Video Article

Metabolomic Analysis of Rat Brain by High Resolution Nuclear Magnetic Resonance Spectroscopy of Tissue Extracts

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Abstract

Studies of gene expression on the RNA and protein levels have long been used to explore biological processes underlying disease. More recently, genomics and proteomics have been complemented by comprehensive quantitative analysis of the metabolite pool present in biological systems. This strategy, termed metabolomics, strives to provide a global characterization of the small-molecule complement involved in metabolism. While the genome and the proteome define the tasks cells can perform, the metabolome is part of the actual phenotype. Among the methods currently used in metabolomics, spectroscopic techniques are of special interest because they allow one to simultaneously analyze a large number of metabolites without prior selection for specific biochemical pathways, thus enabling a broad unbiased approach. Here, an optimized experimental protocol for metabolomic analysis by high-resolution NMR spectroscopy is presented, which is the method of choice for efficient quantification of tissue metabolites. Important strengths of this method are (i) the use of crude extracts, without the need to purify the sample and/or separate metabolites; (ii) the intrinsically quantitative nature of NMR, permitting quantitation of all metabolites represented by an NMR spectrum with one reference compound only; and (iii) the nondestructive nature of NMR enabling repeated use of the same sample for multiple measurements. The dynamic range of metabolite concentrations that can be covered is considerable due to the linear response of NMR signals, although metabolites occurring at extremely low concentrations may be difficult to detect. For the least abundant compounds, the highly sensitive mass spectrometry method may be advantageous although this technique requires more intricate sample preparation and quantification procedures than NMR spectroscopy. We present here an NMR protocol adjusted to rat brain analysis; however, the same protocol can be applied to other tissues with minor modifications.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51829/>

Introduction

Murine models have been utilized extensively in brain research¹. Genotype-phenotype correlations have been investigated in mouse and rat brains by studying gene expression at the RNA and/or protein levels on the one hand, and morphological, functional, electrophysiological and/or behavioral phenotypes on the other²⁻⁶. However, to completely understand the mechanisms linking phenotype to genotype, it is imperative to investigate the molecular events downstream of protein expression, i.e. the metabolism of the biochemical substrates upon which enzymes act⁷. This requirement led, over the past 10 to 15 years, to a renaissance of metabolic research in many branches of biology^{8,9}. While classical metabolic studies have often been focused on details of specific pathways, the new metabolomic approach is geared towards an all-encompassing investigation of the global metabolic profile of the tissue under consideration. One consequence of this concept is an obvious need for analytical tools that minimize bias towards specific metabolic pathways and/or classes of compounds. However, a classical biochemical assay is based on a particular chemical reaction of a specific analyte that needs to be specified before the assay is performed. By contrast, spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (i) are based on particular molecular (physical) properties of biochemical compounds, each of which gives rise to one or several distinct signals in a spectrum detected in the course of one experiment; and (ii) detect a large number of different compounds per experiment.

Thus, each spectrum contains the combined information of a whole range of metabolites. For this reason, spectroscopic methods are adequate tools for metabolomics, as no prior selection needs to be made regarding the nature of the analyte to be measured⁸. As a consequence, these techniques naturally lend themselves to exploratory studies because they greatly facilitate the detection of unexpected metabolic changes.

Although NMR spectroscopy and MS can be used interchangeably for the analysis of many metabolites, each method possesses specific advantages and disadvantages that have recently been reviewed¹⁰. Briefly, NMR spectroscopy can usually be performed from crude extracts and does not require chromatographic separation of sample compounds before analysis. By contrast, MS works with gas or liquid chromatography (GC or LC) separation, except for particular recent developments such as mass spectrometry imaging. In a few special cases such as the analysis of sugars, LC separation may become a necessity for NMR spectroscopy as well, because the resonance lines of different sugars

overlap significantly in proton (^1H) NMR spectra. Nevertheless, ^1H NMR spectroscopy without chromatographic separation remains the most popular, almost universally applied metabolomic NMR method. Generally, sample preparation is more time-consuming and complex for MS than it is for NMR spectroscopy. Serious problems due to matrix effects are much less common in NMR spectroscopy than in MS where they may lead to considerably attenuated signals. Metabolite quantitation can be achieved with either method. However, multiple standard compounds are needed for MS due to variations in matrix effects and ionization efficiencies between metabolites. By contrast, only one standard per sample is needed for an NMR spectroscopic analysis because under appropriate measuring conditions, the latter method is intrinsically quantitative thanks to the strictly linear NMR response by the observed nuclei. A major drawback of NMR is its relatively low sensitivity. MS, in particular LC-MS, is more sensitive than NMR by several orders of magnitude; for this reason, MS is to be preferred over NMR for the analysis of compounds occurring at very low concentrations. On the other hand, the nondestructive nature of the NMR experiment is a clear advantage over MS; in this way, NMR can be performed repeatedly on the same sample, e.g., for different NMR-active nuclei such as ^1H , phosphorus-31 (^{31}P), carbon-13 (^{13}C), fluorine-19 (^{19}F) etc., as no material is consumed by NMR as opposed to MS measurements.

Both NMR and MS can be employed in different modes, each one being optimal for the detection of compounds with particular chemical characteristics. For instance, ^{31}P NMR is often better suited than ^1H NMR for the analysis of moderately concentrated phosphorylated compounds, although almost all phosphorylated metabolites also contain protons. However, their ^1H NMR signals may be obscured by ^1H NMR signals from other, non-phosphorylated compounds, while the latter obviously do not cause background signals in ^{31}P NMR spectra. In an analog situation, ^{19}F NMR analysis is to be preferred for fluorinated compounds, e.g., fluorinated drugs (no background signals from endogenous metabolites), while the special case of ^{13}C NMR is of interest almost exclusively if the fate of ^{13}C -labeled exogenous metabolic precursors needs to be followed, due to the extremely low natural abundance of the ^{13}C isotope (ca. 1%). Many mass spectrometers work in either negative ion mode or positive ion mode. Therefore, it is important to know ahead of the analysis whether the ions to be observed are negatively or positively charged. We focus here on a protocol for the analysis of the brain tissue metabolome by ^1H and ^{31}P NMR spectroscopy because this method yields a large number of important metabolite concentrations at low cost in terms of (i) time needed for sample preparation and (ii) effort required for metabolite quantitation. All experiments can be performed using the equipment of a standard wet-chemistry laboratory and a high-resolution NMR spectroscopy facility. Further requirements are described in the **Protocol** section below.

