and reporting of diagnostic metabolic biomarkers. Important steps from harvesting of tissue material to analysis of the NMR spectra are covered.

2 Materials

Sample collection

The protocol includes materials/equipment needed to handle tissue samples following collection during open surgery, needle biopsy or similar. Equipment needed for surgery/needle biopsy varies between procedures and is not described.

1. Cryo-bucket filled with liquid nitrogen.

2. Safety equipment: Lab coat, safety glasses and disposable gloves (*see* **Note 1**).
3. Tweezers, scalpel, blades and cryo-tubes.

2.2 Sample preparation

1 Safety equipment: Lab coat, safety glasses and disposable gloves (*see* **Note 1**).

2 Cryobucket with liquid nitrogen for sample storage and cap removal.

3 Workstation with liquid nitrogen (*see* **Note 2**) or ice block.

4 Scalpel and blades, tweezers, scale and weighting boats.

5 D_2O (99.8%) with HCOONa (~25 mM). Pipette and tips (pipette volume > 3 µL).

6 HR MAS accessories: (see Figure 1):

a) 4 mm ZrO_2 rotors (either volume 50 or 80 µL) with spinning caps (*see* **Note 3**).

b) Spacers (for 50 µL rotors only).

c) Disposable inserts, plugs and screws (80 µL rotors only).

d) Filling funnel, blunt-ended screwdriver and forked screwdriver. Permanent marker.

7 Rotor cap remover and extraction screw for recovery of the sample after analysis.

8 Chemicals for temperature calibration, magic angle adjustments and stability test: KBr salt, 4% methanol dissolved in methanol-d_4.

2.3 HR MAS NMR acquisition

NMR spectrometer with an HR-MAS probe (*see* **Note 4**). Compatible user interface software, such as Topspin for Bruker instruments (*see* **Note 5**).

2.4 Data Analysis

1. Database and software for identification of metabolites, for example Human Metabolome Database (HMBD), Chenomx or MNova.
2. Software for quantification of metabolites, for example TopSpin, LCModel, MATLAB or R.
3. Software for data analysis for example MATLAB, R, The Unscrambler, SIMCA or Metaboanalyst.

3 Methods

3.1 Sample collection and storage

Tissue samples must be collected under conditions which ensure that the integrity of the tissue metabolite profiles are preserved. However, patient safety is of paramount importance. The sample collection procedure must therefore be adapted to the biopsy procedure/surgical procedure.

1. Collect the biopsy (needle biopsy, surgical biopsy etc) from the organ or tissue of interest. The procedure must be performed by a health care professional. The time from sample collection until freezing should be minimized to avoid tissue degradation. We recommend < 5 minutes from collection until snap freezing. For surgical biopsies, the time from stopping blood circulation to the tissue to snap freezing should be as short as possible.
2. Using a pair of tweezers, put the tissue sample into the cryotube and snap freeze in liquid nitrogen.
3. Store the sample either in liquid nitrogen or at -80 °C until analysis.

3.2 Sample preparation for HR-MAS NMR

The sample needs to be cut with a scalpel to fit into a disposable insert or the rotor itself. To avoid tissue degradation it is important to keep samples cold or, preferably, frozen during sample preparation. We recommend preparing all samples on an ice block or on a workstation filled with liquid nitrogen. We also recommend thorough cleaning of rotors and inserts prior to analysis, and inspection of rotors and inserts to ensure the absence of any contaminants that can interfere with tissue analyses (*see* **Note 6**).

1. Place the insert or the rotor (50 µl) on the scale and tare.
2. Add at least 3.0 µl D_2O with HCOONa (25mM) to the insert (or rotor) and weigh. Tare the scale afterwards.
3. Put the weighing boat either on an ice block or on a workstation with liquid nitrogen.
4. Put the frozen tissue sample on the weighing boat on the iceblock/cold workstation and use a scalpel to carefully cut the sample into an appropriate size (typically between 5 to 45 mg) so that it fits in the sample insert or rotor (*see* **Note 7**).
5. Transfer the sample carefully into the bottom of the insert or into the rotor itself directly using tweezers. Make sure the sample is evenly distributed (*see* **Note 8**) and avoid air bubbles (*see* **Note 9**).
6. Weigh the sample.
7. If sample insert is used, put the plug into the insert using a blunt-ended screwdriver. Place the screw into the plug and tighten (*see* **Note 10**), then put the insert inside the rotor. If a 50 µl rotor is used, put the spacer into the rotor using the screw, and adjust the depth of the spacer using a rotor packer.
8. Put the cap on the rotor. This can be done using a filling funnel.
9. Label the bottom of the rotor using a permanent marker. The sample is now ready for HR-MAS NMR acquisition.

