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Evaluation of a Semipolar Solvent System as a Step toward Heteronuclear Multidimensional NMR-Based Metabolomics for ^{13}C -Labeled Bacteria, Plants, and Animals

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 Supporting Information

ABSTRACT: Nuclear magnetic resonance (NMR) has become a key technology in metabolomics, with the use of stable isotope labeling and advanced heteronuclear multidimensional NMR techniques. In this paper, we focus on the evaluation of extraction solvents to improve NMR-based methodologies for metabolomics. Line broadening is a serious barrier to detecting signals and the annotation of metabolites using multidimensional NMR. We evaluated a series of NMR solvents for easy and versatile single-step extraction using the ^{13}C -labeled photosynthetic bacterium *Rhodobacter sphaeroides*, which shows pronounced broadening of NMR signals. The performance of each extraction solvent was judged using 2D ^1H – ^{13}C heteronuclear single quantum coherence (HSQC) spectra, considering three metrics: (1) distribution of the line width at half height, (2) number of observed signals, and (3) the total observed signal intensity. Considering the total rank values for the three metrics, we chose methanol- d_4 (MeOD) as a semipolar extraction solvent that can sufficiently sharpen the line width and affords better-quality NMR spectra. We also evaluated the series of extraction solvents by means of inductively coupled plasma optical emission spectroscopy (ICP-OES) based ionomics approach. It was also indicated that MeOD is useful for excluding paramagnetic ions as well as macromolecules in an easy single-step extraction. MeOD extraction also appeared to be effective for other bacterial and animal samples. An additional advantage of this semipolar solvent is that it supplements the aqueous (polar) buffer system reported by many groups. The flexible, appropriate application of polar and semipolar extraction should contribute to the large-scale analysis of metabolites.

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful and widely used techniques for determining structure and analyzing the environment and physical properties of molecules. As a method for metabolomic screening, high-throughput one-dimensional (1-D) ^1H NMR, combined with multivariate statistics, is well established.^{1–4} Overlapping signals have been overcome by homonuclear and heteronuclear two-dimensional (2-D) NMR experiments, which are particularly useful for signal isolation and the unambiguous assignment of metabolites.^{5–10} Furthermore, the combination of heteronuclear NMR with stable isotope labeling is one of the most powerful tools in metabolomics.^{11–15} We have been developing a methodology for “multidimensional” NMR-based metabolomics with (occasionally uniform) stable isotope labeling of plants,^{16–20} bacteria^{21,22} and animals.^{23,24} In addition to advantages in terms of sensitivity and monitoring metabolic flux, stable isotope labeling allows the application of three-dimensional (3-D) pulse sequences to increase the reliability of metabolite annotation.²⁴ Several powerful schemes rely on transferring ^{13}C – ^{13}C magnetization in several steps via J couplings.²⁵ However, signal detection in “multidimensional” NMR is sometimes hampered by detrimental line-broadening effects, such as, the coexistence of paramagnetic

ions and/or macromolecules. The extent of the line-broadening effect seems to depend on the origin of the biological samples, although sometimes we need to perform comparative analyses across a variety of organisms without biases due to the quality of spectra. Such effects are dependent on the sample properties, and it has been suggested that the efficiency of magnetization transfer was decreased by short T_2 relaxation values.^{7,8,10} Organisms of interest for metabolomic analysis are becoming more diverse across multiple fields, such as environmental science,^{1,26,27} health care science,^{28,29} and the industrial application of bacterial fermentation.^{30,31} Furthermore, the target of analysis is not limited to one species; even interactions between organisms have been targeted. This necessitates an extraction solvent that results in better-quality spectra, regardless of the target organism.

Extraction methods for NMR-based metabolomic analyses have been examined thoroughly in many studies using various organisms.^{2,4,32–36} Recently, we reported polar and semipolar metabolite profiles of both the solvent extract and extraction

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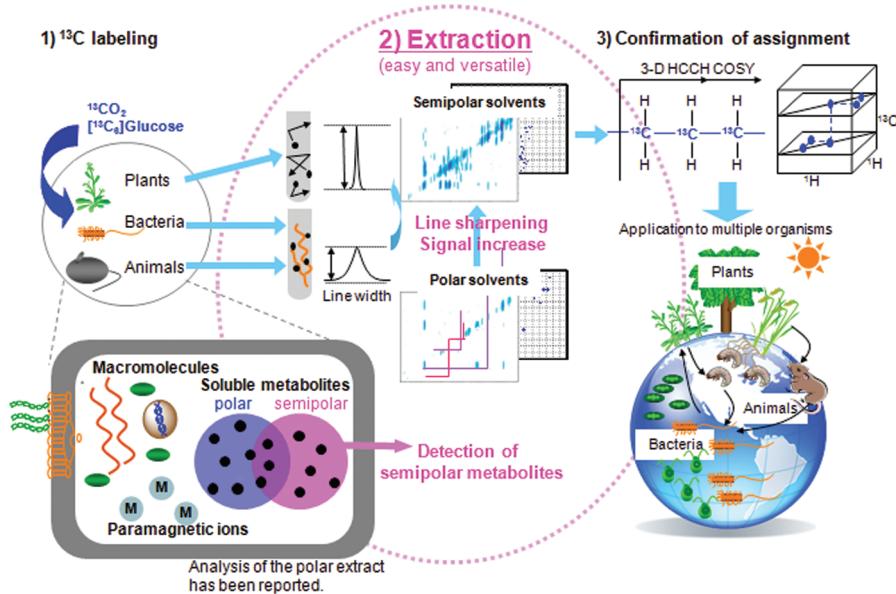


