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Automated Microflow NMR: Routine Analysis of Five-Microliter Samples

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Abstract

A microflow CapNMR probe double-tuned for ^{1}H and ^{13}C was installed on a 400-MHz NMR spectrometer and interfaced to an automated liquid handler. Individual samples dissolved in DMSO-d_6 are submitted for NMR analysis in vials containing as little as 10 μL of sample. Sets of samples are submitted in a low-volume 384-well plate. Of the 10 μL of sample per well, as with vials, 5 μL is injected into the microflow NMR probe for analysis. For quality control of chemical libraries, 1D NMR spectra are acquired under full automation from 384-well plates on as many as 130 compounds within 24 h using 128 scans per spectrum and a sample-to-sample cycle time of ~11 min. Because of the low volume requirements and high mass sensitivity of the microflow NMR system, 30 nmol of a typical small molecule is sufficient to obtain high-quality, well-resolved, 1D proton or 2D COSY NMR spectra in ~6 or 20 min of data acquisition time per experiment, respectively. Implementation of pulse programs with automated solvent peak identification and suppression allow for reliable data collection, even for samples submitted in fully protonated DMSO. The automated microflow NMR system is controlled and monitored using web-based software.

Automated methods for synthesis and compound screening in drug discovery have resulted in an increasing need for high-throughput analytical tools. Nuclear magnetic resonance (NMR) spectroscopy is a very versatile approach with applications ranging from qualitative analysis and purity assessment for single synthetic samples, quality control of compound libraries, and screening for small molecule binding to proteins of pharmacological importance to the analysis of low-molecular-weight metabolites in blood and other biological fluids in search of biomarkers or drug degradation. NMR spectrometers are often interfaced to sample loaders equipped to handle individual glass tubes that are filled manually or with the aid of laboratory robots. Tube-based systems range in size from conventional 5-mm tubes with a 550-μL fill volume down to 1-mm-diameter tubes that accommodate sample volumes as small as 5 μL.[1]

Although the benefits of flow NMR have been recognized, namely, an increased throughput because samples are directly introduced into the detector from 96-well microtiter plates without the need for sample transfer to and from tubes, these systems have until recently required

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SUPPORTING INFORMATION AVAILABLE
Photographs of the system as well as flow diagrams. This material is available free of charge via the Internet at http://pubs.acs.org.

sample volumes between 100 and 600 μL.[2-4] The newest generation of cryogenically cooled probes has accomplished mass sensitivity enhancements of 4-fold relative to conventional flow designs.[5] However, progress in conventional NMR flow analysis has been slowed by sample management challenges, such as peak dispersion, sample dilution, and excessive carryover that are problematic traits common to conventional-scale (several millimeter diameter and higher) fluidic systems. The recent development of capillary-scale microflow probes has provided an ability to accurately flow-inject and analyze sample volumes of several microliters. By combining small radio frequency microcoils that wrap directly around the NMR flow cell for high fill factor with 75-μm-i.d. fluidic transfer lines, these capillary probes provide enhanced mass sensitivity, negligible dispersion, and excellent fluidic performance by maintaining in real time the integrity of the fluidic flow path.[6-15] Microflow cell volumes are typically 3–10 μL with RF observe volumes of 1.5–5.0 μL.[6,8,10,12] These miniaturized, "tubeless" configurations provide spectra from microliters of sample, allowing either low-volume LC/MS vials or low-volume 384-well microtiter plates to be used as sample reservoirs.

Automation of NMR sample management and data acquisition is particularly advantageous for the confirmation of purity and structure of large collections of natural or synthetic compounds by rapid acquisition of one-dimensional NMR spectra.[2,12,16] Because of their low-volume requirement and their high mass sensitivity, microflow NMR probes are ideally suited for the chemical analysis of limited-quantity samples. This includes library compounds synthesized through combinatorial approaches and natural products extracted from plants and other sources[13,14] and the analysis of metabolites in biological materials, such as blood or tissue. For the latter studies, NMR spectroscopy is combined with pattern recognition methods, such as principle component analysis, to evaluate the biochemical variability of biofluids, such as urine, plasma or serum.[17-20] To achieve statistically significant results, proton spectra are acquired on a large number of small samples. Often, the sample volume is very limited; for example, only 50 μL of serum can be obtained from a mouse without killing the animal.