Protocol

NOTE: ANIMAL ETHICS STATEMENT

Animal studies on rats followed the guidelines valid in France, and were approved by the local Ethics Committee (#40.04, University of Aix-Marseille Medical School, Marseille, France).

1. Harvesting and Freezing Rat Brain

1. Prepare items required: liquid nitrogen ($\text{N}_{2\text{liq}}$) in Dewar that is large enough to keep a freezing clamp (at least 2-3 L volume); anesthetic (e.g., isoflurane, or ketamine/xylazine); anesthesia chamber; sterile dissection tools: surgical scissors, scalpel, forceps; tissue wipes and bottle with cleaning alcohol (ethanol); needles (25 G); 1 ml and 10 ml syringes. Label aluminum sheets (ca. 10 x 10 cm) with sticky tape. Use sheets to wrap individual freeze-clamped rat brains.

NOTE: Always wear protective gloves and eye protection goggles or mask when handling liquid nitrogen!

2. Fill Dewar with $\text{N}_{2\text{liq}}$, and place freezing clamp in a Dewar. Make sure the amount of $\text{N}_{2\text{liq}}$ in the Dewar is sufficient for repeated freeze-clamp procedures (several liters; the amount of $\text{N}_{2\text{liq}}$ evaporating during each freeze-clamping depends on the size of the clamp).
3. Anesthetize animal (e.g., by isoflurane, or by intraperitoneal injection of a ketamine/xylazine mixture). Proceed to euthanasia by cardiac puncture to prevent bleeding when scalp is removed and skull is opened. **Steps 1.4-1.7** below describe this standard procedure.
NOTE: In special protocols requiring maximum preservation of glucose and high-energy metabolites, sacrifice rat by funnel-freezing the brain of the anesthetized animal with $\text{N}_{2\text{liq}}$ ¹¹ after retraction of the scalp. Then, dissect brain out of the skull under intermittent $\text{N}_{2\text{liq}}$ to minimize post-mortem metabolism¹².
4. Moisten head of rat with cleaning alcohol. Use surgical scissors to (i) remove scalp, and (ii) open skull along cranial sutures.
5. Use forceps to open skull further, and to remove entire brain from underneath the open skull, positioning the head upside down. Remove quickly any visible traces of blood using tissue wipes. If brain hemispheres are metabolically and morphologically symmetric, it is convenient to use one of the hemispheres for metabolic analysis after separating it from the other hemisphere by incision with the scalpel. If required, use the remaining hemisphere for other brain studies such as histology.
6. Quickly remove freezing clamp from $\text{N}_{2\text{liq}}$ -filled Dewar, place entire or half rat brain on one inner surface, clamp and immediately insert freezing clamp into $\text{N}_{2\text{liq}}$ -filled Dewar while holding clamp firmly compressed. Ensure that **steps 1.3-1.6** take no longer than 60 sec.
7. After 1-2 min remove freezing clamp from the Dewar, open clamp, loosen frozen brain tissue from clamp, and wrap frozen tissue in appropriately labeled aluminum sheet (see 1.1 above). Make sure that the label is legible and stays firmly in place after the sample is wrapped. Quickly place wrapped sample in $\text{N}_{2\text{liq}}$. Perform the entire procedure as quickly as possible to avoid thawing of frozen brain tissue.
8. Store frozen sample in $\text{N}_{2\text{liq}}$ or in a freezer at $-80\text{ }^{\circ}\text{C}$ until metabolite extraction.

NOTE: Whenever a biological sample is to be stored for more than one year, storage at $\text{N}_{2\text{liq}}$ temperature is to be preferred over $-80\text{ }^{\circ}\text{C}$. This also applies to the remainder of this protocol.

2. Preparation of Metabolite Extraction Procedure

1. Prepare tissue homogenizer and matching test tubes (e.g., 10 mm inner diameter, depending on the diameter of the homogenizer shaft; usually made of plastic). Use electrical homogenizers rather than manually driven homogenizers (tissue grinders). Prepare vortexer and laboratory balance.
2. Prepare bucket filled with crushed ice. Keep sufficient quantities of methanol, chloroform and water on ice (4 ml each per 250-350 mg frozen brain tissue).
3. Prepare transfer pipettes and vials.

1. Prepare glass vials (≥ 20 ml volume) with screw caps and place on ice (one vial per tissue extract). Fit screw caps with a Teflon septa resistant to chloroform.
2. Prepare 5 ml plastic pipettes for dispensing methanol and water, and glass pipettes or Hamilton syringes for dispensing chloroform. Prepare additional plastic pipettes (5 or 10 ml volume) for transferring mixtures of tissue homogenate and methanol.
3. Make sure all glassware is thoroughly rinsed with distilled water and carefully dried before use to remove traces of impurities, notably NMR-detectable formate and acetate.
4. Fill porcelain mortar with N_{2liq} , place porcelain pestle in mortar and refill with N_{2liq} . Nitrogen will evaporate until temperature of mortar and pestle is sufficiently low. Keep small amount of N_{2liq} in mortar.