Figure 1. Concept and strategies for our technological advances. (1) ^{13}C labeling to increase the efficiency at detecting signals and the use of multidimensional pulse sequences. (2) Evaluation of extraction solvents to avoid line broadening, which is frequently observed in animal tissue and bacterial samples, and to afford better-quality spectra for assignment. Such extraction solvents are required for comparative analyses across a wide variety of organisms. (3) Peak assignments using 3-D NMR techniques, such as HCCH–COSY. In this work, we examined the second topic, extraction solvents (highlighted in the dotted pink circle). An additional advantage of the selected semipolar solvent (methanol- d_4), which improved NMR spectra regardless of the sample, is that it reinforced the metabolite detection system, in combination with the aqueous (polar) system.

residues of ^{13}C -labeled *Arabidopsis thaliana* during repeated extraction processes.³⁷ Furthermore, we developed a mathematical score, the SpinAssign *p*-value, for annotating metabolites in polar extracts of ^{13}C -labeled plant cells.³⁸ With these developments in mind, we are planning to extend the detectable NMR signals and variety of metabolite diversity. The concept and strategies for our technological advances are summarized in Figure 1: ^{13}C labeling to increase the efficiency of detecting signals and the use of multidimensional pulse sequences, the evaluation of extraction solvents to avoid line broadening and afford better-quality spectra for assignment, and peak assignments using 3-D NMR techniques, such as HCCH–COSY. If this strategy is applicable to a wide range of organisms, comparative analysis becomes possible between various combinations of organisms.

In this study, we focused on the second topic, extraction solvents. Using the ^{13}C -labeled photosynthetic bacterium *Rhodobacter sphaeroides*, we found that deuterated methanol (MeOD) was a suitable semipolar solvent for sharpening line width and detecting more metabolites in ^1H – ^{13}C heteronuclear single quantum coherence (HSQC) spectra^{6,39} in an easy single-step extraction.

■ EXPERIMENTAL SECTION

Chemicals. Methanol- d_4 (MeOD, 99.8% D), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)- d_{18} (98% D), and [$^{13}\text{C}_6$]glucose (>98% ^{13}C) were purchased from Cambridge Isotope Laboratories (Andover, MA), and deuterium oxide (99% D) was obtained from Sigma-Aldrich (St. Louis, MO). The ^{13}C , ^{15}N -labeled algal amino acid mixture (97.4% ^{13}C , 97.9% ^{15}N) was purchased from Chlorella Industry (Tokyo, Japan). The gas mixture containing 31% O_2 , 79% N_2 , and the desired $^{13}\text{CO}_2$ concentration (345–350 ppm by volume) was acquired from Takachiho Chemical Industrial (Tokyo, Japan). The other

chemicals used in this study are listed in Supporting Information Table S-1.

Preparation of NMR Samples . General Procedure. Each labeled sample was collected and used to produce a stock powder or paste following a previously reported procedure.³⁷ Each sample was stored at -80°C until extraction.

Stable Isotope Labeling of Organisms

Arabidopsis thaliana (ecotype Columbia 0). *A. thaliana* (ecotype Columbia 0) was germinated and grown on half-strength Murashige and Skoog agar⁴⁰ [pH 5.7, 0.8% agar (w/v)] supplemented with [$^{13}\text{C}_6$]glucose (1.0% w/v) at 22°C in the dark. Each Petri dish contained 30 mL of medium, and 32 seeds were put on each plate. The sprouting plants were collected on day 50, and the medium was washed away with sterilized water. The washed plants were lyophilized and ground into powder, 10 mg of which was used for extraction.

Poplar. A young branch of hybrid aspen (*Populus tremula* L. \times *tremuloides* Michx.)⁴¹ was grown on half-strength Murashige and Skoog agar [pH 5.6, 0.27% agar (w/v)] supplemented with [$^{13}\text{C}_6$]glucose (0.5% w/v) at 22°C under a 16-h light/8-h dark cycle at a light intensity of $90 \mu\text{mol}/\text{m}^2 \cdot \text{s}$ for 2 months. Leaves of the labeled poplar were lyophilized and ground into powder, 10 mg of which was used for extraction.

Escherichia coli O157. The rifampicin-resistant mutant strain *E. coli* O157:H7 strain 44^{RF} (O157)⁴² was originally isolated from bovine feces and maintained as a 25% glycerol stock. The glycerol stock (4 mL) was transferred into 800 mL of modified M9 medium⁴³ (pH 7.4) containing Na_2HPO_4 (12.8 g/L), KH_2PO_4 (3.0 g/L), NaCl (0.5 g/L), MgSO_4 (2.4 g/L), CaCl_2 (0.011 g/L), FeCl_3 (3.5 mg/L), tyramine (2.4 mg/L), biotin (1.2 mg/L), [^{15}N]ammonium chloride (1.5 g/L), and [$^{13}\text{C}_6$]glucose (0.2%). The inoculated culture was incubated at 37°C in the dark for 24 h on a rotary shaker at 140 rpm. The culture was centrifuged (9400g, 5 min), and the pellet was washed repeatedly with

50 mM potassium phosphate buffer (pH 7.0). The combined bacterial cake was lyophilized, and the freeze-dried bacterial cake was ground into powder, 10 mg of which was used for extraction.

Rhodobacter sphaeroides. *R. sphaeroides* f. sp. *denitrificans* IL 106 was labeled according to a reported procedure,⁴⁴ with slight modification. The organism was precultured under semi-aerobic/dark conditions at 30 °C in M22+ medium⁴⁵ for 3 days. Then, the seed culture (100 mL) was transferred into 1 L of modified M22+ medium supplemented with [¹³C₆]glucose (0.5 g/L) and [¹³C₂]sodium acetate (2.0 g/L) instead of sodium lactate, succinic acid, glutamic acid, aspartic acid, and casamino acid. The culture was incubated for 30 days under the same conditions. The fermentation broth (1 L) was centrifuged (2260g, 10 min), and the pellet was washed repeatedly with 50 mM potassium phosphate buffer (pH 7.0). The combined bacterial cake was lyophilized and the freeze-dried, bacterial cake was ground into powder, 10 mg of which was used for the extraction experiments. Nonlabeled *R. sphaeroides* was prepared by the same procedure using M22+ medium without ¹³C-glucose and acetate.

