

Sample Concentration and Separation for Nanoliter-Volume NMR Spectroscopy Using Capillary Isotachopheresis

Roger A. Kautz¹, Michael E. Lacey^{2,3}, Andrew M. Wolters^{2,3}, Frantisek Foret¹, Andrew G. Webb^{3,4}, Barry L. Karger^{1,*}, and Jonathan V. Sweedler^{2,3,*}

¹Barnett Institute of Chemical and Biological Analysis, Northeastern University, Boston, MA 02115, ²Department of Chemistry, ³Beckman Institute and ⁴Department of Electrical and Computer Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, 61801. *Corresponding authors

Received xxxxxxxx

Full characterization of trace level analytes in complex mixtures is vital to diverse areas such as cellular analysis, natural product extraction, and combinatorial chemistry. Since nuclear magnetic resonance (NMR) spectroscopy can provide structural and dynamic information unattainable by other means, it has found broad utility for compounds ranging from small organic molecules to biopolymers. Despite major advances in performance, NMR systems still require much greater sample amounts than other molecular characterization methods. We present here a technique that concentrates analytes by capillary isotachopheresis (cITP) to generate the highest mass sensitivity ¹H-NMR spectra ever reported from low microliter-volume samples.

While increased magnetic field strengths have yielded major NMR sensitivity gains, escalating costs and significant technical obstacles have motivated additional strategies such as polarization transfer techniques, cryogenic probes, and reduced-diameter radiofrequency (RF) coils.¹ Since mass sensitivity (S_m , S/N per unit amount) is inversely proportional to coil diameter to a first approximation, commercial NMR probes have been designed for mass-limited samples with volumes in the tens of microliters. Solenoidal microcoil probes, which have observed volumes (V_{obs}) of 5 nL - 1 μ L, have shown even further improvements in S_m . However, efficient delivery of nanoliter-volume samples into the microcoil V_{obs} within the magnet remains challenging. Sample handling losses and dilution during transfer are difficult to avoid in this smaller volume regime, so that only a small fraction of the injected sample may reside in the V_{obs} .

We report the first coupling of cITP to NMR. Using cITP, larger (several μ L) sample volumes may be injected and focused to less than 100 nL, within the size regime of a microcoil V_{obs} . This method improves the concentration sensitivity of nanoliter-volume NMR by over two orders of magnitude. Focusing may also purify analytes from contaminants. Individual components of complex samples may be focused into discrete zones, allowing resolution of their NMR spectra.

Sample focusing methods are well established in capillary electrophoresis (CE).² In cITP, the sample is introduced between leading ions, which have an electrophoretic mobility (μ_e) greater than the analyte, and trailing ions of lower μ_e .³ When an electric field is applied, sharp and stable boundaries form between discrete zones of ions with different μ_e . Once established, the bands remain in contact and travel at constant velocity. To maintain constant current throughout the capillary, each zone must contract or expand to adjust its analyte ion concentration proportional to the leading electrolyte (LE). Hence, the focused volume of each zone is determined by the quantity of charged analyte ions present. For cITP-NMR, this would permit focusing an injected sample plug 10-cm-long into a 1-mm-long microcoil V_{obs} . cITP electrolyte systems are available for either anionic or cationic analytes.³ In the following experiments, cationic analytes were focused using 120 mM sodium deuterio-acetate (d_3 , pH 5) in D_2O as the LE and 10 mM deuterio-acetic acid (Dac) as the trailing electrolyte (TE).

The cITP-NMR instrumentation was similar to that used previously for microcoil NMR detection of CE.⁴ All NMR experiments were performed on a Varian 500 MHz spectrometer with a wide-bore magnet, using a novel microcoil sleeve probe in which the RF coil was wrapped around a thin polyimide sheath.⁵ A fused silica capillary was inserted through the sleeve to create a V_{obs} of \sim 30 nL. Polyvinyl-alcohol (PVA) coated capillaries were used to minimize electroosmotic flow, which degrades the boundaries between analyte bands. Hydrodynamic flow was used for sample injection and for positioning the focused sample band in the coil V_{obs} . Injection and separation conditions to focus a sample into a sharp band in 510 minutes were determined by observing the charged visible dye methyl green (MeG) in a capillary on a benchtop.

Figure 1 compares a spectrum of 5 mM tetraethylammonium bromide (TEAB) acquired without pre-concentration (Figure 1A) to a stopped-flow cITP-NMR spectrum of 8 μ L of TEAB injected at 200 μ M initial concentration (Figure 1B).⁵ The S/N for the latter was twice as great as in Figure 1A for the same 10 s NMR experimental time and data processing parameters. Based upon integrated peak area, cITP focusing in Figure 1B resulted in \sim 100-fold increase in concentration, with an

(2) *High Performance Capillary Electrophoresis: Theory, Techniques, and Applications*; Khaledi, M.G., Ed.; Chemical Analysis: A Series of Monographs on Analytical Chemistry and Its Applications, Vol. 146; John Wiley & Sons: New York, 1998.

