

# A Simple Approach for Obtaining High Resolution, High Sensitivity $^1\text{H}$ NMR Metabolite Spectra of Biofluids with Limited Mass Supply

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*A simple approach is reported that yields high resolution, high sensitivity  $^1\text{H}$  NMR spectra of intact biofluids with limited mass supply. This is achieved by spinning a capillary sample tube containing a biofluid at the magic angle and at a spinning rate of about 80Hz. A 2D pulse sequence called  $^1\text{H}$  PASS is used to produce a high-resolution  $^1\text{H}$  NMR spectrum that is free from magnetic susceptibility induced line broadening. Using this new approach a high resolution  $^1\text{H}$  NMR spectrum of biofluids with a volume less than  $1.0\ \mu\text{l}$  can be achieved at a magnetic field strength as low as 7.05T. Furthermore, the methodology facilitates easy sample handling and is suitable for high throughput investigations. In this paper first results are shown obtained at a magnetic field of 7.05T on urine samples collected from mice using a modified commercial NMR probe.*

**Key words:**  $^1\text{H}$  NMR, biofluid, metabolites, slow magic angle spinning, capillary sample tube.

## 1.0 Introduction

Metabolites are chemical compounds that are classified as reactants (substrates), intermediates, or byproducts in a cellular metabolic pathway, and include carbon compounds with a molecular weight typically in the range 100-1000. Metabolomics, the characterization of the metabolic composition of a biological specimen, and metabonomics, the quantitative study of the changes in the metabolic profiles of living systems in response to stimuli, are rapidly expanding areas of biomedical and biochemical research and complement genomics and proteomics. Metabolic changes are the earliest cellular response to environmental or physiological changes such as toxin exposure or disease state, so metabol(n)omics of, e.g. body fluids, can assist in detecting and diagnosing a disease or evaluating the efficacy of therapy in an early stage [1].

Nuclear Magnetic Resonance (NMR) spectroscopy, a quantitative, non-destructive method that requires minimal sample preparation, is one of the most powerful analytical tools for metabol(n)omic research [2,3]. A NMR spectrum of a biofluid or tissue consists of many lines, the intensities of which relate to the concentrations of metabolites in the sample, thus providing a biochemical fingerprint of an organism [4,5].  $^1\text{H}$  NMR is very attractive because of its large NMR sensitivity. However, in standard NMR experiments of fluids the lines of  $^1\text{H}$  spectra can be broadened due to variations in the bulk magnetic susceptibility between the fluid and, e.g., the sample tube, the air above the fluid, or the NMR coil. This gives rise to a reduced spectral resolution that hampers the spectral analysis. Special probes have been developed in order to reduce susceptibility broadening. One type is a flow injection probe that utilizes probe materials with a similar susceptibility as the sample and a receiver coil that is made of special magnetic susceptibility matched wires to minimize the susceptibility fields arising from this coil [6]. A second probe is the Nano-probe, where the sample is rotated at the magic angle to remove the susceptibility broadening [7-10]. Nano-probes utilize spherical sample holders with typical volume of about 30 to 40  $\mu\text{l}$  into which a capillary tube is attached for sample loading, which is placed inside a larger rotor. With these probes spinning rates of about 2 kHz are used to eliminate spinning sidebands that would occur in the spectra if lower spinning speeds are employed. This requires that the capillary hole must be blocked. However, air pockets can be created in the sample that can give rise to additional

line broadening when there is not enough fluid to fill the sample cell and then the liquid is moving in the sample holder during the spinning.

In this work an alternative method is reported that allows spinning at much lower frequencies while still eliminating the spinning sidebands in the spectrum. This has been achieved by combining the slow magic angle spinning with a high-sensitivity R.F. pulse sequence called PASS (phase-adjusted spinning sidebands) [11]. PASS was originally developed in solid state NMR and has recently been successfully extended for use on biological systems, i.e., excised tissues, organs [12], dense bacterial cell systems or bacterial cells attached to solid surfaces [13], food and food seeds [14]. In the present work PASS is applied successfully for the first time to study biofluids. It will be shown that a standard MAS probe can be used containing a simple capillary sample tubes inserted into the rotor, and that even with the present inefficient set-up, where the NMR coil is much larger than the sample and where a relatively low external field of 7.05 T is employed, PASS can provide high-quality spectra of a urine sample with a volume as small as 2.5  $\mu\text{l}$ . It is expected that with a more efficient instrument this volume can be reduced to 1  $\mu\text{l}$  or smaller. This is important, e.g., for applications on mice. The pharmaceutical industry is increasingly using mouse models for toxicological screening or disease evaluation [3], as it is important to minimize the quantity of synthesized (expensive) drugs that is required. One of the possible ways to investigate, e.g., drug toxicity, is to determine the metabolic changes in the urine after the drug administration [15], and even with our present setup it is possible to utilize mice.

