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Reducing the NMR sample volume using a single organic liquid: Increased sensitivity for mass-limited samples with standard NMR probes

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ABSTRACT

A simple inexpensive protocol for confining an aqueous sample to the active region of a standard NMR probe is examined for high-resolution NMR. The aqueous sample is sandwiched between an inert perfluorinated organic liquid that has been exploited in the design of micro-coil NMR probes. The procedure is demonstrated with 3 mm NMR tubes at ambient and elevated temperatures but should be equally applicable to smaller diameter tubes. It is shown that confinement has minimal effects on line shape and provides at least a two fold increase in sensitivity over a conventional sample, for the same mass of solute.

1. Introduction

The poor sensitivity of NMR spectroscopy has led to the development of strategies to increase sensitivity for mass-limited samples. In addition to the design of micro-coil and cryogenic probes there has been continued interest in concentrating the sample of interest to the active region of the receiver coil. Shigemi tubes, where solvent susceptibility-matched glass components are used to confine the sample to the active receiver volume, are widely used to increase sensitivity for mass-limited samples. These tubes however, are costly, not readily available for NMR tubes of smaller diameters (<3 mm), and suffer from the potential presence of air bubbles especially at elevated temperatures which reduces field homogeneity. Solid susceptibility-matched plugs [3] can also experience similar problems. Recently Lippens and co-workers have reported that Nujol mineral oil and chloroform can be used to constrain aqueous samples to the active region of the receiver coil for NMR tubes with diameters as small as 1 mm [1,2]. The magnetic susceptibility differences between the sample's three phases are sufficiently small that the effect on spectral resolution of sealing the sample between the organic phases was minimal. However, this "sandwich" technique suffers when the Nujol oil phase is within the receiver coil. The large Nujol signal requires that the receiver gain be reduced and significant spectral distortions arise in the aliphatic region. The need to enhance sensitivity for glycan structural analysis of small quantities of biosynthetic intermediates prompted us to investigate a similar approach in which an aqueous sample is sandwiched between two "plugs" of the highly fluorinated organic fluid FC-43 (Fluorinert, Perfluorotributylamine, PFTBA, Tris(nonafluorobutyl)amine, $N(CF_2CF_2\ CF_2CF_3)_3$, Sigma–Aldrich, MO). Since the proton background is minimal, this approach does not suffer in performance even when the organic phase is substantially within the receiver coil. The choice of the organic phase originated from the work of Webb and co-workers who utilized an inert polyfluorinated organic liquid to compensate magnetic susceptibility differences arising in micro-coil probes for aqueous mass-limited samples [4]. In the latter study, the aqueous phase extended 1.4 times the RF coil length. It should be noted that Pruessmann and co-workers have recently discussed the optimization and exploitation of single-liquid plugs for susceptibility compensation and sample confinement in measuring magnetic fields and field dynamics in MR systems [5].

2. Comparison with a standard sample in a 3 mm NMR tube

FC-43 has a boiling point of 174 °C, density of 1.86 g/ml and solubility in water of <5 ppm. Its magnetic susceptibility is 0.93% that of D_2O [4] so that the effect of the aqueous to organic phase transition is expected to be small. Sample preparation is straight forward with 60–80 μl of FC-43 placed in a 3 mm NMR tube, followed by 65–70 μl (active volume of the probe) of the aqueous sample and then 60–80 μl of FC-43 is layered onto the aqueous phase using a long pipette. The volumes of FC-43 above and below the aqueous sample are chosen to approximately center the sample in the receiver coil and to meet the vendor's specifications for optimal sample height. That FC-43 is denser than water is problematic for 5 mm NMR tubes but obtaining a sample at 3 mm and, by inference, smal-