(3) (a) Bocek, P.; Deml, M.; Gebauer, P.; Dblnik, V. *Analytical Isotachopheresis*; VCH Publishers: New York, 1988. (b) Foret, F.; Szoko, E.; Karger, B.L. *Electrophoresis* **1993**, *14*, 417-28. (c) Enlund, A.M.; Schmidt, S.; Westerlund, D. *Electrophoresis* **1998**, *19*, 707-11.

(4) Olson, D.L.; Lacey, M.E.; Sweedler, J.V. *Anal. Chem.* **1999**, *71*, 3070-6.

(5) Coated 50- μ m diameter round Cu wire was used to wrap an 18-turn solenoid around a 370 μ m i.d./420 μ m o.d. polyimide tube. Static analyses yielded linewidths between 1-2 Hz at 500 MHz. Focusing was performed in an 85-cm-long, 200 μ m i.d./360 μ m o.d. fused silica capillary internally modified with a covalent PVA coating (Goetzinger, W.; Karger, B.L. US Pat. #5840388, 1998). The inlet was placed directly in the TE at the anode 20 cm below the magnet. The outlet capillary, extending 12 cm beyond the NMR coil, was connected to the cathode reservoir of LE by 22 ga Teflon tubing. The tubing and capillary were soaked with 0.1% Triton X-100 detergent in D_2O for 30 min after every 3 runs. Before each injection, the capillary was flushed with LE. Sample injection was hydrodynamic: 4-6 μ L TE, 5-20 μ L sample in 50% TE, 6 μ L TE. The initial injection of TE aided rapid formation of the LE/sample boundary; the latter injection moved the sample plug closer to the V_{obs} . The applied potential was 20 kV. Since the LE has much greater conductivity than the TE, the relative position of the focused sample can be estimated by the electrophoretic current. Extended NMR acquisitions were obtained by discontinuing the applied electric field and using a stopcock near the cathode vial to stop the flow.

(1) (a) Ernst, R.R.; Bodenhausen, G.; Wokaun, A. *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*; Oxford University Press: Oxford, 1991. (b) Navon, G.; Song, Y.Q.; Room, T.; Appelt, S.; Taylor, R.E.; Pines, A. *Science* **1996**, *271*, 1848-51. (c) Flynn, P.F.; Mattiello, D.L.; Hill, H.D.W.; Wand, A.J. *J. Am. Chem. Soc.* **2000**, *122*, 4823-4. (d) Dorn, H.C.; Gu, J.; Bethune, D.S.; Johnson, R.D.; Yannoni, C.S. *Chem. Phys. Lett.* **1993**, *203*, 549-54. (e) Lacey, M.E.; Subramanian, R.; Olson, D.L.; Webb, A.G.; Sweedler, J.V. *Chem. Rev.* **1999**, *99*, 3133-52.

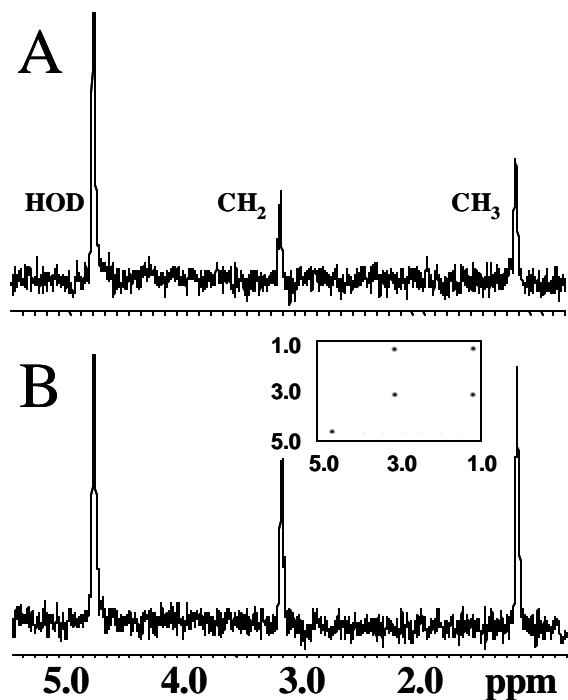


Figure 1. $^1\text{H-NMR}$ spectra of TEAB. A.) Capillary filled with 5 mM TEAB without sample stacking (S/N of peak at 1.2 ppm = 13). B.) 8 μL of 200 μM TEAB injected; spectrum after sample stacking by cITP (S/N of peak at 1.2 ppm = 30). The inset shows a COSY spectrum. 1D NMR acquisition parameters: SW = 5000 Hz, NP = 12420, AT = 1.242 sec, 55° pulse, delay time=0 s, NT = 8, total NMR experimental time = 10 sec. Data processing conditions: two ZF, linear back prediction (LN) of 4 pts based on first 64 data pts, LB = 2 Hz. COSY acquisition parameters: SW = 2271.4 Hz, NP = 1024, AT = 0.225 s, delay time=1 s, NT = 4, NI = 256, time increment change = 0.44 ms, total NMR experimental time = 22 min. COSY processing conditions: magnitude mode, one ZF along indirect dimension, sine-bell multiplication in both dimensions.

overall increase in sample observation efficiency from 0.5% to ~50%. Similar concentration factors also were obtained for 5 to 20 μL injections of analytes at initial concentrations of 30 to 200 μM .

