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Comparison of metabolic profiling of *Daphnia magna* between HR-MAS NMR and solution NMR techniques

Seonghye Kim, Sujin Lee, Wonho Lee, Yujin Lee, Juyoung Choi, Hani Lee, Youzhen Li, Seulbin Ha, Suhkmann Kim*

Department of Chemistry and Chemistry Institute for Functional Materials,
Pusan National University, Busan 46241, Republic of Korea

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Abstract *Daphnia magna* is used as target organism for environmental metabolomics. The metabolome of *D. magna* was studied with NMR spectroscopy. Most studies used the extract of *D. magna*, but the reproducibility cannot be obtained using extracted sample. In this study, lyophilized *D. magna* samples were analyzed with two different ^{1}H NMR techniques, HR-MAS on intact tissues and solution NMR on extracted tissues. Samples were measured three times using 600 MHz NMR spectrometer. Metabolite extraction required more than twice as many *D. magna*, but the metabolite intensity was lower in solution NMR. In the spectra of HR-MAS NMR, the lipid signal was observed, but they did not interfere with metabolite profiling. We also confirmed the effect of swelling time on signal intensities of metabolites in HR-MAS NMR, and the results suggest that appropriate swelling should be used in lyophilized *D. magna* to improve the accuracy of metabolite profiles.

Keywords *Daphnia magna*, Metabolomics, Nuclear magnetic resonance (NMR), High resolution magic angle spinning (HR-MAS)

Introduction

Metabolomics which analyzes metabolite profiles altered by internal or external factors has emering as an efficient method to understand environmental or toxic stress on organisms. Metabolites, the end product in the central dogma, are affected even at low concentrations that do not cause mortality or morphological disturbances. Therfore, metabolomics can provide insights into how effect of toxicity at sub-lethal levels.

Daphnia magna (water flea) are organism most widely used to evaluate aquatic toxicity or water quality.[1] *D. magna* are sensitive to enviromental stress and have short reproductive cycle. In addition, *D. magna* have advantages of low cost, ease to maintain in a controlled laboratory setting.

Most studies were conducted to analyze the metabolites of *D. magna* using nuclear magnetic resonance (NMR) spectroscopy.[2-4] NMR is a widely utilized tool for detecting metabolites in metabolomics because of its high reproducibility, easy preprocessing and fast measuring time. Almost metabolomics studies with *D. magna* used solution ^{1}H NMR and it generally requires metabolite extraction. More metabolites can be identified in the extracted sample, but chemical extraction of molecules is time consuming and have potential problems such as experimental errors and losses during the extraction process.

* Address correspondence to: **Suhkmann Kim**, Department of Chemistry and Chemistry Institute for Functional Materials, Pusan National University, Busan 46241, Republic of Korea, Tel: 82-51-510-2240; Fax: 82-51-516-7421; E-mail: suhkmann@pusan.ac.kr

Using high resolution magic angle spinning (HR-MAS) NMR, the intact tissue can be measured non-destructively and intactly without extraction.[5] This technique allows intact sample to be recognized like solution state by rotating the sample rapidly (typically 2000 Hz) at the magic angle (about 54.71 degree) and this reduces the line-broadening effects of inhomogeneous samples.

In this study, metabolic profiles of *D. magna* were performed using ^{1}H HR-MAS NMR and ^{1}H solution NMR, and the differences between the two NMR techniques were compared.

Experimental Methods

Culturing of Daphnia magna- *D. magna* were obtained from Gwangju Institute of Science and Technology (GIST, Gwangju, Korea) in 2015 and they have been subcultured continuously in laboratory. *D. magna* were maintained in container filled with 4 L culture media. Room temperature was kept at 20 ± 2 ℃ and light/dark period was set at a ratio of 16/8-h with a light intensity of approximately 800 lx.