Bombyx mori. Silkworm (*B. mori*) eggs were purchased from Ehime Sanshu (Ehime, Japan), and the larvae were reared on an artificial diet, Silkmate 2S (Nihon Nosen Kogyo, Yokohama, Japan), at 26 °C under a 10-h light/14-h dark cycle. The fourth instar larvae were reared on the artificial diet supplemented with an aqueous solution (200 μL/1 g Silkmate 2S) containing 2% (w/w) [¹³C₆]glucose and 1% (w/w) [¹³C, ¹⁵N]amino acid mixture for 12 days under the same conditions. The larvae molted to the fifth instars during the labeling experiment. Hemolymph was collected from a wounded proleg, and the residual body was lyophilized. The head and silk gland were removed from the freeze-dried body, and the body wall containing skin and associated muscles was ground to afford a paste material, 25 mg of which was used for extraction.

Mus musculus (female BALB/cAJcl). Female BALB/cAJcl mice were purchased from CLEA Japan (Tokyo, Japan) and used for labeling experiments. During the labeling experiments, the mice were raised in a metabolism cage (CM10S; CLEA Japan). [¹³C₆]Glucose was dissolved in tap water (0.7% w/v) and autoclaved. The mice (22–23 weeks old) were fed the sterilized glucose solution with mouse diet CA1 (CLEA Japan) for 1 week. Typically, a mouse drank 5–6 mL of glucose solution per day. The isolated liver was washed with phosphate-buffered saline (PBS) and lyophilized. The freeze-dried liver was ground into powder, 30 mg of which was used for extraction.

Extraction. The powder or paste (in the case of *B. mori*) of each labeled organism was suspended in 500 μL of each solvent. The mixture was heated at 50 °C for 5 min in a Thermomixer comfort (Eppendorf AG, Hamburg, Germany) and then centrifuged (9060g, 5 min). The supernatant was used for the NMR experiments. The solvents used in the extraction experiments are listed in Supporting Information Table S-2. The reproducibility and stability of the metabolites with this extraction method have been assessed previously.³⁷ For example, the ratio of the standard deviation to the average of observed signals and total intensity were 0.002 and 0.02 in MeOD, and 0.05 and 0.04 in D₂O. The errors in the signal intensity of each metabolite were also reported in the previous paper.³⁷ In this study, we discuss a rough tendency beyond the range of error. It was also found that the metabolites in the MeOD and D₂O extracts were stable over the duration of the experiment.³⁷

NMR Analyses . Solution NMR. Sample solutions were transferred into 5-mm φ NMR tubes. NMR spectra were recorded on an

Avance-700 spectrometer (Bruker, Billerica, MA), equipped with an inverse triple resonance CryoProbe with a Z-axis gradient for 5-mm sample diameters operating at 700.153 MHz for ¹H and 176.061 MHz for ¹³C or an Avance-500 spectrometer (Bruker), equipped with an inverse probe with a triple axis gradient, operating at 500.132 MHz for ¹H and 125.764 MHz for ¹³C. The temperature of all NMR samples was maintained at 298 K. The chemical shifts were referenced to the methyl group of the sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) internal standard.

Two-Dimensional ¹H–¹³C HSQC Spectroscopy. Two-dimensional ¹H–¹³C HSQC spectra were collected using echo/antiecho gradient selection (the hsqcetgp pulse program in the Bruker library). The proton 90° pulse value was checked and set for each sample. In all cases, the carbon 90° pulse was 15.0 μs long. Relaxation delay values of 1.2 s were used and 128 complex f1 (¹³C) and 1024 complex f2 (¹H) points were recorded with eight scans per f1 increment. The spectral window and offset frequency in the f1 dimension were 40 ppm and 68 ppm, respectively. The spectral window in the f2 dimension was 16 ppm. The offset frequency in the f2 dimension was set to each residual solvent signal. ¹³C-Decoupling during acquisition was performed using GARP sequence (pulse length 75 μs).⁴⁶ To normalize the signal intensities in the ¹H–¹³C HSQC spectra, they were standardized against the noise level of each spectrum. To reduce total signal values for ease of subsequent calculations, these signal-to-noise values were divided by 1000 (SN × 1/1000).

Three-Dimensional HCCH–COSY Spectroscopy. Three-dimensional HCCH–COSY spectra were acquired using the States-TPPI method (the hcchcog3d pulse program in the Bruker library). The proton and carbon 90° pulse values were set to 10 and 15.0 μs, respectively. Relaxation delay values of 1.2 s were used and 2048 complex f1 (¹H), 96 complex f2 (¹³C), and 100 complex f3 (¹H) points were recorded with 16 scans per increment. The spectral window in the f1 and f3 dimensions was 16 ppm. The offset frequency in the f1 and f3 dimensions was 3.3 ppm (MeOD/HEPES-d₁₈) and 4.8 ppm (hexafluoroacetone (HFA)/HEPES-d₁₈), respectively. The spectral window and offset frequency in the f2 dimension were 75 ppm and 80 ppm, respectively.