3.3 HR-MAS NMR acquisition

The following section describes how 1D and 2D ^{1}H and ^{13}C HR-MAS NMR spectra from tissue samples can be acquired. Before acquiring MR spectra, it is important to calibrate the temperature (*see* **Note 11**) and to ensure that the magic angle is adjusted correctly (*see* **Note 12**). We recommend to perform temperature calibration and magic angle adjustment at least once per week during the analysis period. We also recommend analysis of a standard

sample weekly and log the results (*see* **Note 13**), to detect any changes in NMR system performance.

1. Transfer the sample into the magnet.
2. Spin the sample at 5000 Hz.
3. Set the temperature to a desired value based on the last temperature calibration (*see* **Note 14**). Wait until temperature is stable.
4. Tune and match the probe for the nuclei of interest (^{1}H, ^{13}C etc). Use the rods that extend from the bottom of the HR-MAS probe labelled "T" (for tuning) and "M" (for matching) (*see* **Note 15**).
5. Lock the B_0 field using D_2O (*see* **Note 16**).
6. Acquire a spectrum with a fewer number of scans (NS ≈ 4) without applying line broadening. Measure full width at half maximum (FWHM) from one peak (*see* **Note 17**).
7. If FWHM > ~1.5 Hz, perform shimming by adjusting the current in the shim coils: X, XZ, XZ^2 and Z^4 (or Y, YZ, YZ^2 and Z^4, depending on the probe orientation) (*see* **Note 18**).
8. Determine the 90° ^{1}H pulse (P1) by acquiring an NMR spectrum using the one pulse sequence (*see* **Note 19**).
9. Determine the optimal frequency for water suppression (O1) using an NMR sequence with water suppression (*see* **Note 20**).
10. Set up NMR experiment using an appropriate pulse sequence and insert optimized parameters (pulse angles, transmitter frequency offset (O1) etc.). Some commonly used pulse sequences are described in the next section.

3.4 NMR pulse sequences and acquisition parameters

The aim of this section is to briefly describe some of the most commonly used NMR pulse sequences used during tissue analysis. Each NMR pulse sequence is a series of RF pulses, delays and gradient pulses designed excite nuclei and create desired signals from samples. The resulting NMR signal is detected by the receiver coil, digitized and stored as a free induction decay (FID) on a computer. After Fourier transformation, this FID is transformed into a one dimensional (1D) NMR spectrum. By introducing a second time variable in the pulse sequence (i.e. an incremented delay) and double Fourier transformation of the data collected as a function of two time variables two-dimensional (2D) NMR spectrum is generated. 2D NMR spectra are useful for identifying unknown metabolites. Generally, the pulse sequences used for HR-MAS NMR are the same as used for liquid samples, but some sequences require special consideration due to sample spinning. This is especially important for TOCSY experiments, where rotor synchronization is recommended.

3.4.1 **One pulse sequence:** One pulse sequence is the simplest of the NMR pulse sequences. It consist of a single 90° pulse that flips the z-magnetization to the xy-plane. The freely evolving signal is then detected as FID and processed to get 1D spectrum.

3.4.2 **One pulse sequence with presaturation:** The is the same as one pulse sequence, but has an additional low power pulse to saturate the signal from water abundantly present all tissue samples. Effectively suppressing water signal is critical for detecting low concentration metabolite signals from tissue.

3.4.3 **1D noesy with presaturation:** 1D nuclear Overhauser effect spectroscopy (NOESY) is probably the most frequently used experiment in NMR-based metabolomics studies. It offers better water signal suppression; it consists of

three 90^o RF pulses apart from a long presaturation pulse for water suppression. Gradient pulses are used in the sequence to purge residual water signal.