Adult daphnia, the 21 th day of birth, were used in this study. For NMR analysis, daphnids were rinsed three times with distilled water and flash-frozen in liquid N_2 , and then lyophilizated for 24 hours.

Sample preparation for HR-MAS ^{1}H NMR analysis- For HR-MAS NMR measurements, one sample included fifteen daphnids with three replicates. Each *D. magna* was directly inserted in a nano tube (Agilent Sample Tube, 4 mm) with phosphate buffer (pH 7.4) in deuterated water (D_2O) containing 2 mM sodium 3-trimethylshily-2,2,3,3-d_4-propionate (TSP-d_4).

HR-MAS ^{1}H NMR analysis- Samples were analysed with a 600 MHz HR-MAS NMR spectrometer with a nano-NMR probe (Agilent Technologies, Santa Clara, CA, USA). The spinning rate was 2050 Hz with a degree of magic-angle (54.74 °) at a temperature of 25 ℃ (298 K). For excluding the macromolecule and water peaks, the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was applied. Spectra were acquired under conditions of 5.95 μs 90° pulse, 1.5 s relaxation delay and 3 s acquisition time with 128 total transients. Total acquisition time was 13 min 9 s.

Sample preparation for ^{1}H NMR analysis- Polar *D. magna* metabolites were extracted for ^{1}H NMR analysis. Thirty *D. magna* were used for each sample with three replicates. This extraction method is a modification of Bligh and Dyer's method using methanol and chloroform.[6] The polar layer supernatant of each sample was transferred to vial and lyophilized. After lyophilization, completely dried samples were dissolved with 700 mL phosphate buffer (D_2O, pH 7.4) containing 2 mM TSP-d_4 and transferred to 5 mm NMR tubes.

^{1}H NMR analysis- All extracts of *D. magna* were measured using 600 MHz Agilent NMR spectrometer with a solution probe. The CPMG pulse was applied and spectra were acquired using an 9.8 μs 90° pulse 1.5 s relaxation delay, 3 s acquisition time, and 13 min total acquisition time. A total of 128 scans were acquired for each sample at a spectral width of 24,038.5 Hz.

NMR data analysis- The acquired spectra were phased, and the baseline was corrected and referenced to the TSP-d_4 peak (chemical shift of 0 ppm) using VnmrJ 4.2 software (Aligent Technologies, Santa Clara, CA, USA). Identification and quantification of metabolites were conducted using the Chenomx NMR Suite 8.4 professional (Chenomx Inc., Edmonton, Canada) based on a 600 MHz NMR library database. The reference compound for quantification was 2 mM of TSP-d_4, and the concentrations of metabolites were calculated from the TSP peak integral.