3. Extraction of Metabolites

1. Remove frozen brain tissue sample from storage (N_{2liq} tank or -80°C freezer). Then, immediately transfer tissue sample to mortar partially filled with N_{2liq} .
2. Use the N_{2liq} -cold pestle to break the frozen brain tissue into smaller pieces that easily fit into the test tubes used for tissue homogenization. To prevent pieces of frozen tissue from being projected out of the mortar in the process, break up the tissue while still being wrapped in aluminum sheet. Don't grind frozen tissue to powder as this would make transfer to the test tube more difficult (increased risk of water condensation). IMPORTANT: Throughout the entire procedure, add N_{2liq} to mortar as needed to keep the sample deep frozen.
3. Mix small pieces of frozen brain tissue thoroughly, weigh out 250-350 mg and transfer to a test tube filled with ice-cold methanol (4 ml for 250-350 mg brain tissue). Every time pieces of frozen tissue are added, homogenize these immediately with the tissue homogenizer. NOTE: Complete this entire procedure quickly to avoid (i) significant condensation of water on the sample which would lead to an overestimation of the sample weight, and (ii) heating and thawing of the sample. Individual tissue pieces should be in a frozen state at the beginning of the homogenization process.
4. After the last piece of the frozen rat brain sample has been added to the test tube and homogenized, transfer the homogenate to a ≥ 20 ml volume glass vial, close screw cap and let stand on ice for 15 min. If the volume of the test tube is not sufficiently large for ≥ 4 ml methanol, use a smaller methanol volume to homogenize a part of the frozen brain tissue, transfer the mixture to the glass vial and continue homogenizing the residual tissue pieces with fresh methanol. Make sure the total methanol volume is 4 ml per 250-350 mg brain tissue.
5. Add the same volume of ice-cold chloroform (*i.e.*, 4 ml per 250-350 mg brain tissue) to homogenate, vortex thoroughly and let stand on ice for 15 min.
NOTE: Always use ventilated chemical hood when handling volatile solvents, notably chloroform!
6. Add the same volume of water (*i.e.*, 4 ml per 250-350 mg brain tissue) to homogenate, vortex thoroughly and let stand at -20°C overnight.

4. Preparation of Phase Separation and Solvent Evaporation

1. Prepare cold centrifuge (4°C , 13,000 x g at maximum radius) and centrifuge tubes. Ensure that the latter are ≥ 20 ml volume, resistant to chloroform, and can withstand centrifugal forces of 13,000 x g. Use dedicated glass centrifuge tubes but rinse (as all glass ware) with distilled water before use (see 2.3).
2. Prepare thoroughly rinsed glass Pasteur pipettes and an appropriate propipettor (bulb, manual pipette pump, automatic pipette aid/pipettor, etc.).
3. Prepare two additional thoroughly rinsed tubes (≥ 15 ml volume) per brain sample, one of which needs to be resistant to chloroform (made of glass or Teflon), the other one to methanol (made of plastic resistant to methanol, or glass).
4. Prepare solvent evaporation apparatus (commercially available or homebuilt). Ensure that this device provides a finely controlled stream of dry nitrogen gas that is directed onto the surface of an extract solution containing volatile solvents (methanol, chloroform).
5. Prepare two additional trays or buckets filled with ice: one for sample transport on ice, and another one for keeping samples cold during the evaporation process.
6. Prepare lyophilizer (freeze-dryer) and materials needed for lyophilization: (i) one 50 ml centrifuge tube or vacuum round bottom flask per extract, and (ii) N_{2liq} to freeze the aqueous phase of samples (≤ 0.3 L per sample). If centrifuge tube is used, also prepare wide-neck vacuum filter bottle suitable for lyophilizer.

5. Phase Separation and Solvent Evaporation

1. For complete phase separation, transfer the methanol/chloroform/water/brain tissue homogenate (see 3.6) to a chloroform-resistant centrifuge tube (≥ 20 ml volume) and centrifuge at 13,000 x g and 4°C for 40 min.
NOTE: Two phases will form, separated by a layer of precipitated protein. The lower (heavier) phase consists of methanol, chloroform and dissolved lipids, whereas the upper (lighter) phase consists of water, methanol and dissolved water-soluble metabolites.
2. Use a Pasteur pipette to transfer the upper phase to an appropriate ≥ 15 ml tube (plastic resistant to methanol, or glass). Keep on ice.
3. Use a fresh Pasteur pipette to transfer the lower phase to an appropriate ≥ 15 ml tube (glass or Teflon). Keep on ice.
4. Store the layer of precipitated protein at -80°C if it is to be used for determination of total protein; or else discard.
5. Keep the tube with the water/methanol phase (see 5.2) on ice and evaporate methanol by directing a dry nitrogen stream onto the surface of the extract solution. Alternatively, gently bubble nitrogen through the extract solution. Terminate the evaporation process when nitrogen bubbling no longer causes volume reduction in the extract solution. At this time, continue with lyophilization (see 5.7), or freeze and keep sample at -80°C with the tube closed (screw cap or Parafilm) until ready for lyophilization.
6. Place the tube with the methanol/chloroform phase (see 5.3) on ice and evaporate methanol by directing a dry nitrogen stream onto the surface of the extract solution. When all solvent is evaporated, terminate the process and keep the sample at -80°C with the tube closed (screw cap or Parafilm) until ready for NMR analysis.
7. Prepare Aqueous Phase for Lyophilization
 1. After the end of the methanol evaporation process (see 5.5), transfer the sample to a thoroughly rinsed 50 ml centrifuge tube (if the sample is frozen, thaw before transfer). Alternatively, transfer to a vacuum round bottom flask.

2. Freeze extract solution by rotating centrifuge tube (or round bottom flask) partially inserted in N_{2liq} such that the inner surface of the tube or flask is progressively covered by frozen liquid. Make sure no N_{2liq} can enter the receptacle.
 3. When all the liquid is frozen, cover the tube with a punctured lid or screw cap to allow the vapor to escape, and place the tube in a wide-neck vacuum filter bottle.
 4. Start lyophilization after attaching the flask or bottle to the freeze-dryer.
8. Terminate the lyophilization process when the sample is entirely dry. Keep the sample at $-80^{\circ}C$ in a closed tube (with a tight cap!) until used for NMR analysis.
- NOTE: Usually no more than 24 hr are needed for lyophilization. Several samples can be lyophilized simultaneously, depending on the design of the lyophilizer, and on whether centrifuge tubes in wide-neck bottles or round bottom flasks are used.