An automated, microflow NMR system would provide clear advantages for a wide variety of applications, and here we illustrate the implementation of such a system for (1) automated measurement of 10-μL samples from 384-well plates for the analysis of compound libraries and (2) measurements of individual samples submitted by synthetic chemists in 100-μL sample vials. High-quality proton NMR spectra are obtained using an automated microflow NMR system with web-based control.

EXPERIMENTAL SECTION

Microflow NMR Probe, Autosampler, and Sample Injection. A CTC-PAL autosampler (model HTS, LEAP Technologies, Inc., Carrboro, NC) is connected to a CapNMR probe (Protasis/MRM Corporation, Savoy, IL) and installed on a 400-MHz Bruker DPX spectrometer (Bruker Biospin, Billerica, MA). The NMR probe features a 5-μL flow cell and is double-tuned to ^{1}H and ^{13}C on the same microcoil. The NMR-active volume is 2.5 μL, and the probe includes a deuterium lock channel and *z*-gradient and is configured in a manner that allows both indirect and direct carbon measurements. The spectrometer is controlled by Bruker Biospin XWIN-NMR software on a Windows 2000 PC. A chromatographic-grade, high-pressure, low-volume pump (High Throughput Sample Loader or HTSL, Protasis Corporation, Marlboro, MA) delivers solvent from a reservoir to the autosampler and probe. The autosampler includes three temperature-controlled drawers designed to hold up to six microtiter well plates or a combination of standard or deep 96-well microtiter plates, high-density 384-well plates, or trays for vials. The autosampler has a wash station module for rinsing the syringe in two different fluids if desired. The wash station also serves as a source of fresh system solvent for the syringe. Accessories are available to expand the autosampler to accommodate a maximum

of 6 drawers and 12 sample trays. Photographs of the system as well as flow diagrams are available as Supporting Information.

In this study, samples are provided either in low-volume 384-well plates (Greiner Bio-One, Longwood, FL, custom plate) or in vials specifically designed for microliter-volume samples. The vials (Polypropylene Limited Volume 11-mm SnapnCrimp 100-μL vials, Thomson Instrument Company, Oceanside, CA, Part no. 30312) hold a maximum of 100 μL and have a sharp taper to allow, if desired, for nearly complete withdrawal of samples with a syringe and needle. The vials are covered with clear snap caps (Thomson Instrument Company, Oceanside, CA, Part no. 70502).

Samples are picked up from their vial or well by the autosampler's 25-μL syringe and loaded directly into the injection port of a six-port, two-position valve fitted with a 5-μL stainless steel sample loop (Valco, Houston, TX, Part no. SL5CW). This approach of sample loop and valve is common in flow injection and provides a reliable means of sample introduction without incurrence of air bubbles. In this work, the CTC-PAL is set to fill and empty the syringe three times with 8 μL of sample to ensure adequate mixing of the analyte and solvent in the well then draws 8 μL (pickup volume) of sample from the well or vial and loads this volume into the 5-μL, stainless steel sample loop. The pick-up volume can be set to any volume ranging from a few tenths of a microliter to the maximum volume of the syringe, and with the appropriate choice of well plate or vial and needle, the autosampler is capable of picking up virtually all of the sample contained in a well or vial. Our choice of allocating 10 μL of sample in the well or vial and a pickup volume of 8 μL for NMR analysis proved to be reasonable starting parameters and worked well with the fluidic components chosen for this study. The ability to adjust sample loop parameters (material, inner diameter, and volume), syringe size (as small as 1 μL) and flow loading conditions provides experimental flexibility to accommodate the sample type and performance optimization as defined by the user using simple, drop-down menus and form fields within the graphical user interface of the software.