Data Processing. The spectra were processed with NMRPipe and analyzed using NMRDraw.⁴⁷ All of the ¹H–¹³C HSQC spectra were Fourier-transformed with the Lorentzian-to-Gaussian window function (10 Hz Lorentzian line width and 15 Hz Gaussian line width for the f1 dimension; 5 Hz Lorentzian line width and 10 Hz Gaussian line width for the f2 dimension). The data were zero-filled to 1024 (f1) and 4096 (f2) data points. Subsequently, automatic polynomial baseline correction was applied in both dimensions, and linear prediction to 1024 points was applied to the f1 dimension. The 3-D HCCH–COSY spectrum was Fourier-transformed with the Lorentzian-to-Gaussian window function (7 Hz Lorentzian line width and 5.8 Hz Gaussian line width for the f1 and f3 dimensions; 17.6 Hz Lorentzian line width and 14.7 Hz Gaussian line width for the f2 dimension). The data were zero-filled to 256 points in f2 and f3 dimensions. Automatic polynomial baseline correction was subsequently applied in all dimensions, and linear prediction to 2048 and 96 points was applied to the f1 and f2 dimensions, respectively.

Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) Analysis. All test tubes, pipet tips, and syringes employed were polypropylene. The powder (40 mg) of non-labeled *R. sphaeroides* was suspended in 1.5 mL of each solvent. For ICP-OES analysis, corresponding nondeuterated solvents were used for extraction. The mixture was heated at 50 °C for 5 min in a Thermomixer comfort and then centrifuged (9060g, 5 min).

The supernatants, except for the solvents corresponding to 3, 6, and 9, which contained DMSO, were concentrated to dryness in a centrifugal evaporator (EYELA CVE-3100 with a cold-trap UNITRAP UT2000, Tokyo, Japan). The extracts corresponding to the solvent 3, 6, and 9 were lyophilized to give a dried residue. The residue was suspended in 10 mL of aqueous nitric acid (6.9% v/v), and the insoluble materials were removed by centrifugation (5800g, 3 min). The supernatant was filtered through a Millex GS filter (0.22 μ m, Millipore, Billerica, MA), and the filtrate was used for ICP-OES analysis. ICP-OES analysis was performed with SPS5520 (SII NanoTechnology, Chiba, Japan). The ICP-OES operating conditions were as follows: power 1.2 kW, nebulizer gas flow 15 L min⁻¹, and auxiliary gas flow 1.5 L min⁻¹. Concentration values were statistically corrected using Z score normalization to account for the large dynamic range of elemental concentrations.

Chemical Shift-Based Profiling of ^1H – ^{13}C HSQC spectra. Chemical shift-based profiling of ^1H – ^{13}C HSQC spectra using hierarchical clustering was performed as described previously,³⁷ with slight modification (Supporting Information Figure S-6).

Collection of Standard Chemical Shifts. Standard chemical shifts were collected using MeOD/HEPES- d_{18} buffer, which was prepared as described previously.³⁷ The concentration of each standard compound was less than 1 mM.

Preparation of HFA/HEPES- d_{18} Buffer. The HFA/HEPES- d_{18} buffer was prepared by mixing 90:10 (v/v) of HFA and 400 mM HEPES- d_{18} solution in D₂O (adjusted to pH 7.0 with 30% NaOD in D₂O) and DSS (final concentration 1 mM), affording HFA/HEPES (40 mM) solution.

Annotation of Signals. Candidate metabolites for each peak were selected from standard compounds by comparing the chemical shift difference. A compound was selected when the chemical shift difference between the standard and queried peak was less than 0.03 ppm and 0.53 ppm for ^1H and ^{13}C , respectively. The annotation was confirmed by 3-D HCCH–COSY analysis.

■ RESULTS AND DISCUSSION

Choice of Extraction Solvent for the Bacterial System. The effects of solvent and sample type on line broadening are significant (Figure 2). An overlay of the ^1H – ^{13}C HSQC NMR spectra of the trideuterated hexafluoroacetone (HFA)/HEPES- d_{18} extracts of ^{13}C -bacteria (*R. sphaeroides*), ^{13}C -plant (*P. tremula* L. × *tremuloides*), and ^{13}C -animal (*B. mori*) are shown in 2A, while 2B shows that of the MeOD/HEPES- d_{18} extracts. One-dimensional slices at δ_c 65 ppm are shown above the HSQC spectra. Given the strong tendency of hydrated fluoroketones and fluoroalcohols to form hydrogen bonds, these are considered as good solvents exhibiting a strong solubilization capacity.⁴⁸ However, the HFA extraction sometimes causes significant broadening of NMR signals and decreases detectable signals. In fact, when the HFA extract of *R. sphaeroides* was redissolved in MeOD after removing the HFA by evaporation, the ^1H – ^{13}C HSQC signals were sharpened and hindered metabolites could be detected (Supporting Information Figure S-1A,B). This indicates that although HFA can extract a large number of metabolites, signal detection was hampered by some line-broadening effects, such as coexistence of paramagnetic ions and/or macromolecules, which decreases the efficiency of magnetization transfer by short T_2 relaxation values. Comparison of 1-D ^1H and Carr–Purcell–Meiboom–Gill (CPMG) spectra⁴⁹ of the HFA extract suggested that the broad line widths are partially attributed to overlapping