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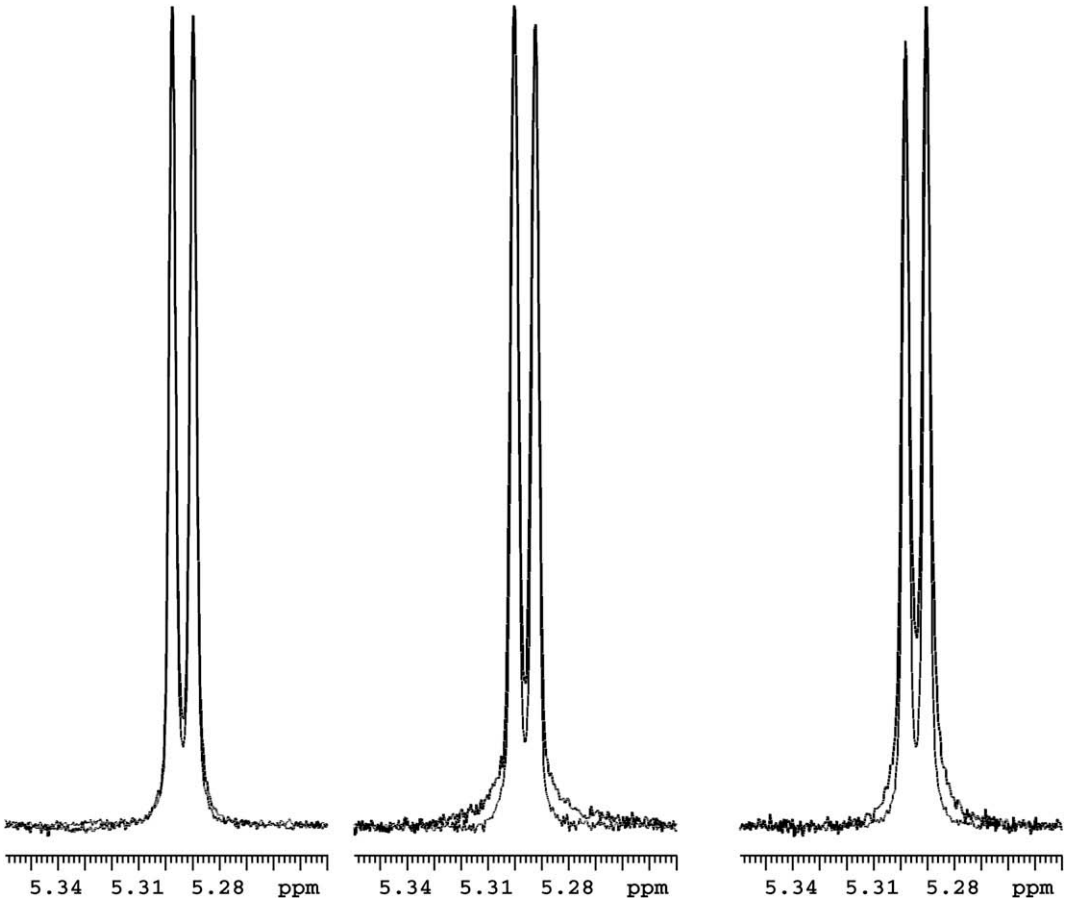

Fig. 1. Comparison of 500 MHz ^{1}H NMR spectra of 1.9 mM sucrose in D_2O acquired with 180 μl standard (thin line) sample and (thick line) confined between two 80 μl volumes of FC-43 in 3 mm (o.d.) NMR tubes. (A) 65 μl at 25 °C. (B) 65 μl at 60 °C and (C) 40 μl at 25 °C. Samples were gradient shimmed with the same shim map except at 60 °C. Spectra were acquired with identical conditions, on an INOVA 500 spectrometer and a 3 mm probe with water presaturation and processed with identical parameters. No line broadening was used in the processing.