Results and Discussion

Comparison of NMR spectra- To compare the

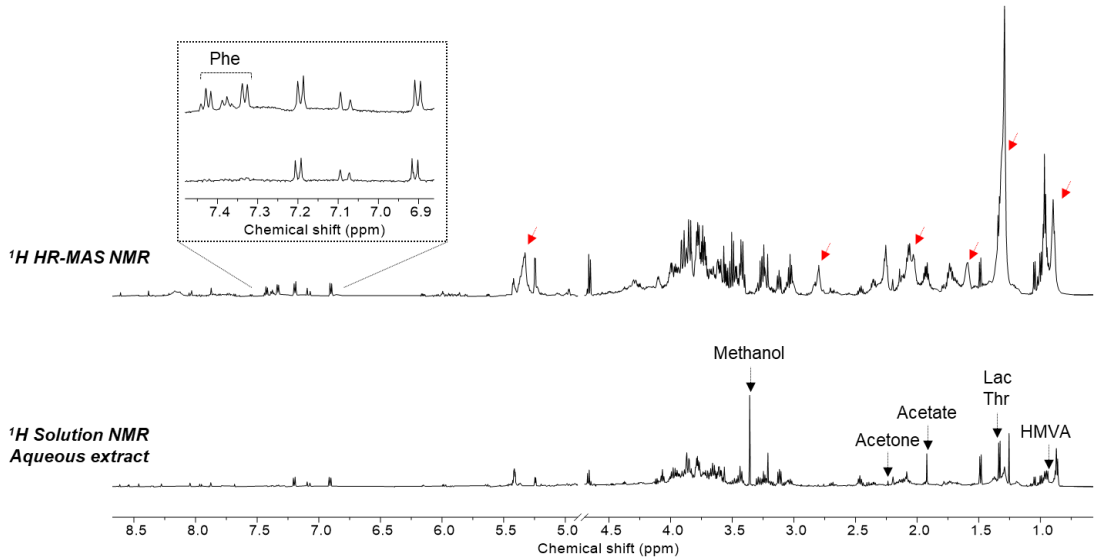

Figure 1. A representative ^{1}H NMR spectra of *D. magna* obtained using two different techniques, HR-MAS NMR and solution NMR spectroscopy. Lac, Lactate; Thr, Threonine; HMVA, 2-Hydroxy-3-methylvalerate; Phe, Phenylalanine. The red arrows indicate lipid signals.

difference of metabolic profiles between two different ^{1}H NMR techniques, HR-MAS and solution NMR, a total of 135 *D. magna* were analyzed. The 45 *D. magna* were analyzed with HR-MAS as intact tissues and 90 *D. magna* were extracted and then analyzed by solution NMR. The both representative NMR spectra with 0.3 Hz line-broadening are shown in Figure 1.

In the case of HR-MAS NMR, spinning side bands were observed but they did not interfere with metabolite profiling. For solution NMR, a single peak of methanol used for extraction was observed in the spectra at 3.34 ppm. In addition, *D. magna* were used two times more in each sample for solution NMR than HR-MAS NMR, however, the signal intensities of metabolites in solution NMR were lower than HR-MAS NMR.

Since *D. magna* have contains the lipids for growth[7], the most apparent difference between two NMR techniques is a removal of lipid signal by extraction. The peaks of lipid molecules were detected only in HR-MAS NMR spectra. As lipids were removed by extraction, small amounts of some metabolites in the range of 0.8-1.5 ppm were observable in solution NMR spectra (Fig. 1).

Table 1. List of (A) metabolites identified in both techniques and metabolites identified only in (B) solution NMR and (C) HR-MAS NMR

	Identified Metabolites
(A) Both	1,3-Diaminopropane, AMP, Alanine, Arginine, Asparagine, Aspartate, Betaine, Choline, Citrate, Formate, Glucitol, Glucose, Glutamate, Glutamine, Glycine, Histidine, Isobutyrate, Isoleucine, Lactate, Leucine, Lysine, Maltose, Methionine, O-Phosphocholine, Phenylalanine, Proline, Putrescine, Succinate, Threonine, Tyrosine , UDP-glucose, Valine, sn-Glycero-3-phosphocholine
(B) Solution NMR	2-Hydroxy-3-methylvalerate (HMVA), Acetate, Acetone, Pyruvate
(C) HR-MAS NMR	Phenylalanine, Tryptophan

Comparison of identified metabolites-

Peak assignments of each metabolites were verified as reported in the literature.[6] HR-MAS NMR and solution NMR analysis identified 35 and 37 metabolites in *D. magna*, respectively. The metabolties indentified in HR-MAS NMR , except phenylalanine and tryptophan, were also identified in spectra of solution NMR (Table 1). The signals of phenylalanine and tryptophan showed in solution

NMR spectra but they were not quantifiable.

Acetate, acetone, pyruvate, and 2-hydroxy-3-methylvalerate (HMVA) were obviously detectable in solution NMR because the overlapped lipid signals were excluded (Fig. 1). Although these metabolites were not identified, most of the fundamental metabolites could be analyzed in HR-MAS NMR spectra. Therefore, we suggest that HR-MAS NMR is more cost- and time-effective considering the number of *D. magna* used for NMR analysis.