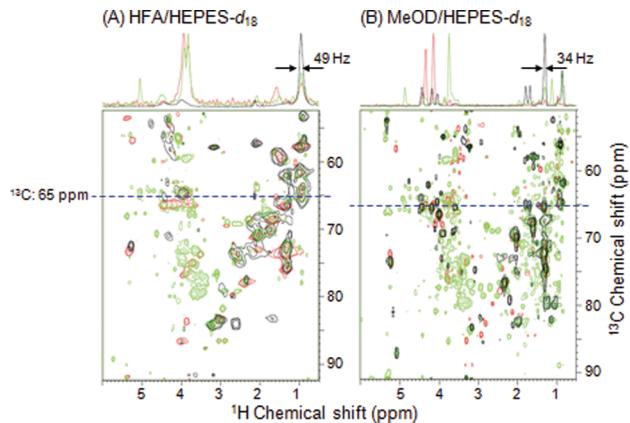


Figure 2. ^1H – ^{13}C HSQC spectra of ^{13}C -bacterium (*R. sphaeroides*, black), ^{13}C -plant (*P. tremula* L. × *tremuloides*, green), and ^{13}C -animal (*B. mori*, red) as examples of the effect of extraction solvent on line width and detectable signals. (A) ^1H – ^{13}C HSQC spectra of the HFA/HEPES- d_{18} extract (500 MHz at 298 K). (B) ^1H – ^{13}C HSQC spectra of the MeOD/HEPES- d_{18} extract (500 MHz at 298 K). One-dimensional slices at δ_c 65 ppm are shown above the HSQC spectra with the line width at half height of the largest peak of *R. sphaeroides*. The spectral window in the f2 dimension was 16 ppm. The offset frequency in the f2 dimension was 3.3 ppm for the MeOD/HEPES- d_{18} extracts and 4.8 ppm for the HFA/HEPES- d_{18} extracts. The spectral window and offset frequency in the f2 dimension were 40 ppm and 68 ppm, respectively.

with the signals of the molecules having short T_2 relaxation values (Supporting Information Figure S-1C). Such line broadening seemed to be larger in animal tissues and bacterial samples. Because a future goal of metabolomics is to perform comparative analyses across a variety of organisms, a suitable extraction solvent is required to afford constant quality of NMR spectra, regardless of sample type.

Thus, we evaluated a series of NMR solvents using ^{13}C -*R. sphaeroides*, which showed the most marked line broadening with HFA extraction, based on three metrics: (1) the distribution of the full-width-at-half-maximum (fwhm) of signals observed in ^1H – ^{13}C HSQC spectra (Figure 3A); (2) the number of observed signals; (3) the total observed signal intensity (Figure 3B). Detailed values are summarized in Supporting Information Table S-2. Semipolar organic solvents, such as MeOD, acetone- d_6 , 2-propanol- d_8 , and acetonitrile- d_3 showed sharpening of the line width (~12 Hz narrower in ^1H line width than the HFA or HFA/HEPES- d_{18} extract). Furthermore, we evaluated the solubilization capacity of these solvents based on the number and total intensity of the ^1H – ^{13}C HSQC signals measured using both 700-MHz NMR with a cryogenic probe and 500-MHz NMR with a conventional probe (Figure 3B). While few differences in the distribution of line width were observed among the four semipolar solvents listed above, MeOD dissolved up to 2-fold more metabolites than the three other solvents. It was shown that the mixed chloroform solvents, which are commonly used, were also effective. Extractions based on various methanol/chloroform/water ratios are an efficient way to detect signals, and they are widely used for both NMR and GC-MS based metabolomic analysis. However, one of the goals of this work is to advance a single solvent step extraction method to make these types of analyses easier to perform. We ultimately chose MeOD as a candidate semipolar extraction solvent based on the total rank values for the three metrics (Supporting Information Table S-3). The 3-D HCCH–COSY

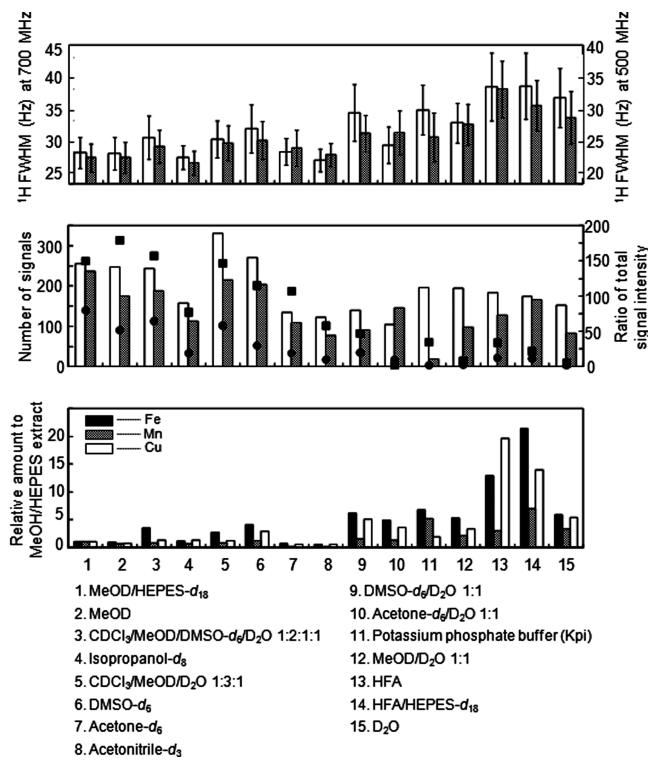


Figure 3. $^1\text{H}-^{13}\text{C}$ HSQC analysis of the extraction solvent for ^{13}C -R. *sphaerooides* (10 mg of the bacteria in 500 μL of NMR solvent). The solvent number corresponds to the rank of the solvents, based on the total rank values of the three metrics. (A) Distribution of the full-width-at-half-maximum (fwhm) of the $^1\text{H}-^{13}\text{C}$ HSQC signals of ^{13}C -R. *sphaerooides* in various solvents: average of the ^1H fwhm at 700 MHz with a cryogenic probe (open rectangles) and 500 MHz with a conventional probe (gray rectangles); standard deviation of the fwhm of all signals in a spectrum (bar). (B) Comparison of the number of signals and ratio of the total intensity of the $^1\text{H}-^{13}\text{C}$ HSQC spectra of ^{13}C -R. *sphaerooides* in various solvents: the number of signals observed at 700 MHz with the cryogenic probe (open rectangles); the number of signals observed at 500 MHz with the conventional probe (gray rectangles); the total intensity observed at 700 MHz with the cryogenic probe (solid squares); the total intensity observed at 500 MHz with the conventional probe (solid circles). (C) Relative amount of Fe (solid rectangles), Mn (gray rectangles), and Cu (open rectangles) to those in MeOD/HEPES- d_{18} extract, detected by ICP-OES measurements. For ICP-OES analysis, corresponding nondeuterated solvents were used for extraction.