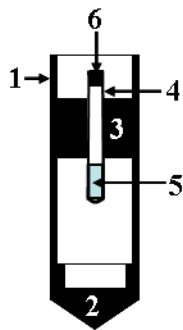
## 2.0 Experimental

### 2.1 Urine Samples

Urine samples were collected from female BALB/c mice (about 40 days old and ~16g in weight). Mice were first administered anesthetic by interperitoneal injection at 1.4  $\mu\text{l}$ /g body weight with a mixture of 0.3ml of Xylazine at a concentration of 20mg/ml and 1.0ml of Ketamine at a concentration of 100 mg/ml. For mouse #1 a urine sample was collected about one hour after the administering the anesthesia and while the mouse was still anesthetized. For mouse #2 the urine sample was collected about three hours after administering the anesthesia at which time the mouse had recovered from anesthesia. The mice were sacrificed by CO<sub>2</sub> asphyxiation and the urine samples were immediately collected into open (both ends) capillary tubes. For mouse #1, about 4  $\mu\text{l}$  of urine was obtained while about 14  $\mu\text{l}$  was obtained for mouse #2. The urine samples were immediately stored at -80°C until experiments. Prior to NMR measurements, the urine samples were thawed and transferred (using a plastic gel-loading micro pipet with a disposable tip of approximately 0.5mm in OD) into other capillary sample tubes that have one end fused. The fused glass capillary sample tube has an OD of 1.5mm and ID of 1.1mm. Each urine sample was centrifuged for about half a minute at 100g to eliminate air bubbles and locate all the sample at the bottom of the tube. A net volume of 2.5  $\mu\text{l}$  urine sample was successfully transferred into the capillary tube for mouse #1 while about 13  $\mu\text{l}$  urine from mouse #2 was used. All experiments were performed at room temperature, i.e., 25 °C.

### 2.2 NMR Experiments

All <sup>1</sup>H NMR experiments were performed on a Chemagnetics 300 MHz Infinity spectrometer, operating at a proton Larmor frequency of 299.982 MHz. A standard Chemagnetics CP/MAS probe with a 7.5-mm pencil type spinner system was used. Figure 1 shows the modification where the capillary sample tube is inserted into the rotor. In order to spin at low frequencies the rotor was equipped with a flat drive tip and an airflow restriction was utilized in the driver channel. Further, the rotor was marked with three equally paced precision markers so that three TTL pulses were generated during each rotation of the sample. The spinning rate was controlled using a commercial Chemagnetics MAS speed controller. It was found that with this controller the frequency stability at 40 to 200Hz Hz is better than  $\pm 0.3$  Hz, which is sufficient for our experiments.