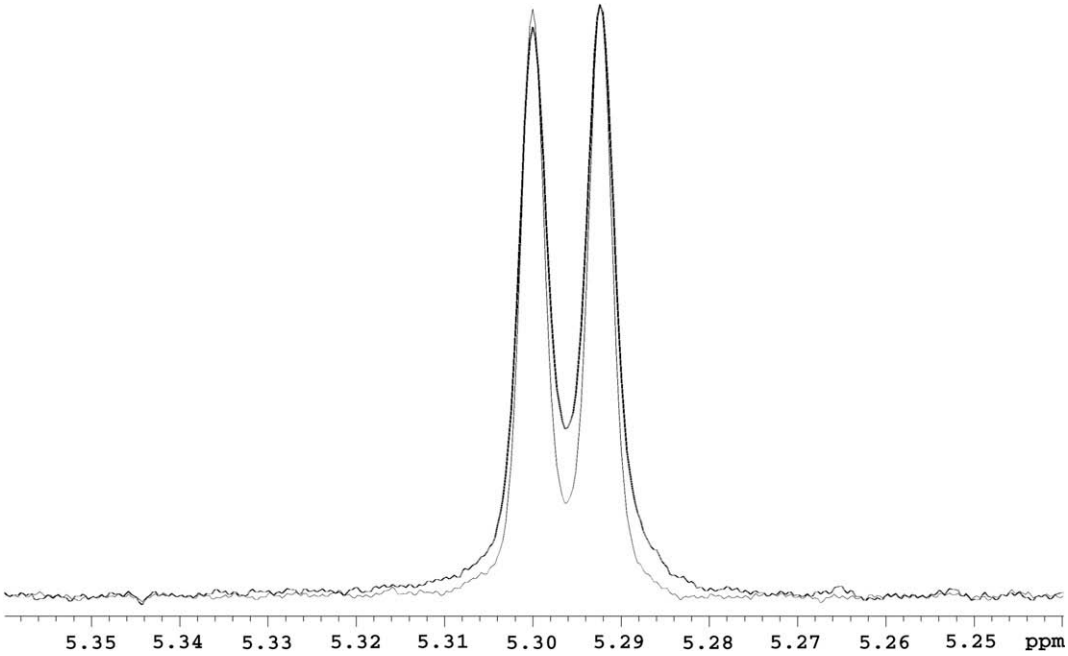

Fig. 2. Comparison of 500 MHz ^{1}H NMR spectra of 1.9 mM sucrose in H_2O (90%)/D_2O (10%) containing 170 mM NaCl and acquired with 180 μl standard sample (thin line) and 65 μl confined between two 80 μl volumes of FC-43 (thick line) in 3 mm (o.d.) NMR tubes at 25 °C. Samples were gradient shimmed with the same shim map. Spectra were acquired with identical conditions as in Fig. 1 on an INOVA 500 spectrometer with water presaturation and processed with identical parameters. To reduce the effects of truncation of the residual water signal, a 0.1 Hz Gaussian line broadening was used during data processing.

ler diameters is straightforward. Fig. 1A compares standard ^{1}H NMR spectra of a 1.9 mM sample of sucrose in D_2O from a standard 180 μl and a "sandwiched" 65 μl configuration. Based upon the vendor's (New Era, NJ) NMR tube specification (i.d. 2.36 mm) and the RF coil length of 16 mm (Varian 3 mm ^{1}H{13C/31P} PFG probe), the receiver volume is estimated to be 70 μl so that the organic phase is within the RF coil. Inspection of the anomeric proton resonance shows that the sandwiched sample geometry has minimal loss in line shape as compared with the longer standard sample geometry. It should be emphasized that no special effort in shimming of the samples was used. All samples (except at elevated temperature) were gradient shimmed using the same shim map as that for the 180 μl sample. The S/N ratio of the anomeric signal was 204:1 and 187:1 for the standard and sandwiched geometries, respectively. Correcting for the filling factor (70/65 μl) gives an adjusted S/N ratio of 201:1 for the sandwich geometry. Similar minimal loss in line shape with the sandwich geometry is obtained at elevated temperature (Fig. 1B), conditions frequently used in glycan structural studies. At 60 °C the S/N ratio of the anomeric signal was 138:1 and 126:1 (corrected for the filling factor) for the standard and sandwiched geometries, respectively. When the aqueous volume is reduced to 40 μl there is a greater effect on the line shape with the line width at half height increasing by ~29% (Fig. 1C) and the resulting S/N was 147:1 (corrected for filling factor, 70/40 μl). The background signals from the organic phase (5.04, 4.93, and 0.72 ppm) are relatively broad and become potentially problematic when the sample concentration approaches 50 μM. However, at these concentrations recourse to a smaller diameter tube/RF coil combination would alleviate the problem.

Samples of 1.9 mM sucrose in aqueous (90% H_2O/10% D_2O) 170 mM NaCl with the two sample geometries were compared to explore performance with aqueous phases with reduced D_2O content (Fig. 2). The 65 μl sample had a line width at half height that was 20% greater than that of the standard sample. Increasing the confined aqueous volume to 75 μl gave a line width at half height that was 13% greater than that of the standard sample. The increased line width for the 65 μl sample, relative to that of the 180 μl standard sample, reflects the poorer match between the magnetic susceptibility of the organic phase and that of the aqueous saline solution. By increasing the volume to 75 μl the organic-aqueous interface is moved further from the active volume of the RF coil leading to some improvement in line shape. The corresponding S/N ratios for the anomeric proton of the confined samples were 75:1 (65 μl) and 86:1 (75 μl) compared with 90:1 for the standard geometry.