6. Preparation of NMR Samples

1. Store dried and lyophilized extracts at very low temperature ($\leq -80^{\circ}C$). Re-dissolve lyophilizates for preparation of NMR samples *immediately* before NMR experiment.
NOTE: Storing samples in solution and/or near room temperature may result in sample degradation!
2. Prepare an aqueous 200 mM solution of the chelating agent, trans-1,2-cyclohexyldiaminetetraacetic acid (CDTA), as follows:
 1. Add pure water (e.g., 20 ml) to a test tube or centrifuge tube, and add the amount of CDTA powder necessary to generate a 200 mM solution.
NOTE: A substantial proportion of CDTA will not be soluble, because the pH is very low since the acid form of CDTA was used rather than a CDTA salt.
 2. Carefully add, step by step, increasing amounts of CsOH powder to the CDTA solution, and vortex thoroughly after each addition.
NOTE: The soluble CDTA fraction will increase with increasing CsOH content in the aqueous solution. Avoid "overtitration", i.e. add less than the stoichiometric amount of CsOH to the CDTA solution.
 3. When nearly all the CDTA powder is dissolved, start measuring pH after each CsOH addition and thorough vortexing. Terminate CsOH addition when the final pH is reached (**Table 1**).
NOTE: At this time, all the CDTA will be dissolved. The use of Cs^{+} as a counterion to CDTA is generally preferred over Na^{+} or K^{+} . Cs^{+} is a soft Lewis acid due its large ionic radius, as opposed to Na^{+} and K^{+} that have smaller ionic radii and are hard acids. Consequently, Cs^{+} forms complexes with phosphates (hard bases) less readily than do Na^{+} and K^{+} . This is advantageous for ^{31}P NMR spectroscopy of phosphorylated metabolites because complexation tends to increase NMR linewidths, notably under conditions of slow to intermediate ion exchange.
3. For ^{31}P NMR analysis of phospholipids (PL), dissolve dried lipids (see **5.6**) in 700 μl of a solvent mixture consisting of deuterated chloroform ($CDCl_3$), methanol and the CDTA solution prepared as described in **6.2** (5:4:1 volume ratio). Transfer the sample to a microcentrifuge tube. Use direct-displacement (or positive-displacement) micropipette with chloroform-resistant tips in both steps.
NOTE: Keep in mind that changing any of the following parameters will affect the appearance (chemical shift and line widths) of PL ^{31}P NMR spectra¹³⁻¹⁷:
 - (i) volume ratio $CDCl_3$: MeOH : CDTA solution
 - (ii) total solvent volume used
 - (iii) pH of the aqueous component of the solvent
 - (iv) CDTA concentration of the aqueous component of the solvent.
 NOTE: In general, fine-tuning of these sample parameters (**Table 1**) is not necessary, and should be performed with extreme care if desired in special cases. Changing the volume ratio between solvents easily results in the formation of a system consisting of two phases instead of one homogenous phase! The one-phase system was found to be more practical than the two-phase system in most applications^{13,14}.
4. For 1H NMR analysis of water-soluble metabolites, dissolve lyophilizate (see **5.8**) in 700 μl deuterium oxide (D_2O) containing 3-(trimethylsilyl)propionic-2,2,3,3-d $_4$ acid sodium salt (TSPd $_4$) in the millimolar range (D_2O containing 0.05% TSPd $_4$ is commercially available). Transfer the sample to a microcentrifuge tube.
5. Adjust the pH of the resulting aqueous extract solution to 7.3 by adding small amounts (ca. 2 μl) of deuterium chloride (DCI) and sodium deuterioxide (NaOD) solutions. First, start with 0.02 N DCI or NaOD solutions. If 2 or 3 subsequent additions do not cause sufficient pH change, continue with 0.2 N DCI or NaOD solutions. Be careful not to overtitrate.
NOTE: The overall amount of DCI or NaOD solution needed depends on the amount of brain tissue extracted; note this value for precise metabolite quantitation. In most cases the combined volumes of the added DCI and NaOD solutions should be virtually negligible (close to 1% of NMR sample volume).

7. Performance of the ^{31}P NMR Experiment for Brain Phospholipid Analysis^{13,14}

1. For best results, use a multinuclear high-resolution NMR spectrometer (≥ 9.4 Tesla field strength, corresponding to 162 MHz ^{31}P , or 400 MHz 1H resonance frequencies). Besides the ^{31}P coil, ensure that the ^{31}P NMR probe possesses a 1H coil for proton decoupling. Set temperature regulation of the NMR probe to desired target value (usually $25^{\circ}C$).
NOTE: Probe temperature may need 10-20 min to stabilize!
2. Centrifuge PL extract solution in microcentrifuge tube (see 6.3) at $4^{\circ}C$ and $11,000 \times g$ for 30 min to spin down solid residues in the sample. Transfer 600 μl of the supernatant to a high-quality NMR tube (5 mm outer diameter).
3. Prepare appropriate coaxial insert stem filled with an aqueous 20 mM methylenediphosphonate (MDP) solution at pH 7.0 for chemical-shift referencing and quantitation. Place this insert in the NMR tube filled with PL extract solution.
4. Fit the NMR tube with the appropriate spinner and transfer to the NMR magnet. Now spin the sample at 15-20 Hz and wait until the sample has adjusted to the set temperature (ca. 10 min).
5. Carefully minimize magnetic-field inhomogeneity across sample by adjusting on-axis and off-axis shim coil currents¹⁸.
6. Set NMR spectrum acquisition parameters to optimal values, which may vary as a function of magnet field strength (for a 9.4 T system, see **Table 1** for recommended parameter values).