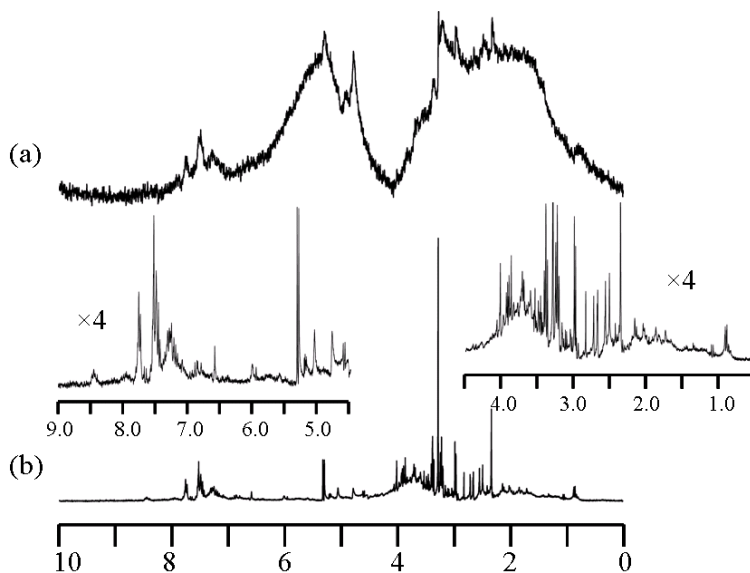
**Figure 1.** A capillary sample tube with OD of 1.5mm and ID of 1.1mm is inserted into the commercial 7.5mm Chemagnetics MAS rotor. The Chemagnetics MAS rotor has an OD of 7.5mm and ID of 6mm. **1:** The cylinder of the rotor; **2:** Flat drive tip; **3:** Teflon spacer for holding the capillary tube; **4:** Capillary sample tube with OD of 1.5mm and ID of 1.1mm, respectively; **5:** Sample; **6:** Cap of the sample tube.

PASS is a one-rotor-period, constant evolution time 2D experiment, during which five  $\pi$  pulses are applied, with time intervals  $tm_1$  to  $tm_6$  [11,12]. In PASS the center band spectrum and the spinning side band (SSB) spectra are separated by order. This is achieved by acquiring the signal after a series of PASS experiments with different values of the time intervals  $tm_1$  to  $tm_6$ . Each combination of time intervals has been chosen in such a way that the contribution of the signal in the observed free induction decay (FID) in the acquisition dimension ( $t_2$ ), arising from the center band and SSBs, is proportional to a phase factor given by  $\exp(-ik\Theta)$ , where  $k$  denotes the sideband order and  $\Theta$  is a variable called “pitch”. Then after 2D Fourier transform with respect to  $t_2$  and  $\Theta$  a series of spectra is obtained that separates the contributions for each  $k$  value, i.e. it separates the center band and side band spectra. The details on the implementation of the PASS sequence and water suppression can be found in refs.[11,12] (Note: The PASS pulse sequence on both Chemagnetics and Varian spectrometers is available upon request).

### 3.0 Results and Discussions

Figure 2 shows the water-suppressed  $^1\text{H}$  NMR spectra obtained on the  $2.5\ \mu\text{l}$  urine sample from mouse #1 and at a sample spinning rate of 80Hz using  $^1\text{H}$  PASS. The intensity of the peak corresponding to the water signal in urine is usually about a factor of 10000 times more intense than those from many of the metabolites, necessitating the suppression of the water signal because of the limited dynamic range of the spectrometer digitizer. Several results can be deduced from Figure 2. (a) The spectral resolution is poor when the spectrum was acquired on a static sample (Fig. 2a). The static spectrum in Fig. 2a consists of several relatively sharp peaks on top of very broad peaks. There are two sources contributing to the broad features, the relatively large widths of the metabolite lines resulting from the susceptibility differences between the sample, the glass capillary tube, and the MAS rotor, and a residual water signal that extends into the metabolite spectral range and cannot be suppressed without suppressing the metabolite signals. (b) A high quality, high resolution, high sensitivity isotropic metabolite spectrum is obtained from the center band  $^1\text{H}$  PASS spectrum at a sample spinning rate of 80Hz (Fig. 2b). For instance, the line width of the most intense peak at 3.286ppm is only about 0.8Hz. Moreover, the  $^1\text{H}$  PASS spectrum was acquired without locking the main magnetic field during data acquisition. Deuterium lock of the main field is currently unavailable in the MAS probe used. Thus a better spectral resolution is potentially available if field-locking is employed. (c) A careful evaluation reveals that the peaks with lowest intensities (located at about 8.5 ppm) are about a factor of 12,000 times lower than the water signal. Assuming that the density of water is 1g/ml (or 1mg/ $\mu\text{l}$ ), the total mass of water in  $2.5\ \mu\text{l}$  urine is approximately 2.5mg. This means that, a compound of about 210 nano-grams in mass ( $\sim 2.5\text{mg}/12,000$ ),