Optimization of swelling time for HR-MAS ^1H NMR-

The swelling time can affect on the spectral resolution and signal-to-noise ration in HR-MAS NMR. Because of the increase of the molecular mobility within the heterogeneous matrix, the more samples are swollen, the greater the signal intensity.[8]

The *D. magna* samples for HR-MAS NMR were lyophilizated in this study, therefore, the sufficient sample swelling is needed. The D_2O buffer was used as a swelling solvent.

The first spectrum was obtained 20 minutes after adding D_2O buffer to the sample. As expected, HR-MAS NMR spectra obtained from soaked samples for 30 minutes and 1 hour showed higher spectral resolution and signal-to-noise ratio than the first spectrum. Areas under most metabolites signals were increased, especially arginine (3.2 ppm) and methionine (2.1 ppm) increased more than three times in the soaked sample. The arginine signal (3.2 ppm) was not observed due to the low resolution in the first spectrum, but was clearly observed after swelling. The peak of arginine is highlighted in Figure 2E. In addition, it was confirmed the change of lipid signals induced by sample swelling. We identified peaks from lipid molecules according to the literature[4] (Fig. 2G-I). Interestingly, the lipid peak was not significantly affected by swelling. The lactate signal overlapped with lipid region can be more observable after swelling (Fig. 2H).

Although we did not observe changes after swelling more than 1 h, the results demonstrated that a swelling time of 30 minutes had a significant effect on the NMR spectra. Comparing the spectra of lyophilized *D. magna* samples 30 minutes and 1 hour soaked, the spectra resolution and peak intensities were comparable.

Therefore, we suggest that sufficient swelling in lyophilized *D. magna* samples should be processed for a more accurate metabolic profile, and at least 30 minutes of swelling time will be required.

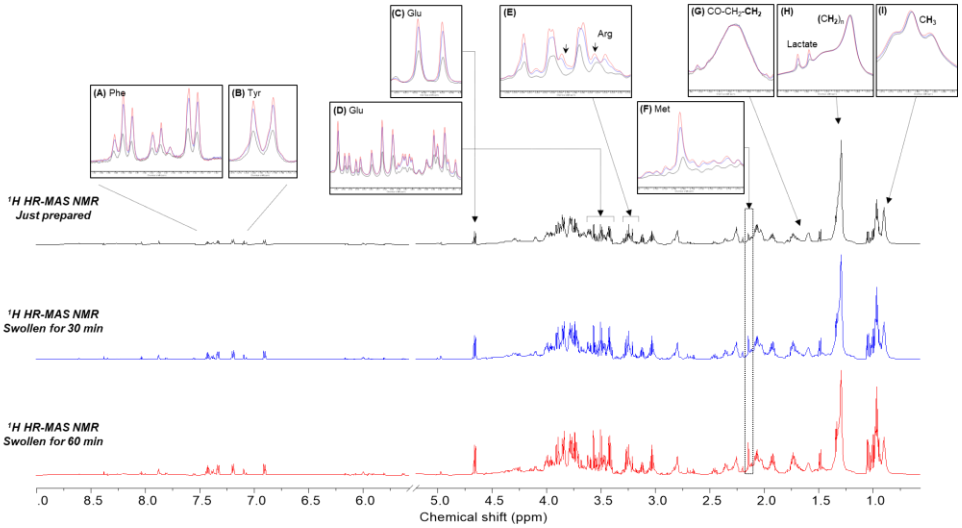

Figure 2. ^1H HR-MAS NMR spectra of lyophilized *D. magna* with different swelling times. The spectra were processed using Mnova 10.0. The colors (black, blue, and red lines) of spectra represents the swelling times. The peak of major metabolites (aromatic metabolites (A, B) , glucose (C, D), amino acids (E, F) and lipids (G, H and I)) are depicted in the figure. The arrows highlight the influence of the swelling time (E).

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