3.4.4 Carr-Purcell-Meiboom-Gill pulse sequence with presaturation: Macromolecules (mainly lipids in tissue) will cause broad peaks in the NMR spectra. The Carr-Purcell-Meiboom-Gill (cpmg) sequence is designed to suppress NMR signals from lipids. This sequence uses a 90° pulse, and series of 180° pulses to create spin echos. Signals from molecules with short T_2 relaxation (large molecules such as lipids) decay faster and consequently suppressed in the NMR spectra. The pulse sequence is generally used with presaturation to suppress signal from H_2O

3.4.5 COrrelated SpectroscopY (COSY): COSY is a homonuclear two-dimensional experiment, used to identify scalar coupled ^1H nuclei in the sample. There are many variants of COSY pulse sequences available, all having their pros and cons. In its most basic form, the COSY experiment consists of a 90° pulse, a t_1 delay which is increased stepwise and a second 90° pulse. The FID is acquired after the second 90° pulse. A problem with the basic COSY is that diagonal- and crosspeaks have a 90^o phase difference, meaning that large dispersive tails from diagonal peaks could mask nearby crosspeaks. This could be remedied by using double-quantum filtered (DQF)-COSY, where both diagonal- and crosspeaks have the same phase. DQF-COSY also suppresses signals from singlets (including water) and provides information of J-couplings from the crosspeak fine structure. The downside of DQF-COSY is a reduction of sensitivity (theoretically 50%) compared to conventional COSY and it requires high resolution to obtain multiplet fine structure.

3.4.6 TOtal Correlated SpectroscopY (TOCSY): TOCSY is similar to COSY spectra, however, it shows cross peaks between all sequentially connected spins. The pulse sequence for TOCSY is similar to that of COSY; in place of the second 90° pulse, TOCSY uses spin-lock pulses to transfer magnetization between all sequentially connected spins. Selecting the appropriate spin-lock duration is crucial for determining how far the magnetization will be transferred through the spin system. Because of faster relaxation, however, it is recommended to use a shorter spin-lock time in HR-MAS NMR compared to liquid state NMR. The optimal value will depends on the sample type, but a compromise value of 80 ms should be a reasonable starting point for tissue samples [7]. The choice of spin-lock sequence is also of importance, and it is recommended to use an adiabatic pulse scheme to better handle RF field inhomogeneity.

3.4.7 ^{1}H J-resolved: The 2D ^{1}H homonuclear J-resolved pulse sequence produces a NMR spectrum with chemical shift values along the first axis and ^{1}H-^{1}H couplings along the second axis. Briefly, the projection along the first axis is a decoupled proton spectrum, while the second axis shows the splitting patterns from ^{1}H-^{1}H couplings.

3.4.8 1D ^{13}C pulse sequence: Despite the low sensitivity, ^{13}C NMR has several advantages over ^{1}H. Firstly, a large chemical shift dispersion in 1D ^{13}C NMR allows easy identification of metabolites with less overlap problem (~200 ppm for ^{13}C, compared to ~15 ppm for ^{1}H). In addition, ^{13}C NMR is most suitable for isotopic labeling studies to track the passage of labeled ^{13}C through metabolic pathways in cell cultures, animal models and even humans. ^{13}C NMR spectra can be acquired using a single pulse sequence with no need for water

suppression. With off resonance-decoupling, ^{13}C peaks show multiplicity due to *J* coupling with ^{1}H. Proton decoupling removes ^{13}C-^{1}H *J* couplings. Power gated decoupling, where decoupling is on during the entire experiment, is commonly used. This leads to nuclear Overhauser enhancement (NOE) to carbon signals disproportionately and hence such spectra are not quantitative. On the other hand, inverse gated decoupling, in which decoupling is on only during acquisition removes the NOE effect and provides quantitative spectrum. Use of shorter (30°) excitation pulse allows the use of shorter relaxation delay and more rapid pulsing compared to 90° pulse.

3.4.9 Heteronuclear Single Quantum Coherence (HSQC): 2D ^{1}H-^{13}C HSQC NMR spectroscopy provides single bond correlations between ^{1}H and ^{13}C nuclei. The experiment is based on proton-detection and hence it offers higher sensitivity compared to carbon-detected experiments. The spectral resolution in the indirect dimension (^{13}C) is usually kept low to shorten experiment time, and closely spaced peaks may be difficult to distinguish if they are not resolved well in the direct (^{1}H) dimension. Carbon atoms not bound to hydrogen (i.e. carbonyls) are not detected by the HSQC experiment. Sensitivity enhanced HSQC sequence with a second refocusing period doubles the theoretical signal to noise ratio. For HR-MAS NMR, this prolonged pulse sequence could actually provide lower signal to noise, due to faster relaxation. In general, we suggest using the sensitivity enhanced version, but for samples with broader peaks, a sequence without the additional refocusing is recommended for better results (*see* **Note 21**).