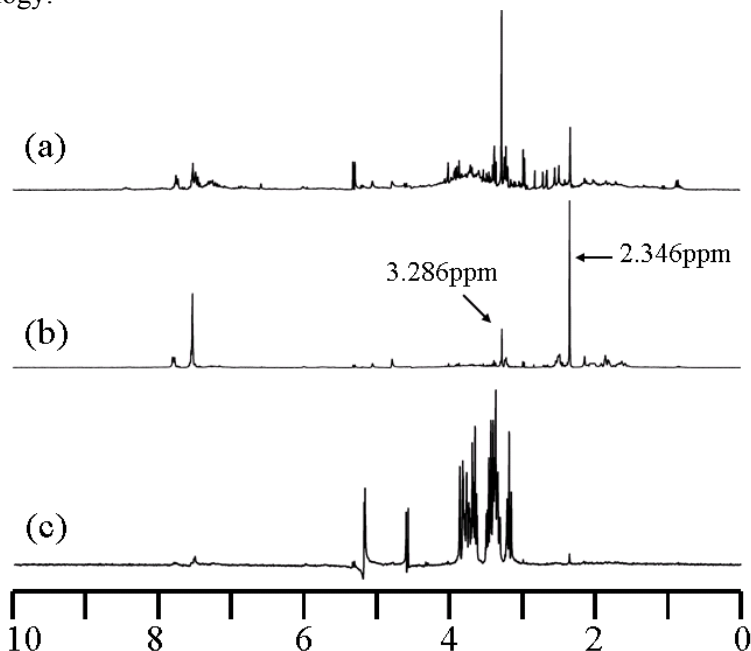
dissolved in the  $2.5 \mu\text{l}$  water sample can already be detected with the present setup. Furthermore, the relative filling factor of the current capillary sample is only about 0.0043, which is estimated using the dimensions of the RF coil of the Chemagnetics MAS probe (ID of 8 mm and length of 10 mm) and the actual capillary sample tube (ID of 1.1 mm and sample height of 2.3 mm). Hence, if a micro coil is used, for example with an ID of 2.5 mm, the filling factor can be easily made as high as 0.2 assuming the length of the coil is 2.3 mm. The corresponding volume of the micro coil would only be 0.022 that of the currently used coil. Since S/N is proportional to the filling factor times the square root of the volume of the RF coil [17, 18], a gain that is about a factor of 6.8 times in sensitivity would be possible if the quality factor of the circuit can be made the same. This would reduce the detection threshold down to about 30 nano-grams. (c) Water suppression becomes much easier in a slowly spinning sample than its corresponding static case, which in part accounts for the high quality spectrum given in Fig. 2b.



**Figure 2** The water suppressed 300 MHz  $^1\text{H}$  NMR spectra obtained from the  $2.5 \mu\text{l}$  urine sample (mouse #1). (a) The static spectrum. This spectrum was acquired using the first increment of PASS where the five  $\pi$  pulses were spaced evenly over 12.5 ms rotor period and the accumulation number was 192 scans. All the other parameters are the same as 2D-PASS. (b) The centerband of  $^1\text{H}$  PASS spectrum. The 2D – PASS data were acquired at a sample spinning rate of 80Hz and using sixteen evolution increments and each with 192 scans and a recycle delay time of 1s. The  $\pi/2$  pulse width was  $3.7 \mu\text{s}$ . The DANTE sequence [16] used for water suppression contained 6000 pulses of pulse width of  $0.2 \mu\text{s}$  and spaced by  $100 \mu\text{s}$ . The carrier frequency for water suppression was set at the centerband of the water peak. The total experimental time, including the recycle delay time and the DANTE period, was approximately 82 minutes. The inserts show spectral regions that are 4 times vertically expanded.

As a typical example of application we have investigated the effect of anesthesia at two different time intervals after the administration of the anesthesia in mice using present experimental setup. The results are summarized in Figure 3. Fig. 3a is a duplicate spectrum given in Figure 2b, i.e., the 80Hz- $^1\text{H}$  PASS centerband spectrum of the  $2.5 \mu\text{l}$  urine sample from mouse #1, where the urine sample was collected about one hour after the administration of the anesthesia. Fig. 3b was acquired under the same experimental conditions as those in Fig. 3a on the same sample but this sample was spiked with about  $1.5 \mu\text{l}$  of antheridia (from 0.3ml Xylazine at a concentration of 20mg/ml + 1.0ml of Ketamine at a concentration of 100mg/ml) prior to data acquisition. Fig. 3c was acquired on a urine sample collected about three hours after the administration of the anesthesia from a different mouse, i.e., mouse #2, of the