spectrum of MeOD/HEPES- d_{18} extract was shown to afford several times as many signals as the HFA/HEPES- d_{18} extract (Supporting Information Figure S-2). To examine whether MeOD/HEPES- d_{18} extraction was applicable to other organisms, the quality of the $^1\text{H}-^{13}\text{C}$ HSQC spectra was compared between HFA/HEPES- d_{18} and MeOD/HEPES- d_{18} extracts of ^{13}C -labeled plant, bacterial, and animal samples. The line width was markedly sharper and the detectable signals were increased with MeOD/HEPES- d_{18} extraction (Supporting Information Figure S-3), especially the bacterial and animal samples. Compared with these semipolar organic solvents, polar aqueous solvents such as Kpi and mixtures containing D₂O seemed to suffer sensitivity loss in a 700-MHz NMR with a cryogenic probe. This may be attributable to the dielectric loss of ionic aqueous solutions, especially at higher magnetic fields.^{50,51} To take full advantage of recent and future improvements in NMR hardware

and techniques, such as high magnetic field strengths and cryogenically cooled probes, careful consideration of the sensitivity loss is essential in sample preparation.

Many factors could potentially influence the line widths observed in the various solvents, including macromolecules (e.g., proteins) and variation in soluble paramagnetic ions. As supplementary data, the protein content in these solvent extracts was roughly compared using the modified Lowry method⁵² (Supporting Information Figure S-4). The increase in line width seemed to partially correspond to the protein content in the extract. Although estimation of the protein content in the HFA and HFA/HEPES- d_{18} extracts failed, because it was difficult to redissolve the extract with the aqueous solution used in the protein assay, HFA is a solvent for solubilizing membrane proteins, such as human erythrocyte-membrane proteins,⁵³ and the light-harvesting antenna proteins of photosynthetic bacteria.⁵⁴ Conversely, it is also known that the semipolar solvents that produced sharpening of the line width are used for protein precipitation. Furthermore, we performed ultrafiltration of the MeOD and D₂O extracts, which are the examples showing sharp and broad line width. After ultrafiltration using 30 kDa molecular weight cutoff membrane, the broad signals could be removed from the ^1H and $^1\text{H}-^{13}\text{C}$ HSQC spectra of the D₂O extract while the spectra of MeOD were almost the same before and after the filtration. This indicates that D₂O extract contained more macromolecules than MeOD extract (Supporting Information Figure S-5). The low concentration of macromolecules in semipolar extracts may partially contribute to the increased quality of the spectra. We also carried out ICP-OES-based ionomics approach in the 15 extracts. The emission lines are listed in Tables S-4. The content of the major paramagnetic ions, Fe, Mn, and Cu, in each solvent are shown in Figure 3C. In addition, hierarchical cluster analysis (HCA) and principle component analysis (PCA) were carried out to show the distribution of the 20 trace elements exhibited over quantitative detection limits among 71 comprehensive elemental analyses (Figure 4). Paramagnetic ions were more distributed in HFA and water-containing solvent extracts, which showed broad signals, than in other organic solvent extracts. These results strongly supported that the observed line broadening could be significantly attributed to the paramagnetic ions. Note that other metals, alkaline metals, and nonmetal ions were little distributed in semipolar solvent systems, which can avoid electrostatic interaction between anionic metabolites and cationic ions in such low dielectric environments. Furthermore, maintaining such a low dielectric environment in semipolar solvents systems can contribute to an enhanced signal-to-noise ratio when used in the cryogenically cooled probe.⁵⁰

Chemical Shift-Based Profiling of $^1\text{H}-^{13}\text{C}$ HSQC Spectra To Evaluate the Relative Abundance of Aliphatic, Hydrophilic, and Aromatic Regions. Because the detectable metabolites are biased, depending on the polarity of the extraction solvent, the efficacy of extraction should be evaluated further based on the variation in signals. The relative abundance of aliphatic, hydrophilic, and aromatic regions was shown using a clustered heat map, based on the chemical shifts and peak intensities in the $^1\text{H}-^{13}\text{C}$ HSQC spectra (Figure 5). Acetone- d_6 , acetonitrile- d_3 , and 2-propanol- d_8 were rich in the aliphatic region, while the hydrophilic region was abundant in D₂O-containing solvents. Compared with these biased extractions, the signals in the MeOD, DMSO- d_6 , and mixed chloroform solvents seemed to be relatively balanced in terms of the three regions.