3.4.10 Heteronuclear Multiple Bond Correlation (HMBC): 2D ^{1}H-^{13}C HMBC NMR is similar to 2D ^{1}H-^{13}C HSQC NMR. However, in HMBC cross peaks for

two, three, and sometimes four bond ^{1}H-^{13}C can be detected, unlike HSQC. HMBC is useful for the assignment of quaternary and carbonyl carbons, and protons bound to other nuclei than ^{13}C. Above all, it is very useful for identifying chemical structure for unknown metabolites. There are many variants of HMBC. It has been shown that constant-time (CT) HMBC performs well under HR-MAS conditions [8]. The intensity of cross peaks depends on the ^{1}H-^{13}C coupling constants and a delay (Δ) in the pulse sequence. Although, longer delays are useful for smaller ^{1}H-^{13}C *J* couplings, the prolonged pulse sequence due to longer delays may cause signal loss due to extended relaxation. As a compromise, a delay of 70 ms is often used, which is optimal for couplings of 7 Hz, even though most coupling constants are smaller than 5 Hz. In HR-MAS, relaxation effect can be more pronounced than for liquid samples, and it might be necessary to reduce delays for better sensitivity. The low resolution in the ^{13}C dimension can sometimes make it difficult to distinguish closely spaced peaks; as an alternative, selective HMBC experiment, which excites a part of the ^{13}C region (e.g. carbonyls) and provides higher resolution for the selected spectral window.

3.5 Choice of acquisition parameters

This section provides a practical guide for various acquisition protocols to get high-quality HR-MAS NMR spectra with examples for trade-off between experimental duration and signal-to-noise ratio.

3.5.1 Non Uniform Sampling (NUS): Data acquisition time for multi-dimensional NMR can be reduced using NUS, which only acquires a subset of the data

points in the indirect dimension and, using these points, the remaining points can be predicted. Sampling only a fraction of the data points reduces the experiment time by the same fraction. The time saved using NUS can be reinvested in more scans, allowing better signal to noise for weak samples.

3.5.2 **Number of scans (NS):** NS to be set depends on the amount of sample and the pulse sequence. For ^{1}H NMR spectra using noesy and cpmg pulse sequences (5-40 mg samples), 128 or 256 scans are commonly used. For 1D ^{13}C NMR acquisition, typical NS is between 4k and 32k.

3.5.3 **Transmitter frequency offset (O1)**: It is important to set the correct O1 value in the ^{1}H direction for optimal water suppression.

3.5.4 **Pulse angle:** For ^{1}H experiments, determine the 90^{o} pulse length to set correct pulse angles. This should also be done for other nuclei utilizing the ^{1}H channel. The ^{13}C pulse angle, however, is much less effected by the sample, and hence it is not calibrated each time.

3.5.5 **Parameters for Quantitative NMR spectra:** These spectra are typically acquired using a 1D pulse sequences such as noesy for ^{1}H, and zgig for ^{13}C. For quantitation, long repetition time (TR) need to be used to avoid T_1 relaxation bias. The T_1 relaxation effect is negligible when TR is more than five times T_1 (*see* **Note 22**). If a short TR is used it is also possible to compensate for this to achieve absolute quantification, as described in [9]. For absolute quantification, spectra from calibration standards (e.g. creatine or formate) need to be obtained as described in section 3.8.

3.5.6 **Receiver gain:** The receiver gain (RG) should be same for all samples if quantitative NMR spectra shall be acquired (*see* **Note 23**).

3.5.7 Repetition time: For 1D NMR spectra the repetition time (TR) can be estimated by summing the relaxation delay and the acquisition time (D1+AQ).

3.6 Processing of NMR spectra

Preprocessing of spectra can be performed using Topspin or other software such as SpinWorks and MNova. Post-processing is typically performed in generic software like MATLAB or R. We describe below pre-processing using Topspin user interface and post-processing using a customized MATLAB pipeline.