7. Set number of transients per experiment (= NS).
 1. Set NS to about 80 (total duration of data acquisition ca. 20 min) if only the most prevalent PLs are to be quantitated with precision (phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), ethanolamine plasmalogen).
 2. Set NS to about 100-200 (total duration of data acquisition 1-2 hr) if less concentrated PLs are to be quantitated (alkyl-acyl-phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidic acid).
 3. Set NS to about 1,500-2,000 (total duration of data acquisition 7-10 hr; overnight experiment) if very-low-concentration PLs are to be quantitated, e.g. phosphatidylinositol mono and diphosphates (PtdIP and PtdIP₂), cardiolipin, lyso-PLs, alkyl-acyl-phosphatidylethanolamine, and occasionally others.
8. After the end of spectrum acquisition, process free induction decay (FID) using optimized parameters. The values of these parameters vary as a function of magnetic-field strength, shim quality, and the PL signal to be quantified.
 1. To obtain best results for the whole range of PLs, repeat processing using multiple (at least two) different filtering procedures. Use strong resolution enhancement for strongly overlapping signals, e.g., for PtdCho and PtdEtn regions.
 2. Use strong filtering (weak or no resolution enhancement) for weak signals.
 3. Filter very weak and broad signals without significant overlap by apodization (e.g., LB = 3 Hz) to increase signal-to-noise ratio, e.g. PtdIP and PtdIP₂. See **Table 1** for characteristic processing parameters.
9. Quantify the area of each PL signal with respect to the area under the signal of the reference compound (MDP) in the same spectrum.
10. Calibrate the signal of the reference compound (MDP) in a separate experiment, using a 5 mm NMR tube filled with (i) a phosphorus compound of known concentration, and (ii) the same coaxial insert stem used in PL ³¹P NMR experiments (see **7.3** and **7.9**).
11. Calculate individual PL concentrations based on relative areas of PL signals on the one hand (see **7.9**), and on calibration value obtained for MDP from coaxial insert on the other (see **7.10**). Take into account that the number of phosphorus nuclei contributing to a particular ³¹P NMR signal may vary as a function of the molecular origin of that signal.
NOTE: The MDP phosphonate signal (usually referenced to 19.39 ppm) represents two phosphorus nuclei, as does the cardiolipin phosphate signal. All other PL ³¹P NMR signals detected in brain extracts represent one phosphorus nucleus each.
12. Use statistics software as needed to compare brain PL levels between groups of animals.

8. Performance of the ¹H NMR Experiment for Analysis of Water-soluble Brain Metabolites

1. Set temperature regulation of the ¹H NMR probe to desired target value (usually 25 °C). See also remarks in **7.1**.
2. Centrifuge the aqueous extract solution in microcentrifuge tube (see **6.5**) at 4 °C and 11,000 x g for 30 min to spin down solid residues in the sample. Transfer 600 µl of the supernatant to a high-quality NMR tube (5 mm outer diameter).
3. Transfer NMR tube to NMR magnet, shim and set NMR spectrum acquisition parameters to optimal values as explained in **7.4-7.6**. See also **Table 1** for recommended parameter values.
4. Set the number of transients per experiment to about NS = 32 (total duration of data acquisition ca. 13 min). To obtain good signal-to-noise ratios for very weak signals, notably in the aromatic region, use NS = 64 (total duration of data acquisition ca. 26 min) or more.
5. After the end of spectrum acquisition, process free induction decay (FID) using optimized parameters. The values of these parameters vary slightly as a function of magnetic field strength, shim quality, and the metabolite signal to be quantified.
NOTE: In most cases, it is sufficient to process each spectrum once, employing a set of processing parameters presenting a good compromise for all metabolite signals. See **Table 1** for characteristic processing parameters.
6. Quantify the area of each metabolite signal (often a multiplet, sometimes overlapping with other singlets or multiplets stemming from different molecules) with respect to the area under the signal of the reference compound (TSP-d₄) in the same spectrum.
7. Calculate individual metabolite concentrations based on TSP-d₄ concentration (see **6.4**). Take into account that the number of protons contributing to a particular ¹H NMR signal may vary as a function of the molecular origin of that signal. The TSP-d₄ trimethyl signal (referenced to 0.0 ppm) represents nine protons.
8. Use statistics software as needed to compare brain metabolite levels between groups of animals.

Representative Results

To obtain best resolution in metabolic NMR spectra of brain and other tissue extracts, it has long been common practice to remove or mask metal ions (most importantly: paramagnetic ions) present in extract solutions. This has been achieved either by adding a chelating agent such as EDTA or CDTA to the extract¹⁹, or by passing the extract through an ion exchange resin such as Chelex-100²⁰. The results presented in **Figure 1** demonstrate that this step is not necessary for ¹H NMR spectroscopic analysis if brain extracts are carefully prepared according to the above protocol. Here, extremely narrow spectral lines were obtained for all spectral regions analyzed. Even in very crowded regions, e.g., for glutamine/ glutamate and myo-inositol/glycine, peaks at a distance of <0.01 ppm are almost completely separable at 400 MHz (**Figure 1**, bottom). As a consequence, qualitative, and quantitative analysis of the numerous small, but currently unassigned, peaks visible in the center and bottom panels of **Figure 1** can be envisaged in the future.

Figure 1. Subregions of a typical ^1H NMR spectrum (400 MHz) of the aqueous phase of a brain tissue extract from a female Lewis rat. All three panels demonstrate the extremely high resolution obtainable using the protocol presented in this paper. Neither chelating agent nor ion exchange resin has been used during sample preparation. Center and bottom panels show the existence of many unassigned low-intensity peaks that hint at the huge dynamic range covered by high-resolution ^1H NMR spectroscopy of tissue extracts if performed using optimized experimental parameters. These weak but well detectable signals can potentially be identified and quantified in the future. Several detected metabolites are specific of brain tissue, e.g., the neuron marker NAA or the neurotransmitter GABA; however, most compounds are involved in a broad spectrum of metabolic pathways that are common to mammalian cells, such as amino acid, branched-chain organic acid, polyol, (phospho)lipid and energy metabolism as well as in glycolysis and glutaminolysis, and in functions such as osmoregulation, cell growth and proliferation. The asterisk denotes the methyl resonance stemming from a methanol impurity. Abbreviations: ala, alanine; lac, lactate; threo, threonine; BHB, β -hydroxybutyrate; val, valine; ile, isoleucine; leu, leucine; AAB, α -aminobutyrate; AHB, α -hydroxybutyrate; tau, taurine; scy-Ins, *scyllo*-inositol; myo-Ins, *myo*-inositol; GPC, glycerophosphocholine; PC, phosphocholine; cho, choline; crn, creatinine; Cr, creatine; GABA, γ -aminobutyrate; asp, aspartate; NAA, *N*-acetylaspargate; gln, glutamine; glu, glutamate; suc, succinate; NANA, *N*-acetylneuraminic acid; ac, acetate; gly, glycine. The small peaks at the base of the ala doublet stem from the lactate ^{13}C satellite doublet (top panel). Reprinted under the Creative Commons Attribution License (CCAL) terms from Lutz N.W. *et al.* (2013) Cerebral biochemical pathways in experimental autoimmune encephalomyelitis and adjuvant arthritis: a comparative metabolomic study. PLOS ONE 8(2): e56101. [Please click here to view a larger version of this figure.](#)