After the CTC-PAL loads the sample loop, it triggers the HTSL pump to dispense a specified volume of push solvent at a designated flow rate to transport the sample plug into the center of the NMR detection coil. The delivery volume required to move the sample from the loop to the probe was determined empirically to be 25 μL at a delivery rate of 30 μL/min or 20 μL/min for D_2O or DMSO-d_6, respectively. While the sample is transferred through FEP Teflon tubing (75-μm i.d., 1/32-in. o.d.) to the NMR magnet, the syringe and injector port of the autosampler are rinsed three times with 12.5μL of DMSO-d_6. A 90-s equilibration period prior to NMR data acquisition is essential if the samples are stored at low temperature in the sample drawers but optional for room-temperature applications. During the NMR acquisition time, the rinsing steps are completed, and the next sample is loaded into the loop, which minimizes overall cycle time.

To avoid clogging and maintain flow path integrity, a 2-μm filter (Optimize Technologies, Oregon City, OR; Part no. 10-04-03357), with a 0.2-μL dead volume, is installed at the inlet of the NMR probe. An additional filter can be added at the port on the injector valve of the autosampler. Solvent drawn up from a reservoir by the HTSL passes through a 2-μm filter (Upchurch Scientific, Oak Harbor, WA, Part no. A-314). A mixture of (nondeuterated) acetone/chloroform/DMSO (v/% of 40/20/40) is injected just like a sample as preventive maintenance every 25 samples. The flow probe is cleaned monthly with 3% hydrogen peroxide in H_2O (45 °C for 12 h), and filters are replaced every 400 samples.

Software. Instrument control is coordinated by a web-based software package called One-Minute NMR (Protasis, Marlboro, MA). Its graphical interface (Figure 1) allows the user to readily change a wide array of operational and control parameters specifically dedicated to the

CapNMR microflow probe, HTSL pump, and CTC-PAL autosampler as well as some basic NMR parameters, such as number of scans, lock solvent, spectral width, and center. NMR experiments are defined via the spectrometer console, and each NMR experiment is selected and triggered via One-Minute NMR.

NMR Data Acquisition and Processing. All 1D NMR spectra shown here were acquired with 8192 complex points, a spectral width of 17.95 ppm and a recycle delay of 2 s. For the CapNMR probe, the ^{1}H 90 degree pulse width (τ_{90}) is approximately 6.7 μs (B_1 = 37.3 kHz) at a ^{1}H transmitter power setting of 0.2 W (20 dB attenuation setting on a Bruker 20 W ^{1}H transmitter), which is ~2 orders of magnitude less power than required by conventional probes for comparable τ_{90}. A unique feature of the CapNMR probe technology is that the pulse width can be further shortened to provide very rapid pulses due to the available transmission power. In automation, NMR spectra in DMSO-d_6 are acquired using a standard LC NMR Bruker release 1D NOESY pulse program with double presaturation (LC1D12).[21,22] Only the tallest solvent peak, usually residual water in the push solvent, is automatically identified and suppressed using low-power presatu-ration pulses.

For samples in protonated DMSO, WET solvent suppression[23] (LC1DWTDC) or a 1D version of NOESYPRTP (LC1DCWPS) with shaped pulses for solvent suppression at multiple solvent frequencies is employed. In these experiments, the two tallest solvent peaks are automatically identified and suppressed using shaped pulses (Squa100.1000) of 100 ms length during recycling and mixing time. ^{13}C satellites are decoupled at 40 ppm using GARP.[24] A mixing time of 100 ms is used in all 1D NOESY experiments. All other pulse programs can be used, as well. NMR spectra were processed in XWIN-NMR 3.1 (Bruker Biospin, Billerica, MA) applying an exponential line-broadening function of 0.5 Hz to all 1D spectra and referenced relative to DMSO at 2.50 ppm. All spectra were acquired at 25 °C.

Preparation of Individual Samples. Microgram quantities of synthetic compounds are dissolved in DMSO-d_6 and dispensed into low-volume LC/MS vials. A 20-μL portion of sample was selected as a reasonable total volume for separate LC/MS and NMR analyses from the same vial, with 10 μL being allocated for each analysis type. DMSO-d_6 was chosen as the system solvent because it dissolves most chemical compounds.