3.6.1 Pre-processing of 1D spectra

1. **Zero filling, apodization and Fourier transformation:**
    a) Zero filling refers to adding additional zero data points to the FID before Fourier transformation. In Topspin, this is done by increasing the size of real spectrum (SI) in the ProcPars tab (*see* **Note 24**). Zero filling is used to create spectra with more defined multiplets, but does not add any additional information to the dataset.
    b) Apodization involves applying a window function to the FID before Fourier transformation. The goal is either to enhance the SNR or resolution in the NMR spectra. Most commonly used window function for enhancing SNR is the exponential multiplication (EM) function using a line broadening (LB). The magnitude of line broadening (LB) can be specified (*see* **Note 25**). The second group of window functions aim to enhance resolution at the expense on SNR. Most commonly used such window functions are Gaussian (GM) and sinebell (SINE) multiplication functions.

c) Resulting FID must then be Fourier transformed to generate the NMR spectrum.

2. **Phasing:** Automatic phase-correction can be done in Topspin. However, NMR spectra from tissue samples may in some cases benefit from manual adjustment. Manual phasing needs to be done in two steps. First phase the reference peak (typically the tallest peak in the spectrum, but not the residual water peak) using zero order phase correction. Then phase the remaining peaks of the spectrum using first order phase correction.
3. **Baseline correction:** The spectra can be baseline corrected automatically in Topspin. Manual baseline correction is also possible using different baselines functions (polynomial, sine and exponential).
4. **Chemical shift calibration:** Proper chemical shift calibration is important. Select a reference peak, zoom into the peak, use the cursor to define the middle of the peak and set its chemical shift value (in ppm) (*see* **Note 26**).

3.6.2 Processing of 2D NMR spectra

1. **Fourier transformation and apozidation**

a) The FID must be Fourier transformed in both dimensions to generate the NMR spectrum.

b) Window functions can be applied in 2D NMR spectra. Commonly used windows functions for 2D are sine-bell squared (QSINE) and sine-bell (SINE).

2. **Phasing:** We recommend manual phasing for 2D NMR spectra. Define two or three rows/columns that contain peaks (right click using the cursor and click add). Then select phase correction for columns/rows. Perform zero order phase correction on the first peak, and first order phase correction on the other peaks. HMBC spectra are not phase sensitive and phasing can therefore be omitted.
3. **Chemical shift calibration:** Use a peak in the 2D spectrum with known ppm values for both dimensions. Select calibrate axis and right click in the middle of the peak and set the correct ppm values.

3.7 Post-processing of 1D NMR spectra for metabolomics analyses:

To evaluate multiple NMR spectra, peaks in the spectra must be aligned and the spectra normalized.

3.7.1 **Peak alignment**:

a) Choose a method for peak alignment that is suitable to your data, for instance icoshift [10] or COW [11] (*see* **Note 27**). Alternatively, for smaller peak shifts binning procedures may be applied. In binning, small spectral regions are summed into one bin (*see* **Note 28**).

b) Most methods require alignment to a reference spectrum. The spectrum with highest correlation to the remaining spectra might be a good choice. The mean or median spectrum may also be a good candidate [12].

c) After peak alignment, visually inspect the results to ensure that the correct peaks have been aligned to each other.

3.7.2 **Spectral normalization**: In order to correct for differences in tissue amount and tissue content, and to make all spectra comparable, normalization of each spectrum should be performed. Methods used for normalization are scaling to

sample weight, mean area normalization, scaling all spectra to an equal total peak area and PQN normalization [13].

a) Remove spectral regions with no peaks, the residual water peak and potential contaminants (*see* **Note 29**). In addition, remove residual lipid peaks from tissue with high lipid content such as breast tissue.
b) Normalize the spectra using the chosen method.

3.8 Metabolite peak assignment

Identification of most of the metabolites detected in tissue by NMR have been described previously. A number of metabolite databases are now freely available including the Human Metabolome Database (HMDB, www.hmdb.ca) [14], which is currently the largest repository of spectroscopic data from human samples. HMDB includes a search function that allows the user to match peaks from 1D or 2D NMR data with the database; it provides a list of potential candidates. The problem, however, is that most spectral data in the database are from solution state and at different temperature than normally used for HR-MAS NMR. Slight deviation in chemical shifts should therefore be considered in the metabolite search.