A perhaps more interesting example is provided by lysozyme (2 mM in D_2O). A standard ^{1}H NMR spectrum (Fig. 3A) shows that 65 μl lysozyme solution between the FC-43 plugs gives a line shape very similar to that of a standard 180 μl sample of the same solution. S/N ratios of the aromatic region were 323:1(65 μl, corrected for filling factor) and 350:1 (180 μl). Fig. 3B compares NOESY spectra of the lysozyme solution for both sample configurations and

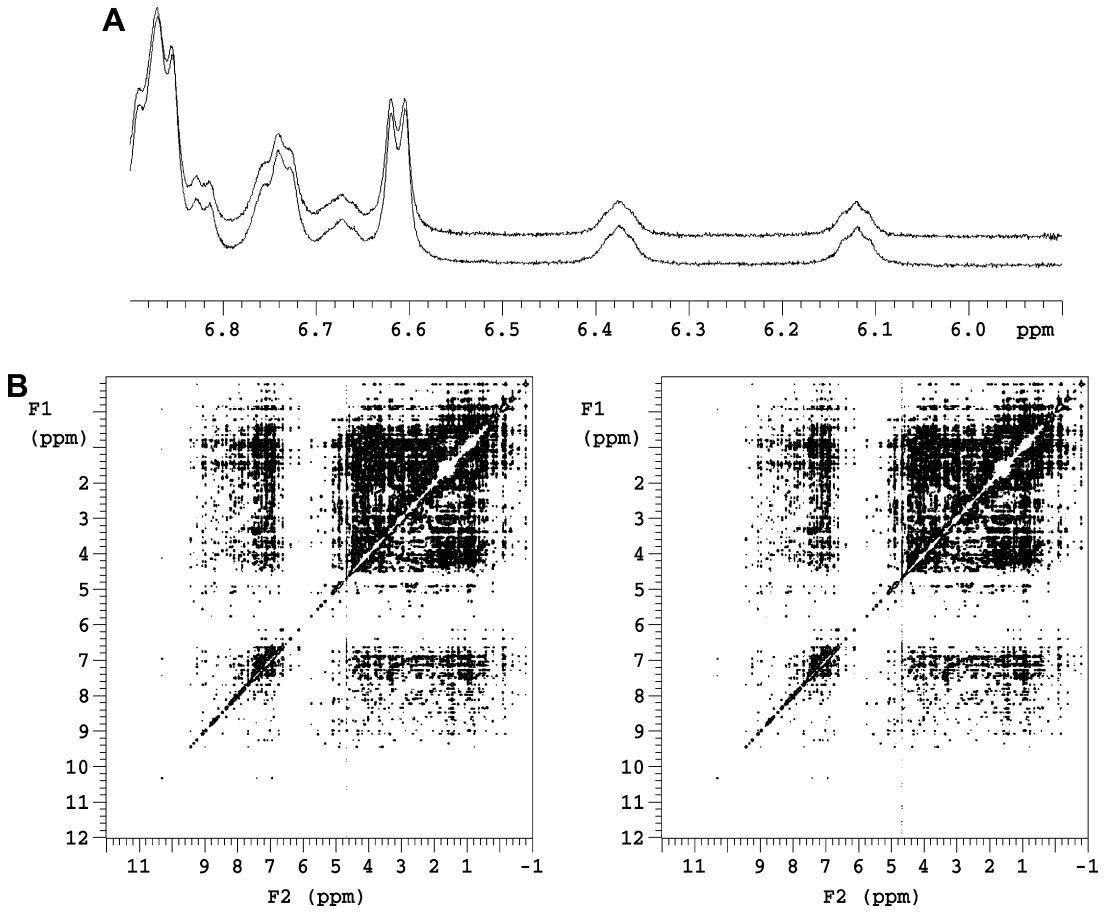

Fig. 3. Comparison of 500 MHz ^{1}H NMR spectra of a 2.2 mM solution of egg white lysozyme (Sigma–Aldrich, MO) in D_2O at 25 °C. (A) Comparison of the 6.9–5.9 ppm region of a standard ^{1}H 1D NMR spectrum for the 180 μl standard(bottom) and 65 μl confined between two 80 μl volumes of FC-43 (top) in 3 mm (o.d.) NMR tubes. Data were acquired and processed identically as in Fig. 1. The vertical scale of the confined-sample spectrum was scaled by 70/65. (B) 500 