Sample Preparation for Quality Control of Library Compounds. Samples originate from 10 mM stock solutions in protonated DMSO that are regularly submitted to the Compound Management Group as part of a sample registration system. A 20-μL aliquot of each compound is transferred into a 384-well plate (Greiner Bio-One, Longwood, FL, custom plate) and dried using a Genevac Speed Evaporator (HT-12 Series II, Genevac, Valley Cottage, NY). Samples are then reconstituted with 10 μL of DMSO-d_6, and the plate is heat-sealed with an aluminum film (ABgene, Rochester, NY, Part no. AB-3738), effectively increasing the sample concentration to 20 mM for easier detection of impurities. Alternatively, plates filled with aqueous samples are sealed with universal optical microplate pressure-sensitive sealing tape (Corning, Inc., Acton, MA, Part no. 6757). The sealed plates are spun at 500 g for 2 min to settle the samples to the bottom of the wells.

RESULTS AND DISCUSSION

An automated microflow injection NMR platform was developed that allows reliable sample pickup from low-volume 384-well plates or LC/MS vials. The CTC-PAL autosampler used here is common in the field of mass spectrometry and offers a wide range of sample volume handling capabilities and rinsing options. Injection and transfer of a sample from a 5-μL loop and subsequent NMR data acquisition are coordinated and controlled by a web-based control interface (Figure 1). The time course of a typical sample handling protocol is shown in Figure

2. Sample pickup and transfer to the NMR requires ~2 min. In contrast to conventional-scale NMR systems, no shimming is required between samples for a given choice of solvent. Data acquisition time depends on sample concentration and the desired NMR signal-to-noise (S/N). In our laboratory, 128 scans collected in just over 6 min results in high-quality spectra for walk-up applications and compound library analysis (see below). Using 128 scans, plus locking, automatic solvent suppression, receiver gain setting and processing, and sample preloading, the cycle time between samples is ~11 min. This could be reduced by decreasing the number of scans and the temperature equilibration time. Carryover between samples is consistently <1% (Supporting Information).

Total consumption of DMSO-d_6 is ~105 μL per sample, which includes three injector wash cycles and solvent overflow in the washstation using a total of ~70 μL, plus 25 μL of push solvent and 10 μL for sample preparation. At $180 per 100 mL of DMSO-$d_6$, this is $0.20 worth of deuterated solvent per sample. Adding the cost of a plastic total-recovery vial and cap at $0.25 each yields a total of $0.45 per sample, which is a greater than 10-fold reduction from an estimated $5 expense per sample for traditional NMR in tubes or flow injection methods with larger flow cells.[4] The One-Minute NMR hardware configuration uses a well-swept, unidirectional flow path which provides minimal sample dispersion and efficient sample handling to preserve the concentration of the original analyte for maximum S/N. Solvent plugs[25] and air gaps[3] to introduce samples into the flow NMR probe are rendered unnecessary, thus simplifying the system and increasing its reliability. Although the system is most often used with DMSO-d_6, we have successfully tested the analysis of D_2O samples employing buffered D_2O as push solvent. Using a second HTSL pump to avoid repriming the pump, the system can be switched over to processing aqueous samples in as little as 10 min.

Applications and Modes of Operation. The automated microflow NMR system is designed for two main applications: (1) open-access or walk-up use for routine chemical analysis of synthetic intermediates or products and (2) quality control of compound collections and libraries. Compound libraries are quality-controlled at a nominal concentration of 20 mM in DMSO-d_6 with sample delivery in 384-well plates. When in use for quality control analyses, the system is simultaneously available for open-access use by synthetic chemists. Their samples are typically submitted in vials filled with at least 10 μL of sample in DMSO-d_6. The One-Minute NMR control software allows open-access users to obtain priority over data collection on compound libraries, at the discretion of the master operator. After data acquisition of walk-up samples in vials is completed, the system automatically resumes analysis of compound libraries from wells.