2D NMR spectra help confirm assignment of 1D NMR spectra and/or elucidate the structure of novel metabolites. In the interest of time, a common practice is to acquire 2D spectra on selected samples after 1D NMR analysis of all samples. Thus selected samples must have high levels of the metabolites that need confirmation/ structure elucidation. Between the two analytic runs, the samples must be stored at -80 ^{o}C or lower to avoid degradation.

Although it is possible to elucidate chemical structures from HR-MAS spectra, usually the data are insufficient for unknown metabolite identification. Tissue extraction and analysis using other spectroscopic methods (usually MS with chromatographic separation) will therefore be required. Alternatively, if the standard compound for the suspected metabolite is available, perform a spike-in experiment to confirm the peak identity; the chemical shifts of the spiked-in compound must match perfectly with the peaks under investigation.

Commercial softwares such as Chenomx, MNova and Amix are also useful tools for assigning peaks using reference spectra.

3.9 Quantification of peaks in NMR spectra

Quantification of individual metabolites is based on accurate measurement of area under the peak. This can be challenging since peak overlap and broad signals from lipids and macromolecules are frequently observed in HR-MAS. Metabolites can be quantified based on either manual peak fitting or automatic fitting [15-18]. For absolute quantification, one possibility is to use the calibrated ERETIC (Electronic REference To access In vivo Concentrations) signal as a reference [16]. The ERETIC signal is calibrated using a solution of standard compound. The inherent stability of modern NMR spectrometers also allow quantification using pulse length based concentration determination (PULCON) [17]. This enables direct quantification based on the spectrum of a reference sample. Other options for quantification are using the BATMAN algorithm in R [19] or commercial softwares such as Chenomx. It should, however, be noted that, absolute quantification may not always be feasible or necessary; depending on the experimental design and research objective, relative concentrations or metabolite ratios may be used.

3.10 Multivariate analysis of ^{1}H NMR spectra

Spectral data are generally complex and hence require specialized analysis methods that can handle such data. The unsupervised method, principal component analysis (PCA), and the supervised, partial least squares (PLS), method are most commonly used multivariate methods for spectral data or quantified metabolites. The analyses can be performed using software such as R, MATLAB (with or without PLS_Toolbox), Unscrambler, and SIMCA.

In PCA, principal component 1 (PC1) explains the direction of largest variation in the data; the second component, PC2, which is orthogonal to PC1, explains the next largest variation, and so on. In PLS analysis, latent variables are defined to maximize the covariance between the input data (spectra or quantified metabolites) and a response variable. PLS regression (PLSR) is used for a continuous response variable, while categorical variables can be modelled by PLS discriminant analysis (PLS-DA). Usually, the first few principal components or latent variables are sufficient to describe important aspects of the data.

The results of PCA and PLS can be visualized in scores and loadings plots. Scores represent the coordinates of the samples in the new coordinate system defined by the model, while loadings represent the weights of each original variable that represent the model.

PCA is particularly useful for an initial analysis of the data. It can detect possible outliers and natural groupings of the samples. Spectral data should be mean-centered before analysis, while quantified data should be either mean-centered or autoscaled (*see* **Note 30**). On the other hand, PLS analysis is used to model the data according to a clinical variable of interest. PLS-R is used for a continuous variable and PLS-DA for categorical variables.

Validation of the PLS model is important as multivariate methods are prone to overfitting. Choose a method for validation suitable for your sample size [20,21].

4 Notes

1. When handling liquid nitrogen you should use cryo-gloves, lab coat with long sleeves, and pants worn outside the shoes.
2. The cooling workstation can be a homemade system containing a styrofoam block containing liquid nitrogen, a cooling block made of metal, and plexiglas for minimizing nitrogen evaporation, as described in [22]. The cooling block must first be cooled by placing it into liquid nitrogen. After 5 -10 min, transfer the cooling block to the top of the workstation. Pour liquid nitrogen into the workstation to keep the cooling block cold for several hours. Place the plexiglas on top to reduce nitrogen evaporation
3. The choice of rotor depends on the design of the HR MAS probe and the type of application. We recommend to use a 50 µL rotor when the availability of tissue is high and if sensitivity is an issue (i.e. 1D ^{13}C spectra). However, 50 µL rotors require thorough cleaning compared to disposable inserts placed in 80 µL rotors. For small samples (<20 mg) we recommend to use disposable inserts placed in 80 µL rotors.
4. The following description pertains mainly to Bruker spectrometers and the TopSpin user interface. However, many of the general features presented also apply to instruments from other manufacturers
5. Table of relevant commands in the Bruker Topspin user interface