same age and nearly the same weight as mouse #1. It follows from Figs. 3a and 3b that the peak at 2.346ppm in (a) is actually from the anesthesia. Even though the anesthesia mixture generates many other peaks, the 2.346 ppm peak is quite distinct and can be easily recognized in Fig. 3a. The most intense peak at 3.286ppm in Fig. 3a has the same absolute spectral intensity as that in Fig.3b within the experimental error range, indicating that the 3.286ppm is not directly from anesthesia. Based on the spectrum given in Fig.3c where both the 2.346ppm peak and the 3.286ppm peak (the 3.286 ppm peak is recognized as a shoulder peak) are nearly proportionally reduced in intensity relative to other peaks, the 3.286ppm peak is considered as a body response to the anesthesia. Clearly at longer times after the administration of the anesthesia, the mouse body is capable of eliminating the injected anesthesia and its associated response and this effect can be easily detected through urine metabolite profiling using the slow-MAS methodology.



**Figure 3** Water-suppressed  $^1\text{H}$  PASS centerband spectra acquired at a sample spinning rate of 80Hz. (a) This spectrum is a duplicate spectrum given in Figure 2b, i.e., the 80Hz- $^1\text{H}$  PASS centerband spectrum of the 2.5  $\mu\text{l}$  urine from mouse #1. (b)  $^1\text{H}$  PASS spectrum of sample (a) + 1.5  $\mu\text{l}$  antheridia. This spectrum was acquired after the experiment in (a) and the same sample was spiked with about 1.5  $\mu\text{l}$  of antheridia (from 0.3ml Xylazine at a concentration of 20mg/ml + 1.0ml of Ketamine at a concentration of 100ml/ml). (c)  $^1\text{H}$  PASS spectrum obtained from the urine sample of mouse #2. Both (b) and (c) were acquired using the same experimental parameters as those given in Figure 2b except that in (c) only sixteen accumulations were acquired for each of the sixteen evolution steps.

## 4.0 Conclusions

It is demonstrated that a high resolution, high sensitivity  $^1\text{H}$  metabolite spectrum can be obtained from biofluids with volumes of a few  $\mu\text{l}$  or less using a combination of  $^1\text{H}$  PASS and slow magic angle spinning of a capillary sample tube with a commercial NMR probe and in a relatively low magnetic field of 7.05T. It is expected that the present development has the potential for detecting compounds with a mass of 30 nano-grams or less if a simple micro RF coil is used. Furthermore, a higher magnetic field can further increase the sensitivity. The sensitivity is roughly proportional to the strength of the field given the fact that the magnetic susceptibility-induced line broadening is proportional to the magnetic field. And an additional four times gain in S/N may be achievable by using a cryogenic probe.

Therefore, the ultimate threshold for a compound in a biofluid that can be detected by the present technique would be as low as a few nano-grams or even less if the combination of a micro RF coil, an ultra-high field, e.g., 21 T, and a cryogenic probe are utilized. However, these options remain to be explored.

The new approach facilitates easy sample handling, i.e., the samples can be directly collected into inexpensive and disposable capillary tubes at the site of collection and subsequently used for NMR measurements. We have also found that slow magic angle spinning improves magnetic field shimming, which is important for high throughput investigations. Since this new approach can be easily applied on a commercial NMR spectrometer equipped with a traditional magic angle spinning probe (even though the conditions are not optimized), it may significantly enhance the NMR based metabolomic research, especially in sample limited situations.

## 5.0 Acknowledgement

The work was supported by PNNL's (Pacific Northwest National Laboratory) Laboratory Directed Research and Development (LDRD), the Environment Biomarker Initiative program. The research was performed in the Environmental Molecular Sciences Laboratory (a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research) located at PNNL, and operated for DOE by Battelle under Contract DE-AC05-76RL01830. The authors are very grateful to Dr. Robert W. Heck for his assistance with the preparing of samples.