Evaluation as an Open-Access System for Single Samples. Using 20-μL aliquots of samples in DMSO-d_6, the synthetic chemists in our laboratory can now choose to obtain a molecular mass for each compound on a LC/MS system and then subsequently acquire a 1D ^{1}H NMR spectrum with the remainder of the sample by transferring the same vial to the microflow NMR system. NMR spectra for a test compound at concentrations of 20, 10, 5, and 3 mM are summarized in Figure 3. For all four concentrations, mass spectra, well-resolved chromatograms, and MW values close to the expected values (data not shown) were obtained prior to NMR data acquisition. NMR with good spectral resolution and signal-to-noise ratios were then acquired from the remainder of the sample: The 20 mM sample (Figure 3A and B) yielded signal-to-noise ratios of ~54 for 32 scans and 223 for 128 scans, respectively. The spectrum of the 3 mM sample (Figure 3E) has a reasonable signal-to-noise ratio of 46 at our default setting of 128 scans. A gradient-selected 2D COSY spectrum on the same sample was acquired in 20 min (Figure 4). Because only ~30 nmol of material is needed for the NMR analysis (~10.5 μg at a typical molecular weight of 350 g/mol), samples are usually not recovered, reducing sample handling time.

Evaluation as a High-Throughput System for Library Compounds. For the quality control of library compounds, dried aliquots are reconstituted in 10 μL of DMSO-d_6 to a final concentration of nominally 20 mM and heat-sealed in 384-well plates. Samples are then automatically picked up from the wells and processed with the One-Minute NMR system. Data acquisition of 128 scans results in spectra of good quality and ensures a throughput of ~5.5 samples/h or 130 samples/day (Figure 5). As with open-access samples, no attempt is made to recover the materials, since only ~200 nmol (70 μg at MW 350) is used for analysis. Finally, all spectra are stored in a chemical database where project chemists can manually inspect their data.

Because the amount of residual HOD varies among samples, the best NMR spectral results are obtained when using a 1D NOESY pulse sequence with presaturation, combined with automatic solvent peak-finding (Figure 5). Alternatively, samples can be prepared in protonated DMSO, and spectra can be acquired using either WET solvent suppression[23] or shaped pulses and ^{13}C decoupling for solvent suppression of residual water and the residual DMSO resonance line (Figure 6). In this case, limited mixing of the sample with some deuterated systems fluid from the dead volumes of the fluidic components and by flow dispersion is beneficial and provides the signal for the frequency lock. Automatic solvent peak-finding and shaped pulse calculations reliably result in high quality spectra, although as expected, resonances near the HOD signal at 3.4 ppm and close to the DMSO residual signal at 2.5 ppm may not be resolvable. In principle, the implementation of these experiments should allow use of protonated DMSO as the system fluid, which would further reduce the cost for deuterated solvent.

In the current configuration, 8 μL of sample is picked up from 384-well plates containing 10 μL per well and loaded into a 5-μL sample loop. Pickup and sample volume and loop size could be reduced further because the active volume of the microcoil probe is only 2.5 μL. The sample loop of 5 μL was chosen to match the volume of the CapNMR probe flow cell. Volumes of 5 μL for the loop and 8 μL for the pick up are reasonable compromises between sample consumption, robustness of the procedure, and resulting S/N in the NMR experiment.

The throughput limit has not been reached in the configuration described here. From the data shown in Figure 3, it is clear that the data acquisition time could be reduced for many samples while retaining an acceptable S/N. Reduction of the number of scans to 32 (1.5 min) is likely sufficient for many applications, rather than the 128 scans typically employed for quality control spectrum of a nominal 20 mM sample of a library compound that may contain impurities that need to be detected. Reducing the number of scans to 32 results in a sample-to-sample cycle time of 6.4 min. Finally, advances in multicoil architectures that employ multiple flow cells in a single probe hold promise for further gain in sample throughput.[26-28]

CONCLUSION

The objective of this study was to interface a microflow CapNMR probe to a CTC-PAL autosampler to implement an automated flow NMR system capable of performing two specific tasks: open-access analysis of single samples from LC/MS vials and high-throughput analysis and quality control of registered medicinal chemistry compounds and compound libraries from 384-well plates. In terms of open-access analysis of single samples, it is possible to obtain LC/MS data and NMR data from the same vial using 20 μL of 3 mM solutions of compounds. Only 10 μL of these samples is required for NMR analysis, and high-quality 1D proton and 2D COSY spectra can be acquired readily. Reasonably high throughput of ~130 samples/day for quality control of medicinal chemistry and library compounds can be accomplished, the biggest advantage being that 70-μg amounts of samples can be prepared in and measured fully automatically from 384-well plates that are filled robotically.