In ^{31}P NMR spectroscopy, masking or removing paramagnetic cations (mostly iron) is unquestionably a necessity because phosphates easily form complexes with divalent and trivalent ions. Addition of CDTA to extracts affects both spectral line widths and chemical shifts; compare **Figure 2**, top and bottom left. Line narrowing by choosing 1,000 mM (bottom left) instead of 200 mM (top left) CDTA was desired, but resulted in superposition of PL signals that should be quantified separately (top left). Therefore, 200 mM CDTA is recommended for the aqueous component of the PL solvent, all other conditions being equal. Moreover, the sample temperature during measurement affects spectral line

widths and chemical shifts; compare **Figure 2**, top and bottom right. Line narrowing by choosing 277 K (bottom right) instead of 297 K (top right) was desired, but resulted in superposition of PL signals that should be quantified separately (top right). Therefore, 297 K is recommended as measurement temperature, all other conditions being equal. Comparison between the two top panels shows that a decrease in CDTA concentration from 200 mM (top left) to 50 mM (top right) only results in a modest increase in line width, and in almost no change in chemical shifts. However, note that the tissue concentration in the 50 mM CDTA extract is half as high as it is in the 200 mM CDTA extract, explaining the relatively narrow lines in the top right panel¹³.

Figure 2. Phosphatidylethanolamine (PtdE) regions of phospholipid ^{31}P NMR spectra (162 MHz) of brain tissue extracts from female Lewis rats. (Top left panel) Brain tissue concentration, 236 mg/ml; CDTA concentration and pH in the aqueous component of the solvent, 200 mM and 7.33, respectively; measurement temperature, 297 K. PtdE_{plasm} and SM signals are well resolved. (Bottom left) Brain tissue concentration, 236 mg/ml; CDTA concentration and pH in the aqueous component of the solvent, 1,000 mM and 7.36, respectively; measurement temperature, 297 K. PtdE_{plasm} and SM signals overlap entirely; they cannot be resolved despite reduced line width, compared with the top left spectrum. (Top right) Brain tissue concentration, 118 mg/ml; CDTA concentration and pH in the aqueous component of the solvent, 50 mM and 7.14, respectively; measurement temperature, 297 K. PtdE and SM signals are well-resolved. (Bottom right) Brain tissue concentration, 118 mg/ml; CDTA concentration and pH in the aqueous component of the solvent, 50 mM and 7.14, respectively; measurement temperature, 277 K. PtdE and SM signals overlap entirely; they cannot be resolved, despite reduced line width compared with the top right spectrum. Abbreviations: PtdE_{plasm}, ethanolamine plasmalogen; PtdE, phosphatidylethanolamine; SM, sphingomyelin; PtdS, phosphatidylserine; PtdC, phosphatidylcholine. Reprinted with permission from Lutz N.W. *et al.* (2010) Multiparametric optimization of ^{31}P NMR spectroscopic analysis of phospholipids in crude tissue extracts. 1. Chemical shift and signal separation. *Anal Chem* 82 (13): 5433-5440. Copyright 2010 American Chemical Society. [Please click here to view a larger version of this figure.](#)

Using the above protocol (one-phase system¹⁴; **Figure 3**, top left), a large number of quantifiable PLs were detected (**Figure 3**, top right), covering a considerable concentration range (note truncated high-intensity signals). Some of these signals are as yet unassigned (U1, U2, U6). If sample preparation, NMR measurement and spectrum processing are performed as indicated in this protocol, spectral resolution is even sufficient to routinely detect partial splitting of certain peaks (PtdS, PtdE, PtdE_{plasm}, AAPtdE; bottom left). As a consequence, qualitative and quantitative analysis of further PL subgroups can be envisaged in the future. **Figure 3**, bottom right, illustrates the power of judiciously chosen processing parameters to partially separate signals of low-concentration compounds (here: PtdC1u, a PL derived from PtdC that is not fully identified, and PtdC_{plasm}) resonating close to very strong signals (here: PtdC).

Figure 3. Phospholipid ^{31}P NMR spectroscopy (162 MHz) of brain tissue extracts from female Lewis rats. (Top left panel) A one-phase system (left) was preferred over a two-phase system (right). The commonly used two-phase system hampers correct PL quantitation because most of the upper phase is located outside the sensitive volume of the coil. (Top right panel) Complete ^{31}P NMR PL spectrum of rat brain. For better visibility of weak signals (PtdIP, PtdG), exponential line broadening (LB = 3 Hz) was applied. In this representation, several PL signals are not well-resolved, notably in the PtdC and PtdE regions. For PLs generating more than one ^{31}P NMR signal, observed nuclei are underlined (PtdIP, PtdIP, PtdIP₂, PtdIP₂). Currently unassigned signals are denoted by "Un" (where $n = 1, 2, \dots$). (Bottom left panel) PtdE and PtdS regions of the same spectrum. For better peak resolution, Lorentzian-Gaussian line shape transformation was applied (LB = -1 Hz, GB = 0.3). Because of these processing parameters, many very weak PL signals are difficult to detect. However, at least two peaks can be discerned for each PtdE, PtdE_{plasm}, AAPtdE, and PtdS. (Bottom right panel) PtdC region obtained with the same processing parameters as the PtdE region. Several signals at the base of the dominating PtdC resonance were detected unambiguously, while they cannot be discerned in the upper spectrum generated with exponential line broadening (AAPtdC, PtdC_{plasm}, PtdC1u). Besides the currently unassigned PtdC analog, PtdC1u, further minor resonances may be present upfield from PtdC. Abbreviations: PtdIP, phosphatidylinositol phosphate; PtdIP₂, phosphatidylinositol diphosphate; PtdA, phosphatidic acid; PtdG, phosphatidylglycerol; CL, cardiolipin; PtdE, sum of PtdE, PtdE_{plasm} and AAPtdE; PtdI, phosphatidylinositol. For further abbreviations see legend to Figure 2. Reprinted with permission from Lutz N.W. *et al.* (2010) Multiparametric optimization of ^{31}P NMR spectroscopic analysis of phospholipids in crude tissue extracts. 1. Chemical shift and signal separation. Anal Chem 82 (13): 5433-5440. Copyright 2010 American Chemical Society. [Please click here to view a larger version of this figure.](#)