MHz ^{1}H NOESY spectrum (300 ms mixing time) for 180 μl standard (left) and 65 μl confined between two 80 μl volumes of FC-43 (right) in 3 mm (o.d.) NMR tubes. Both data sets were acquired with 512 increments in t1 with a spectral width of 10 kHz in both dimensions, 32 acquisitions per increment and the same receiver gain. Data was linear predicted to 2 K complex points in t1, followed by Gaussian broadening and zero filling to 4 K complex points in each dimension prior to Fourier transformation.

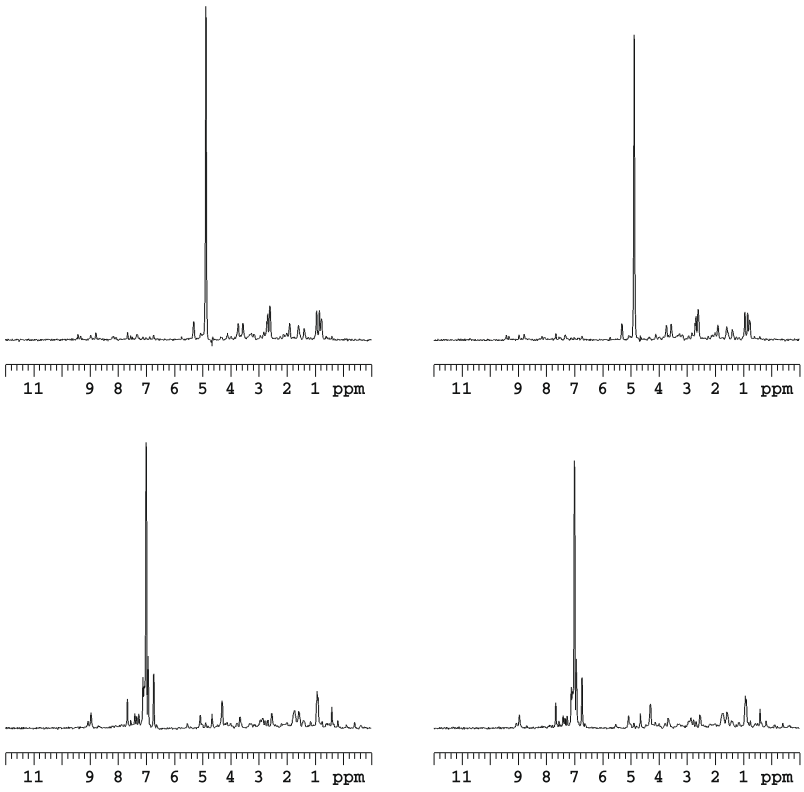

Fig. 4. Comparison of vertical traces from the 500 MHz ^{1}H NOESY spectra in Fig. 3B taken at 7.00 ppm (lower) and 4.88 ppm (upper) for the 180 μl standard sample (left) and 65 μl confined sample (right). The vertical scale of the spectra on the right was increased by a factor of 1.08 over that of the spectra on the left (corresponding to the estimated relative filling factor of the two samples, 70 μl vs. 65 μl).