Because of the high mass sensitivity and low sample consumption of the One-Minute microflow NMR system, and because it can use D_2O or nearly any solvent while still being economical, the automated microflow system described and characterized here also has great potential for metabolite evaluation and other applications of biological interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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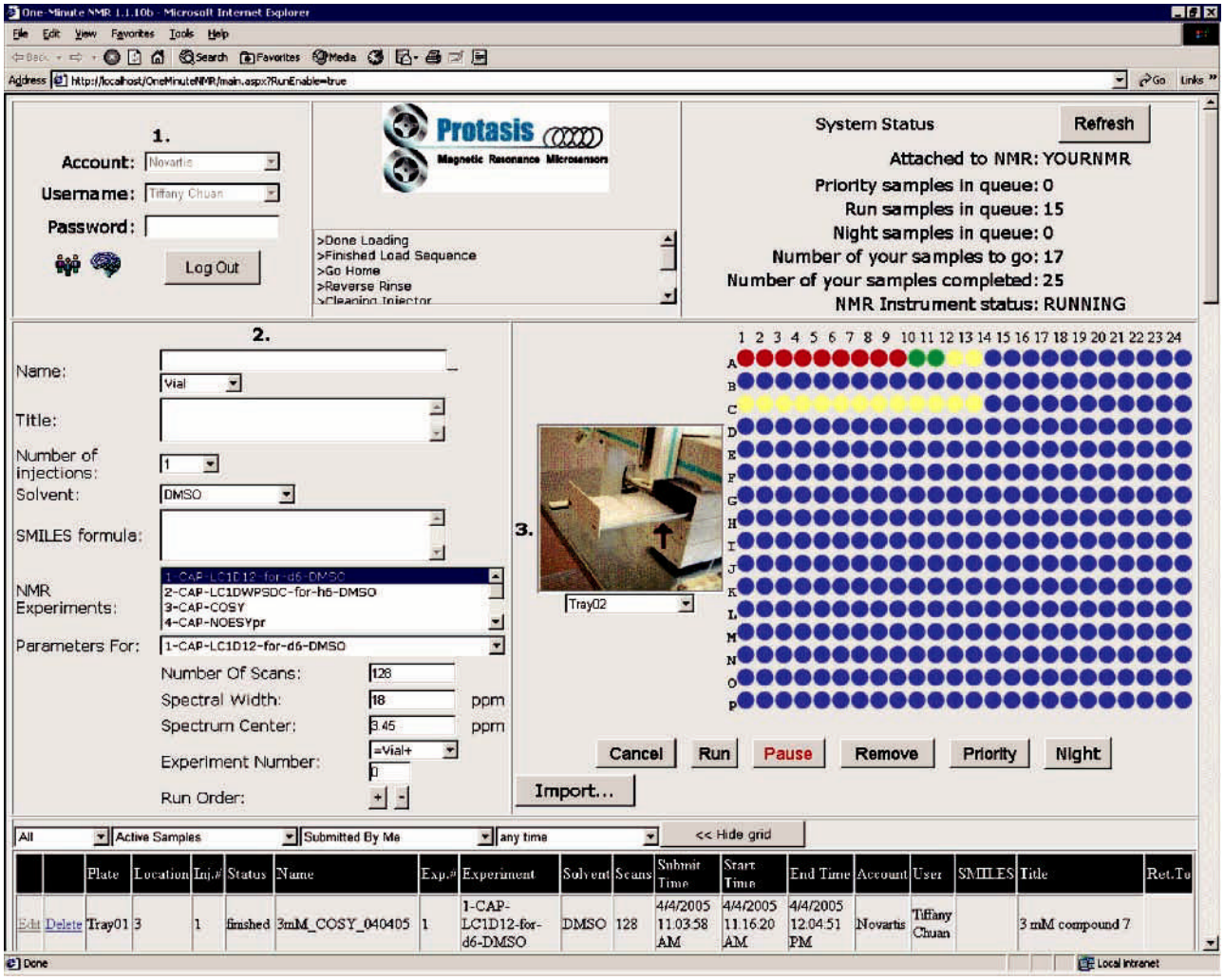