## 6.0 References

- [1] Sinha G. Trying to catch troublemakers with a metabolic profile. *Science* **310**; 965-966 (2005).
- [2] Lindon JC, Holmes E, Nicholson JK. So what's the deal with metabonomics? Metabonomics measures the fingerprint of biochemical perturbations caused by disease, drugs, and toxins *Anal. Chem.* **75**; 384A-391A (2003).
- [3] Reo NV. NMR-based metabolomics. *Drug and Chemical Toxicol.* **25**; 375-382 (2002).
- [4] Bollard ME, Stanley EG, Lindon JC, Nicholson JK and Holmes E. NMR-based metabonomic approaches for evaluating physiological influences on biofluid composition. *NMR in Biomedicine* **18**; 143-162 (2005).
- [5] Nicholson JK, Wilson ID. High resolution  $^1\text{H}$  NMR spectroscopy of biological fluids. *Prog. NMR Spectrosc.* **21**; 449-501(1989).
- [6] Hu JF, Garo E, Yoo H, Cremin PA, Zeng L, Georing MG, O'Neil-Johnson M, Eldridge GR. Application of capillary-scale NMR for the structure determination of phytochemicals. *Phytochem. Anal.* **16**; 127-133(2005).
- [7] Keifer PA, Baltusis L, Rice DM, Tymiak AA, Shoolery JN. A comparison of NMR spectra obtained for solid-phase-synthesis resins using conventional high-resolution, magic-angle-spinning, and high-resolution magic angle spinning probes. *J. Magn. Reson.* **A119**; 65-75 (1996).
- [8] Manzi, A, Salimath PV, Spiro RC, Keifer PA and Freeze HH. Identification of a novel glycosaminoglycan core-like molecule I. *J. Biol. Chem.* **270**; 9154-9163 (1995).
- [9] Chauret DC, Durst T, Arnason JT. Novel steroids from *Trichilia hirta* as identified by nonprobe INADEQUATE 2D-NMR spectroscopy. *Tetrahedron Letters* **37**(44);7875-7878(1996).
- [10] Li JY, Harper JK, Grant DM, Tombe BO, Bashyal B, Hess WM, Strobel GA. Ambuic acid, a highly functionalized cyclohexenone with antifungal activity from *Pestalotiopsis* spp. And *Monochaetia* sp. *Phytochemistry* **56**; 463-468(2001).
- [11] Antzutkin ON, Shekar SC, and Levitt MH. Two-dimensional sideband separation in magic-angle-spinning NMR. *J. Magn. Reson.* **A115**; 7-19 (1995).
- [12] Wind RA, Hu JZ, Rommereim DN. High resolution  $^1\text{H}$  NMR spectroscopy in organs and tissues using slow magic angle spinning. *Magn. Reson. Med.* **46**; 213-218 (2001).

- [13] Hu JZ, Wind RA, McLean J, Gorby YA, Resch CT, and Fredrickson JK, High Resolution  $^1\text{H}$  NMR Spectroscopy of Metabolically Active Microorganisms Using Non-Destructive Magic Angle Spinning, *Spectroscopy* **19**; 98-102 (2004).
- [14] Wind RA, Bertram HC, and Hu JZ, Slow-MAS NMR methods to study metabolic processes in vivo and in vitro, *Advances of NMR in Food Science* (Engelsen SB, Belton PS, and Jakobsen HJ, eds.) Royal Society of Chemistry, Cambridge, 156-165 (2005).
- [15] Waters NJ, Holmes E, Williams A, Waterfield CJ, Farrant RD, and Nicholson JK. NMR and pattern recognition studies on the time-related metabolic effects of  $\alpha$ -naphthylisothiocyanate on liver, urine, and plasma in the rat: An integrative metabolomic approach. *Chem. Res. Toxicol.* **14**, 1401-1412 (2001).
- [16] Morris GA, Freeman R. Selective excitation in Fourier transform nuclear magnetic resonance. *J. Magn. Reson.* **29**; 433-462 (1978).
- [17] Hoult DI and Richards RE, The signal-to-noise ratio of nuclear magnetic resonance experiment, *J. Magn. Reson.* **24**; 71-85 (1976).
- [18] Abragan A, *The principle of nuclear magnetism*, Clarendon Press, Oxford, pp.82-83 (1961).