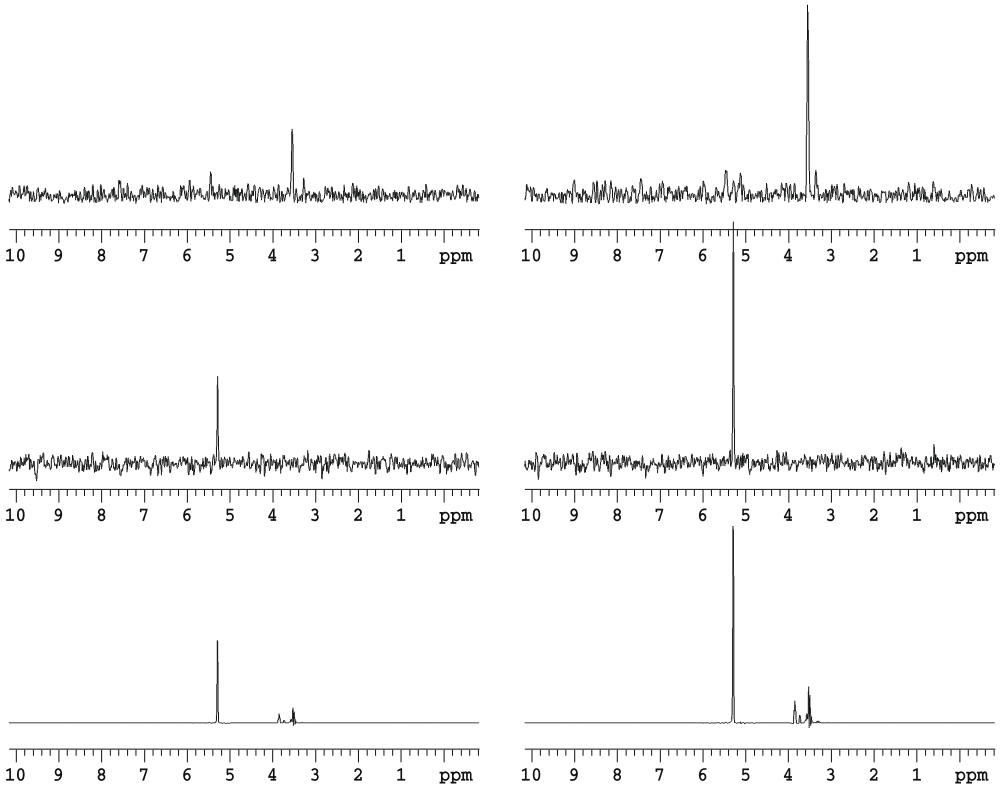

Fig. 5. Traces from ^{1}H TOCSY(bottom), ^{1}H–^{13}C HSQC (middle) and ^{1}H–^{13}C HMBC (top) spectra of a heptasaccharide, maltoheptoase, (93 μg) in 160 μl of D_2O in a 3 mm NMR tube (left) and in 65 μl of D_2O between two 80 μl volumes of FC-43 in a 3 mm NMR tube (right). Traces are plotted at the same vertical scale for the corresponding traces. Data for the two samples were acquired on an INOVA 500 NMR spectrometer under identical conditions and were processed identically.

demonstrates that all regions of the NOESY spectrum are available without interference from the organic phase on "confining" the sample. A comparison of traces from the NOESY spectrum (Fig. 4) illustrates further that there are no significant distortions arising from the organic phase. After one month there was no visual or spectroscopic evidence that the protein was denaturing with prolonged contact between the aqueous solution and the organic phase. Since FC-43 is widely used in ultracentrifugation of proteins [6], denaturation is not anticipated to be a widespread problem.

3. Comparison with a standard sample for a mass-limited sample

That this approach does provide sensitivity gain for mass-limited samples was tested by comparing 93 μg of the heptasaccharide maltoheptaose in 160 μl and in 65 μl of D_2O. Comparison of standard single pulse ^1H spectra acquired and processed under identical conditions gave a 2.3 fold relative increase in S/N for the confined sample which corresponds to that expected (160/70 μl). Traces from a standard ^1H TOCSY spectrum, and standard natural abundance ^1H–^{13}C HSQC, and HMBC spectra for both samples that were acquired and processed under identical conditions are shown in Fig. 5. In all cases, the confined sample geometry gave a >2.3 fold gain in sensitivity. Similar results were obtained at 600 MHz with a 5 mm cryogenic probe (5 mm *Z*-axis gradient HCN probe) and 3 mm NMR tubes where for the maltoheptaose samples a 1.8–1.9 gain in sensitivity were obtained for the same experiments. Assuming that the cryoprobe's coil length is 16 mm, a gain in sensitivity of approximately 2.3 is predicted.

In conclusion, the perfluorinated organic liquid FC-43 may be used with 3 mm or smaller NMR tubes in standard and cryogenic probes. With mass-limited samples, this approach offers a simple and flexible means of increasing sensitivity by a factor of at least two over a standard sample. Aqueous sample recovery is simple and consists of centrifuging or shaking the NMR tube and removing the upper aqueous layer by pipette. The remaining organic phase can be washed with water and dried for reuse.

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