Figure 1.

One-Minute NMR web site and control panel. The three main sections are (1) user log-in; (2) sample identification and NMR experiment selection, including some basic NMR parameter settings; (3) tray holder selection, sample selection and grid, and priority designation. At the bottom is a table summarizing run conditions and status.

Figure 2.

Time course for the injection of samples in DMSO-d_6 using One-Minute NMR. All samples after the first sample are loaded into the sample loop while the prior sample is in the magnet. Injector cleaning of the autosampler is imbedded in the thermal equilibration time of the probe. A 1D NOESY experiment (LC1D12) with 128 scans and automatic solvent peak identification and suppression is used for NMR data acquisition. The NMR data acquisition time of 499 s includes 368 s for data collection (128 scans) and a combined total of ~131 s for locking, solvent peak identification, automated receiver gain setting, and data processing. The complete cycle (after the first sample) includes the transit time to move the sample from the loop to the probe flow cell (79 s), thermal equilibration of the sample in the probe (90 s), and data acquisition for an overall sample-to-sample total cycle time for these conditions of 11.1 min.

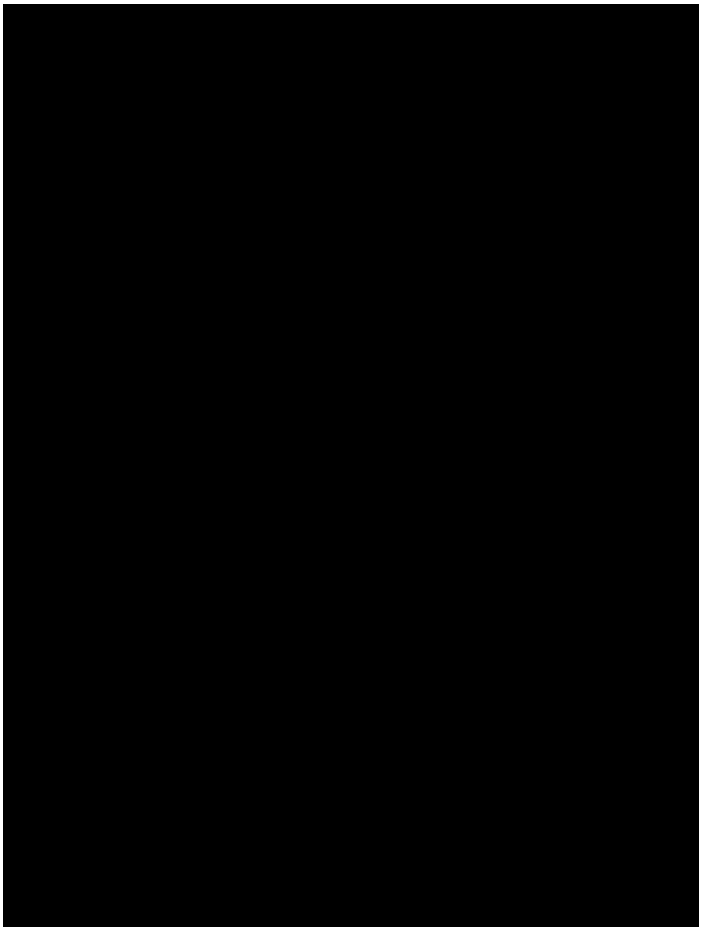

Figure 3.