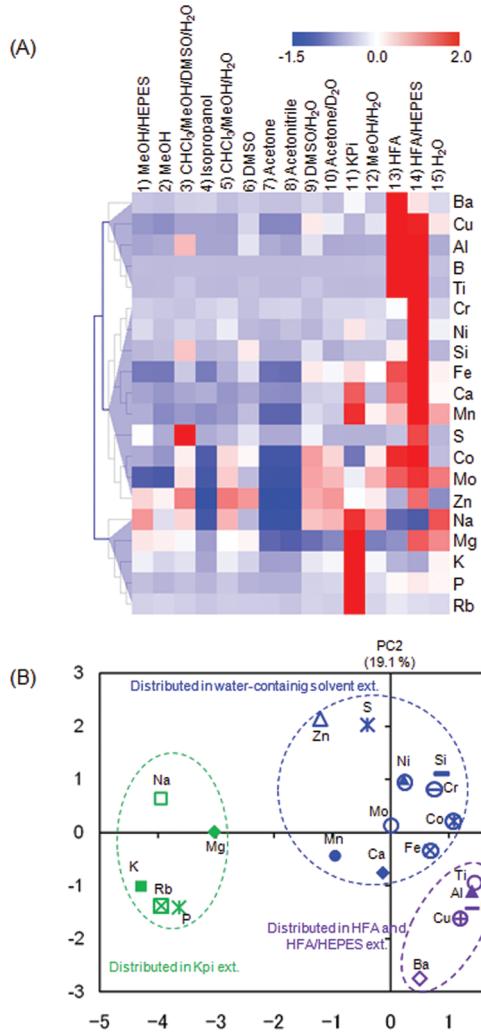


Figure 4. (A) Clustered heat map showing distribution of trace elements. The columns represent the different extraction solvents, and the rows represent the relative amount of each element. Concentration values were statistically corrected using Z score normalization to account for the large dynamic range of elemental concentrations. (B) PCA plot showing the relationships between normalized concentration of 20 trace elements in *R. sphaeroides* samples extracted using 15 solvents as measured by ICP-OES.

Determination of the ^1H and ^{13}C Tolerances for Matching ^1H – ^{13}C HSQC Signals with Standard Chemical Shifts in the Annotation Process. We have previously reported metabolite annotation methods using a polar buffer, 100 mM Kpi (pH = 7.0), in which the queried peaks in the ^1H – ^{13}C HSQC spectra were matched against a reference chemical shift database with tolerances for ^1H (0.03 ppm) and ^{13}C (0.53 ppm). As a next step, we examined the annotation using the selected semipolar solvent, MeOD. Examples of MeOD buffer (MeOD/90 mM KH_2PO_4 = 1:1,⁵⁵ MeOD/100 mM Kpi = 7:3,^{56,57} or MeOD/30 mM Na_2HPO_4 = 7:3⁵⁸) have been reported for the extraction of plants. Because low-conductivity buffers, such as HEPES buffer, have been reported to result in the highest possible sensitivity of cryogenic probes,⁵⁹ we examined buffer systems for MeOD using HEPES- d_{18} solution (pH 7.0). To test the effect of the HEPES buffer on chemical shift fluctuations, nonlabeled *R. sphaeroides* was added to reference compounds (^{13}C , ^{15}N -labeled proteinogenic 16-amino-acid mixture) in MeOD or MeOD/HEPES- d_{18}

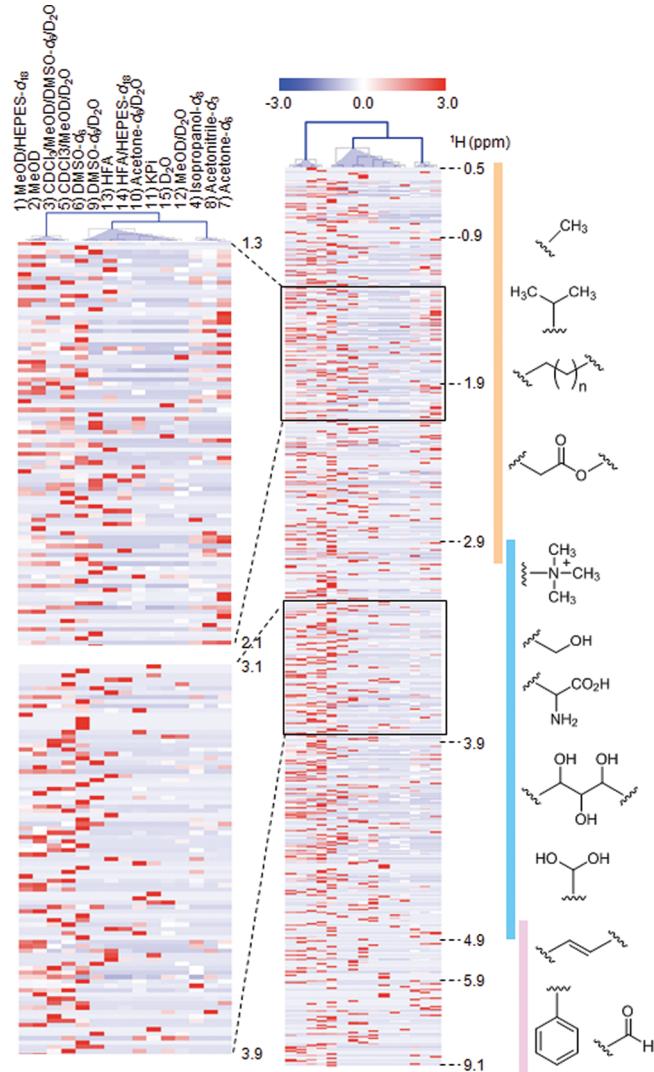


Figure 5. Clustered heat map showing signal variation in the ^1H – ^{13}C spectra of ^{13}C -*R. sphaeroides* in various extraction solvents. An expanded view of the aliphatic and hydrophilic regions is shown on the left. The columns represent different extraction solvents, and the rows represent binned chemical shift data. For each bin, the value used for heat map analysis is the sum of all peaks within that bin (Supporting Information Figure S-6). The solvent number corresponds to the rank of the solvents.

(10 mM) and the peak shifts in the ^1H – ^{13}C HSQC spectra were compared (Supporting Information Figure S-7, Table S-5, S-6). Although the buffer capacity was low in the MeOD/HEPES- d_{18} solution, the chemical shift fluctuations in both MeOD and MeOD/HEPES- d_{18} were small enough. When we collected standard chemical shifts at less than 1 mM in MeOD/HEPES- d_{18} , most of them could be used for matching with metabolites in both MeOD and MeOD/HEPES- d_{18} extracts using the same tolerances as with the polar Kpi system (0.03 ppm for ^1H and 0.53 ppm for ^{13}C).