Harvesting and Freezing Rat Brain	
Dewar for N_2 liq.; freezing clamp (e.g., homemade Wollenberger tongs, refs. 1 and 2, bottom of table); anesthesia chamber; surgical scissors; scalpel; 500 ml bottle with cleaning alcohol	1 of each
Forceps	2
1 ml syringe, 10 ml syringe	1 of each per animal
25 G needles	2 per animal
Sample Preparation	
Typical amount of brain tissue per extraction	250-350 mg
MeOH volume used in homogenizing 250 - 350 mg brain tissue	4 ml
5 ml plastic pipette	3 per extract
10 ml plastic pipette	1 per extract
Glass vial (≥ 20 ml volume)	1 per extract
Chloroform-resistant centrifuge tube	1 per extract
Waiting period after tissue homogenization in MeOH (mixture at 4 °C)	15 min
Water and CHCl_3 volume added to brain tissue homogenized in 4 ml MeOH	4 ml each

Waiting period after mixing homogenized tissue with water and CHCl_3 (mixture at -20°C)	overnight
Centrifugation for separation of aqueous from organic extract phase (glass centrifuge tube)	$13,000 \times g$, for 40 min at 4°C
Concentration of CDTA solution (aqueous phase of solvent mixture for dissolving extracted lipids)	200 mM
pH of aqueous phase of solvent mixture for dissolving extracted lipids	7.4
Volume ratio in lipid solvent A used for ^{31}P PL analysis (CDCl_3 : MeOH : CDTA solution)	5:4:1
Amount of solvent A used to dissolve lipids extracted from 250 - 350 mg brain tissue	700 μl
Centrifugation for spinning down solid particles in NMR sample (microcentrifuge tube)	$11,000 \times g$, for 30 min at 4°C
pH of NMR sample containing water-soluble metabolites	7.3
Sample volume transferred to NMR tube, after centrifugation	600 μl
MDP concentration in coaxial insert stem for ^{31}P NMR	20 mM
NMR Experiments	
Sample temperature during NMR experiment (to be adjusted for minimizing peak overlap)	25°C
Spinning frequency during NMR experiment	15-20 Hz
^1H NMR Acquisition Parameters	
Solvent providing ^2H lock signal	D_2O
Excitation pulse width, P1	11 μsec
Excitation pulse, amplifier attenuation, PL1	0 dB
FID size, TD	32 k
FID acquisition time, AQ	3.283 sec
Relaxation delay, D1	15 sec
Solvent suppression delay, D4	6 sec
Total repetition time, TR	24.3 sec
Spectral width in ppm, SWP	12.47 ppm
Spectral width in Hz, SW	4,990 Hz
Receiver gain, RG	512
Solvent suppression (continuous wave)	CW
Solvent suppression, amplifier attenuation, PL9	50 dB
Number of transients, NS	32 or 64
^{31}P NMR Acquisition Parameters	
Solvent providing ^2H lock signal	CDCl_3
Excitation pulse width, P1	9.5 μsec
Excitation pulse, amplifier attenuation, PL1	4 dB
FID size, TD	16 k
FID acquisition time, AQ	2.019 sec
Relaxation delay, D1	15 sec
Total repetition time, TR	17 sec
Spectral width in ppm, SWP	25 ppm
Spectral width in Hz, SW	4,058 Hz
Receiver gain, RG (maximum)	16,384

Proton decoupling (composite pulse decoupling)	CPD
Proton decoupling, amplifier attenuation, PL13	19 dB
Number of transients, NS	1,500 or 2,000
NMR Data Processing	
<i>¹H Resolution Enhancement, Gaussian-Lorentzian Lineshape Transformation</i>	
Overall evaluation of spectrum (if required, optimize separately for individual spectral regions, using $0.1 < GB < 0.4$, and $-0.6 < LB < -0.2$ Hz)	GB = 0.15, LB = -0.2 Hz
Very weak signals, e.g., aromatic acids (alternatively to apodization with LB ≥ 0.5 Hz)	GB = 0.015, LB = -0.3 Hz
Areas under peaks: use lineshape fits for overlapping resonances	
<i>³¹P resolution enhancement, Gaussian-Lorentzian lineshape transformation</i>	
Overall evaluation of spectrum (optimize separately for individual spectral regions, using $0.05 < GB < 0.2$, and $-3.0 < LB < -1.0$ Hz)	GB = 0.05, LB = -1.0 Hz
Very weak signals, e.g., PtdIP ₂ , PtdA: apodization	LB = 3 Hz
Areas under peaks: use lineshape fits for overlapping resonances	
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Table 1. Experimental parameters for high-resolution ¹H and ³¹P NMR spectroscopy of brain tissue extracts. Typical values for volume ratios and concentrations of solvents and reagents used in brain tissue extraction and NMR sample preparation are presented. Further recommended values relate to sample pH and measurement temperature, as well as NMR acquisition and processing parameters. Minor adjustments may be necessary, in particular if protocol is applied to tissues other than brain. NMR parameters have been optimized for measurements at 9.4 T, and should be adjusted as needed for spectrometers operating at different magnetic-field strengths.

Discussion

NMR spectroscopy is an efficient method for measuring concentrations of chemical compounds in solution in a very reproducible and accurate manner. However, to obtain high-quality data it is necessary to adhere to certain rules concerning sample preparation and analysis. In the determination of metabolite concentrations by NMR spectroscopy, neither the generation nor the reception of the NMR signal dominates the quantitation error, unless the intensity of an observed signal approaches the detection threshold (particularly weak signal). In all other cases biological variability, the sample preparation technique (extraction efficiency, physical and chemical stability of compounds, pipetting and weighing errors etc.), and/or the choice of signal acquisition and processing parameters will determine precision and accuracy of results. Obviously, any metabolic changes occurring during tissue harvest will also be reflected in the metabolite concentrations obtained. Therefore, it is very important to complete tissue harvest as fast as possible, and to apply particular tissue freezing techniques such as funnel freezing if accurate *in vivo* concentrations of rapidly metabolizing compounds are important (notably glucose, lactate, ATP and its catabolites, ADP and AMP).