One potential concern in using focusing methods with NMR is that local differences in the magnetic susceptibility of the focused zones near the NMR coil may degrade spectral resolution. For typical one-dimensional cITP-NMR spectra for the sleeve probe, the linewidths ($w_{1/2}$, full width at half maximum) degraded from 2 Hz to 4 Hz. Microcoils wrapped directly on fused silica capillaries can achieve $w_{1/2} < 1$ Hz;^{1c} however, the sleeve probe described here facilitates capillary exchange. Some broadening is acceptable when focusing makes possible two-dimensional experiments, which use lower digital resolution. The inset in Figure 1B shows a stopped-flow COSY spectrum acquired in 22 min. The increased concentration produced by cITP enabled this spectrum to be collected 10,000 times faster than without sample focusing with this probe. Compared with the smallest-volume (~30 μL) commercially available probes and/or NMR accessories, this approach represents more than an order of magnitude improvement in S_m and a corresponding 100-fold time savings.

CE techniques have found widespread application through their ability to separate minute quantities from complex mixtures with high efficiency.² In addition to sample stacking, cITP is a well-established means to separate

mixtures of analytes with different μ_e .³ Figure 2 shows a stacked plot of NMR spectra from a cITP separation. The 7 μL sample consisted of 200 μM each TEAB, MeG (85% purity), and the dipeptide alanine-lysine (Ala-Lys). The NMR data shown are a series of 40-s-long acquisitions collected sequentially, without interruption of the applied voltage or flow as the focused bands passed through the NMR microcoil via slow hydrodynamic flow. Because of the finite size of the microcoil, adjacent zones overlap in the detector even though the cITP zone boundaries may be sharp. However, the NMR resonances are resolved, and the individual spectra of the four compounds can be seen from their four distinct sets of rise and decay times. Of interest, cITP separated an impurity in the MeG, which migrated as the third component.

cITP-NMR has demonstrated significantly improved sample concentration sensitivity over any prior capillary-scale NMR experiment. This simple yet powerful system takes advantage of electrophoretic methods to effectively focus microliter injection volumes into nanoliter-volume zones of electrophoretically purified analytes. It thus enables nanoliter-volume microcoils, the highest mass-sensitivity NMR probes, to be used for structural elucidation of charged trace components. The strong resonances of TEAB may be used as a marker to trap even lower concentration analytes not detectable in on-flow acquisition. Such a system has immediate applicability to areas of drug discovery and biochemical research where mass-limited analyses of ionizable species are crucial.

Acknowledgment. We acknowledge Dr. Paul F. Molitor (UIUC) and Dr. Tomas Rejtar (Barnett Inst.) for useful discussions of this manuscript. We acknowledge support from N.I.H. (GM53030 to J.V.S. and A.G.W. and GM15847 to B.L.K.), the Camille and Henry Dreyfus Foundation (J.V.S.), a N.S.F. CAREER award to A.G.W. (DBI 97-22320), and an A.C.S. Division of Analytical Chemistry Fellowship sponsored by Merck & Co. to M.E.L. This is contribution number 787 from the Barnett Inst.

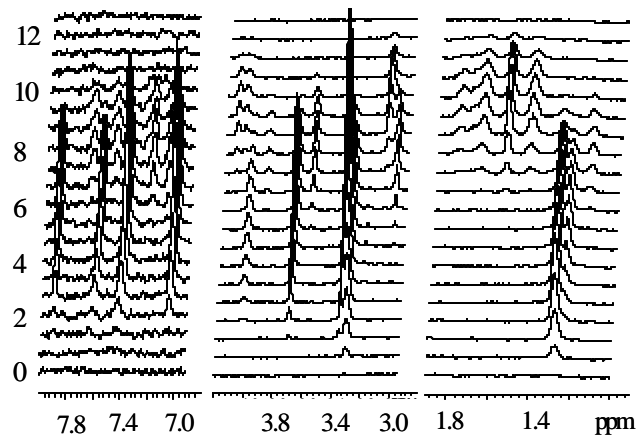


Figure 2. Continuous-flow $^1\text{H-NMR}$ spectra of a cITP separation of TEAB, MeG, and Ala-Lys (200 μM each, 7 μL injected). Spectra were acquired in 10-s intervals; for displayed data, 4 slices were co-added with the separation time (min) labeled on left. TEAB appears from 40 s to 9 min 40 s, MeG from 1 min 20 s to 9 min 20 s, MeG impurity from 5 min 20 s to 10 min, and Ala-Lys from 7 min 20 s to 12 min. The region from 6.80 to 8.00 ppm is scaled 4 times higher than the other regions. Top spectrum is offset by 10% along frequency axis. Spectral parameters: SW = 5000 Hz, NP = 12420, AT = 1.242 s, 55° pulse, delay time = 0 s, NT = 8. Data processing conditions: LN = 2 pts based on first 64 data pts, LB = 4 Hz.