Three-Dimensional HCCH–COSY Analysis for Unambiguous Assignments of ^{13}C -Metabolites. To verify the detectable metabolites in the semipolar MeOD system, we performed metabolite annotation using highly ^{13}C -labeled *A. thaliana* (ecotype Columbia 0). The labeled *A. thaliana* was prepared by feeding with [$^{13}\text{C}_6$]glucose in the dark to avoid dilution by $^{12}\text{CO}_2^{18}$ and to detect ^{13}C – ^{13}C connectivity efficiently in the

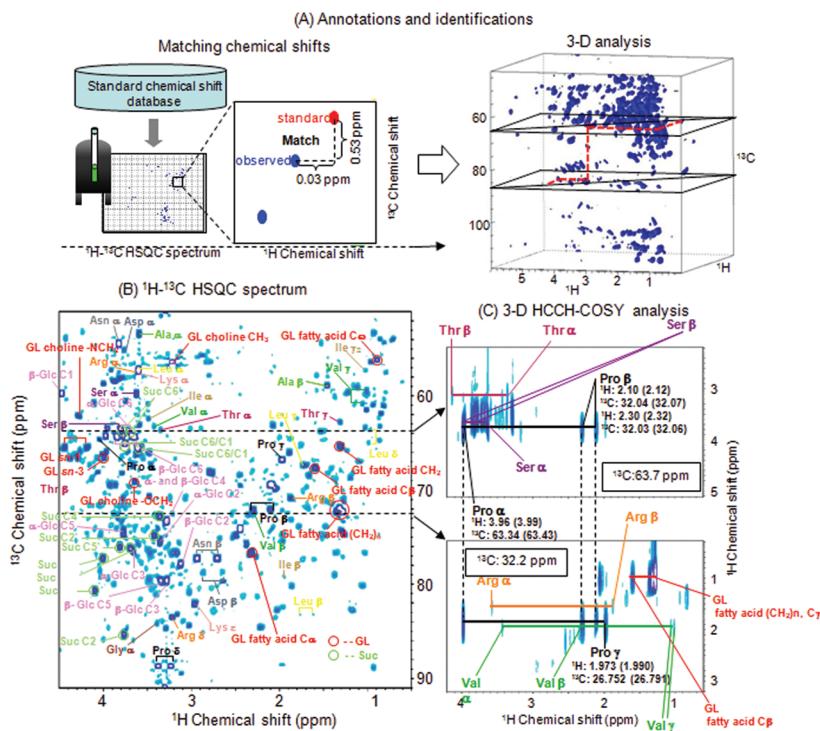


Figure 6. An example of the assignment of metabolites in highly ^{13}C -labeled *A. thaliana*. (A) The annotation method includes matching chemical shifts with the standard database using tolerances of 0.03 ppm for ^1H and 0.53 ppm for ^{13}C . The annotation was confirmed by the 3-D HCCH—COSY analysis. (B) An expanded view of the ^1H — ^{13}C HSQC spectrum of ^{13}C -*A. thaliana* (MeOD/HEPES- d_{18} , 700 MHz with the cryogenic probe). The spectral window and offset frequency in the f2 dimension were 16 ppm and 3.3 ppm, respectively. The spectral window and offset frequency in the f1 dimension were 40 ppm and 68 ppm, respectively. GL, glycerolipid; Glc, glucose; Suc, sucrose. (C) Two-dimensional ^1H — ^1H planes at 32.2 and 63.7 ppm ^{13}C are shown as an example for assignment. The observed and standard (in parentheses) chemical shifts of proline are shown as an example of the chemical shift difference.

3-D HCCH—COSY spectrum. The method used for annotating metabolites is shown in Figure 6A, including systematic batch identification simply by matching the queried observed peaks with peaks in the standard chemical shift database. Using 10 mg of ^{13}C -*A. thaliana*, the ^1H — ^{13}C HSQC spectrum was acquired to afford 317 signals. The ^1H — ^{13}C HSQC spectrum and examples of annotated signals are shown in Figure 6B. When these signals were submitted to SpinAssign with tolerance parameters of 0.03 and 0.53 ppm in the ^1H and ^{13}C dimensions, respectively, 45 candidate metabolites were listed from the standard database. Among these, the H—C—C—H bonds of the 16 metabolites listed in Figure 5B could be checked using the 3-D HCCH—COSY spectrum. Examples for the 2-D ^1H — ^1H plane of the 3-D HCCH—COSY spectrum are shown in Figure 6C. Amino acids and carbohydrates were common to the aqueous and MeOD extracts. Instead of organic acids, which are detectable only in the aqueous extract, glycerolipids could be detected by MeOD extraction.

CONCLUSIONS

To improve metabolite detection systems, several extraction solvents were evaluated using ^{13}C -labeled *R. sphaeroides*, based on the total rank values for line width and detectable signals in ^1H — ^{13}C HSQC spectra. MeOD was found to be an appropriate solvent for line sharpening and increasing the detectable signals in ^1H — ^{13}C HSQC spectra. Furthermore, MeOD extraction appears to be effective for other bacterial and animal samples. Because detectable metabolites are not always extracted completely in the

polar phase, such a semipolar extraction system might contribute to the large-scale detection of various metabolites. In addition, such an easy one-solvent-step extraction would contribute to develop high throughput multispecies platform for metabolomics. Comparative metabolome analysis of bacteria, plant, and animal samples is now in progress using MeOD.

ASSOCIATED CONTENT

S Supporting Information. Additional information is available, as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>

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