Even at the intermediate magnetic-field strength (9.4 T) of a routine high-resolution NMR spectrometer excellent spectra can be obtained. For instance, in ¹H NMR spectra of the aqueous phase of rat brain extracts, signals whose difference in chemical shift, $\Delta\delta$, amounts to ca. 0.005 ppm (corresponding to 2.0 Hz at 400 MHz resonance frequency) can be discerned and quantitated separately. This is illustrated by the partial separation of (i) the lactate and threonine doublets, and (ii) the alanine doublet from the small peaks at its base (lactate ¹³C satellite doublet; **Figure 1**, top). Spectrometers operating at higher fields (14.1 or even 18.8 T) would increase resolution and sensitivity further, although these instruments are less common in NMR laboratories due to their high purchasing cost.

Using ¹H NMR spectroscopy, a broad range of metabolites can be analyzed simultaneously, *i.e.* in one single acquisition. The sensitivity of the experiment can be improved by increasing the number of accumulated transients, although this choice increases measurement time proportionally. (The signal-to-noise ratio is proportional to the square root of the number of transients.) However, the maximum total acquisition time given in the above protocol (20 - 30 min) should be sufficient for virtually all purposes, unless the amount of tissue available for extraction is very limited. Besides making use of the most NMR-sensitive nucleus (proton), metabolomic ¹H NMR spectroscopy has the advantage of comprehensive databases being available for different field strengths and sample pH values¹⁰. Furthermore, software for automatic or semi-automatic spectrum evaluation has become available¹⁰.

While ¹H NMR spectra of tissue extracts can be well resolved without addition of chelating agents, this is no longer true for ³¹P NMR spectra. In fact, the concentration of the chelating agent used (e.g., EDTA or CDTA) has a significant influence on both line width and chemical shift of ³¹P NMR signals stemming from phosphorylated metabolites. Increasing CDTA concentration in an extract decreases ³¹P NMR line widths, but this advantage may be outweighed by smaller differences between chemical shifts, $\Delta\delta$, of neighboring peaks. Therefore, the amount of chelating agent used has to be optimized for both line width and chemical shift together. In lipid extracts, these two spectral parameters are

significantly influenced by the overall sample concentration, *i.e.* the amount of tissue extracted, but also by the pH value and the temperature of the sample during the NMR measurement. Consequently, any optimization of measurement conditions must consider the combined effects of all experimental parameters involved, some of these not being additive. The complexity of this situation is illustrated by the behavior of PL ^{31}P NMR spectra shown in **Figure 2**. Although the lowest tissue concentration (118 mg/ml), the lowest temperature (277 K) and the highest CDTA concentration (1,000 mM) tested would result in lowest line width, the suggested protocol recommends the use of moderate tissue concentration (236 mg/ml), intermediate CDTA concentration (200 mM) and room temperature (297 K) to avoid signal overlap.

Although the conditions of sample preparation and spectrum acquisition are of utmost importance, the role of spectrum processing parameters in extracting a maximum of information from the acquired raw data should not be underestimated. A particular set of processing parameters may be ideal for detecting and quantitating PLs that occur at low concentrations; however, the same set of parameters may obscure individual peaks in 'crowded' spectral regions (**Figure 3**, top right). Conversely, processing parameters providing optimal resolution enhancement in regions of strongly overlapping resonances (**Figure 3**, bottom) would render low-intensity peaks undetectable. Recent developments, including also a comprehensive PL ^{31}P NMR database^{13,14}, greatly facilitate the analysis of NMR spectra^{13,14}.

Ultimately, the objectives of the underlying application must define the criteria for optimization, *i.e.* the desired balance of spectral resolution, sensitivity, precision of metabolite quantification, speed, and other factors. For instance, the recommended one-phase system for PL analysis permits more reliable and efficient PL quantification than two-phase systems (**Figure 3**, top left), and provides high spectral resolution as well as sufficient sensitivity for the quantitation of less-abundant PLs. However, in cases where best signal separation is of much higher priority than efficient and precise quantitation, the use of a higher percentage of chloroform-*d* in the solvent mixture may be attempted, although this easily leads to phase separation in the sample. If this is the case, the volume of the lower phase must be determined to obtain absolute PL amounts (mg PL, per g tissue or mg total protein).

In proton-decoupled heteronuclear NMR experiments, signal intensities may be altered by nuclear Overhauser effects (NOE), in addition to saturation due to rapid acquisition schemes. If uncorrected these effects result in metabolite quantitation errors. There are two alternative strategies to deal with this challenge. In the first approach, individual transients of an acquisition are separated by long delays. This method avoids any saturation or NOE but results in relatively long experiments; it should be used if precise and accurate results are needed. In some cases, priority may have to be given to fast spectrum acquisition, in particular for very dilute samples that can only be measured using a high number of transients. In such cases, addition of paramagnetic relaxation agents to the sample would allow rapid data acquisition without saturation or NOE. Alternatively, fast data acquisition without addition of paramagnetic agents can be employed. The latter method requires correction of signal areas by way of correction factors that have to be determined for each NMR resonance, whereas the presence of relaxation agents causes some broadening of NMR signals that may reduce the precision of metabolite quantitation for crowded spectral regions. Generally, the optimal choice of experimental conditions is not only dictated by the sample type, but also by the information to be extracted from an experiment¹³. If high priority has to be given to fast analysis of rather abundant metabolites, without the need for high precision and accuracy, it may be adequate to measure highly concentrated samples and employ short repetition times, possibly with paramagnetic relaxation agent added to the sample. By contrast, if accurate quantitation of a large number of metabolites is more important than speed, it is preferable to use less concentrated extracts and accept long NMR acquisition times, as demonstrated by the protocol presented here. Furthermore, if a particular research project emphasizes specific metabolites, experimental conditions can be adjusted to achieve optimal spectral resolution for the spectral regions in question. The protocol and discussion presented here may serve as a guide for the optimization of metabolomic analysis of crude tissue extracts by NMR spectroscopy.

Disclosures

The authors have nothing to disclose.

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