Command	**Action**
wobb	Wobbling for tuning and matching magnet
lock	Lock the magnetic field
gs	Repeated execution of the current pulse program
zg	Start acquisition command

zg	Single pulse NMR sequence
zgpr	Single pulse NMR sequence with presaturation
ft	Fourier transform
efp	Fourier transform with window function and phase correction
lb	Line broadening
apk	Automatic phasing of spectra
abs	Automatic baseline correction
cal	Calibration of spectrum chemical shift
xfb	Fourier transform in both directions (2D data)
rsh	Read existing shim file
wsh	Write new shim file

6. We have observed various contaminants in batches of disposable inserts (traces of production oil) and in new rotors. We recommend the following cleaning procedure for the zirconium rotors: wash rotor with dichloromethane (DCM) by pipetting up and down minimum three times, and then place rotor in a glass beaker with DCM for 15 min. Make sure that the rotor is filled with DCM. Repeat the Washing procedure by pipetting DCM up and down three times, and then place rotor in DCM for 15 more min. Afterwards, rinse rotor with DCM and let the rotor dry in fume hood for 30 min.

   For quality control purposes, we additionally recommend to periodically acquire NMR spectrum of a disposable insert from newly procured batches as well as the rotor containing only D_2O to make sure inserts and rotor are devoid of contaminants. Before use, we recommend that inserts are soaked overnight in 100% ethanol and then dry at room temperature for a couple of days.
7. If the tissue sample is too cold it is very difficult to cut and the sample. The trick is to use a warm scalpel (~25 °C) and allow enough time (~1-2 minutes) when cutting the sample

8. We have experienced problems with spinning the rotor if the sample is not evenly distributed in the insert or rotor
9. Shimming will be bad if air bubbles are present
10. Tightening the screw too loose may cause the insert to rotate inside the rotor during spinning. Tightening too much may cause the insert to expand, which can make it difficult to place the insert into the rotor. The trick is to place the insert half way in the rotor while tightening and then screw/adjust until the insert fits perfectly in the rotor without rotating
11. There is usually a discrepancy between the set temperature and the actual temperature inside the rotor. Since temperature will affect the NMR spectra, it is important to know the actual temperature of the sample. This can be done by acquiring NMR spectra from a solution with methanol (typically 4% dissolved in methanol-d_3). The distance between the two methanol peaks in NMR spectrum depends on the temperature. By measuring the distance one can accordingly determine and adjust the temperature inside the sample [23].
12. The sensitivity and spectral resolution is dependent on how accurate the magic angle is (54.7°). It is important to adjust magic angle every time the HR-MAS probe has been changed and otherwise regularly (weekly) during the experiments to achieve high quality NMR spectra. Magic angle can be measured using a rotor filled with solid KBr. The spinning sidebands of KBr are sensitive to magic angle misalignments, and the number and intensities of the sidebands are highest when the magic angle is correctly aligned. Adjustment of magic angle (a rod extends from the bottom of the NMR probe to adjust the angle) should be performed if the intensities of KBr sidebands are low. As a general rule, the intensity of the left sideband peak should be approximately 10-12%

(or more) compared to the intensity of the main peak when the KBr sample is spun at 5000 Hz.

13. We recommend to use an insert filled with 2 mM sucrose in 90 % H_2O and 10 % D_2O. Sucrose can also be used to set up baseline shim values if shim values are far off
14. We recommend sample temperature of ~278 K
15. Tuning adjust the probe so that its frequency corresponds to the transmitter frequency and matching maximizes the power transfer between the probe and the transmitter and receiver. Tuning and matching can be carried out by observing either the wobble curve in Topspin window (see Figure 2), or the green and red LEDs on the High Performance Preamplifier (HPPR) cover display

16. When there is a small B_0 drift, ^2H field lock system is able to detect and automatically compensate for the drift. The B_0 field will thus be kept at a constant value during acquisition. Locking is critical because even small B_0 field drifts will affect the spectral resolution
17. Sodium formate (HCOONa) at 8.44 ppm may be a suitable option to measure the line width.
18. Shimming is important in order to achieve a homogenous magnetic field across the sample. Poor shim values will cause low spectral resolution. This step may be tricky, particularly if the sample contains high amounts of fat. We recommend to shim in "gs-mode" (continuous acquisition of NMR spectra). Adjust the current in the shim coils: X, XZ, XZ^2 and Z^4 until FWHM of HCOONa is less than 1.5 Hz. For a practical guide on how to perform shimming of HR-MAS probes, see [24].
19. Acquire a NMR spectrum using P1 ≈ 90° pulse (pulse program: zg). Phase the spectrum. Then acquire another spectrum using P1 ≈ 360°. Observe if the water peak

in the spectrum is below (too short P1) or above (too long P1) the baseline. Repeat until you see 50% of the signal above the baseline and 50% below the baseline as shown in Fig. 2.

20. Acquire NMR spectrum with water presaturation (pulse program zgpr) using different values for O1. Repeat until suppression of water signal is optimal as shown in Fig. 2.
21. In 2D ^{1}H-^{13}C NMR, signals are detected through the high sensitive ^{1}H nuclei; hence it is beneficial to use this experiment considering the low sensitivity of ^{13}C. Such 2D NMR spectra can be used for semiquantitative analysis of metabolite levels and multivariate analysis, similar to 1D NMR.
22. The T_1 relaxation times for ^{13}C carbonyls are very long often up to 20 s.
23. The observed NMR signal size depends on receiver gain (RG). Too low RG leads to low SNR. If the receiver gain is too high, the signal will exceed the limits of the analogue to digital converter (ADC), and the amplitude of the FID will be clipped. The RG may be automatically optimized by the instrument. We recommend using a fixed RG, which is optimized for the sample type/size used. Tissue with high lipid content might require lower RG setting due to large lipid signals. RG is usually set to maximum value in ^{13}C NMR.
24. On Bruker NMR instruments, when the spectrum size (SI) = time domain points (TD), it means you have zero filled once. If SI=2TD, you have zero filled twice, etc.
25. Typical LB for 1D ^{1}H NMR spectra varies is 0.3 or 1 Hz, and for ^{13}C NMR spectra, 3 Hz. The optimal LB value to achieve the highest possible SNR value is called the matched filter and is defined as the linewidth at half-height.
26. One can use the alanine doublet at 1.48 ppm (middle of the doublet) for ^{1}H spectra, and [3-^{13}C] alanine at 18.9 ppm for ^{13}C NMR spectra for chemical shift scale calibration.
27. Icoshift has been shown to perform well on HR-MAS data

28. Bins may be of fixed width (typically 0.04 ppm) or varying in width such as AI binning [25].
29. Spectra from tissue samples can contain signals from exogenous compounds such as local anesthetics or disinfectants used during sample collection, or from solvents used to clean laboratory equipment. Care must be taken to identify and remove such signals from the spectra before further analyses.
30. Mean-centering is done by subtracting the mean of each variable from each variable. In autoscaling, the mean of the variables is subtracted before each variable is divided by its standard-deviation. After autoscaling, all variables have the same mean and standard-deviation of 0 and 1, respectively, and both small and large variables influence the model equally.

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Figure Captions

Figure 1: Typical accessories used for HR-MAS NMR analysis of tissue samples. 1) Filling funnel; 2) rotor cap remover; 3) narrow pipette tip for adding D_2O with HCOONa; 4) blunt-ended screwdriver; 5) forked screwdriver; 6) rotor packer; 7) extraction screw; 8) tweezers; 9) scalpel; 10) rotor and spinning cap; 11) insert with taper and screw cap.

Figure 2: Some important steps of HR-MAS NMR experiment: Top left: Typical NMR spectrometer equipped with a HR-MAS NMR probe. Top middle: The wobble curve that indicates whether the probe is properly tuned (the dip of the curve is positioned at the red vertical line) and matched (the dip extends all the way down to the x-axis). Top right: Lock display shows the amplitude of the D_2O signal. When the sample is not locked the amplitude of the lock signal will either be very low or a ringing signal is observed. The sample is considered locked when the amplitude of the lock signal (red and green) is high and flat as shown in the figure. Centre: The shim interface in BSMS Control Suite; the shim is optimized by adjusting the current in specific shim coils. Bottom left: Shape of the H_2O signal used to optimize the ^1H 90° pulse length (P1) and the frequency for water suppression (O1). Bottom right: A typical HR-MAS NMR spectrum.