NMR data acquired on four samples submitted in vials after sampling and analysis by LC/MS. Proton spectra for the model compound sulfachloropyridazine in DMSO-d_6 at several concentrations: (A) 20 mM, 32 scans (S/N ~ 54); (B) 20 mM, 128 scans (S/N ~ 223); (C) 10 mM, 128 scans (S/N ~ 97); (D) 5 mM, 128 scans (S/N ~ 61); and (E) 3 mM, 128 scans (S/N ~ 46). Signal-to-noise (S/N) values were calculated on the feature at 6.6 ppm using the signal region from 5 to 8 ppm and noise region from 9 to 11 ppm. Mass spectrometry data (not shown) were obtained using an Agilent 1500 LC/MS. The measured MW for samples in vials A–E were all 285.0 g/mol, in good agreement with the expected value of 284.7 g/mol. See Experimental Section for additional details.

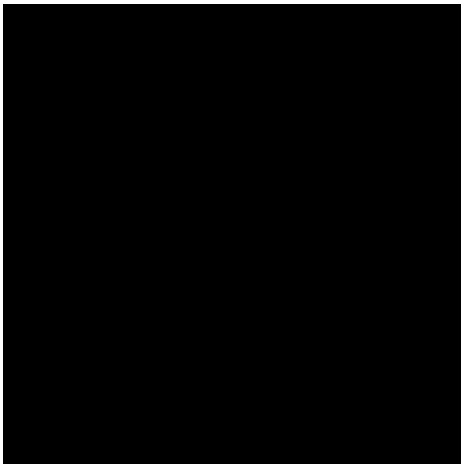

Figure 4.

Gradient-selected COSY[29](cosygpqf) of 3 mM sulfachloropyridazine in DMSO-d_6 (sample in Figure 3E). 256 t_1 increments with two scans each were recorded in 20 min using 2048 complex points in t_2. The spectral width in both dimensions was set to 17.954 ppm, 16 dummy scans with a recycle delay of 2 s at 25 °C. DMSO at 2.50 ppm was used to reference the spectrum. Cross-peaks for residual water and DMSO can be seen at 3.40 and 2.50 ppm, respectively.

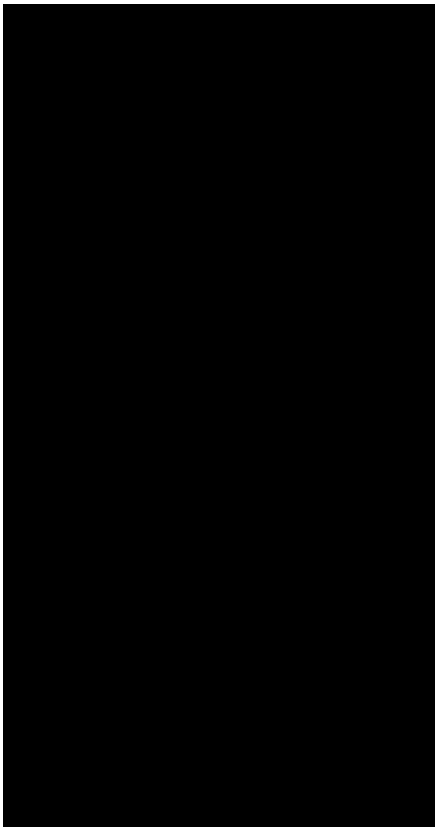

Figure 5.

Automatically acquired proton spectra of library compounds dissolved in DMSO-d_6: (A) sulfadimethoxine, (B) 4-(1-piperazinyl)-1H-indole, (C) sulfadiazine, (D) ethyl 3,5-dimethyl-2-pyrrolecarboxylate, (E) sulfamerazine, (F) gramine, (G) sulfapyridine, (H) sulfamethazine, (I) 6-sulfanilamidoindazole, (J) sulfachloropyridazine. 10 μL of 20 mM solutions in DMSO-d_6; final concentrations were submitted in a low-volume, 384-well plate. Each spectrum was acquired with 128 scans and with a cycle time of 11.1 min between samples. The residual water peak was suppressed using low-power, presaturation pulses after automatic solvent peak-finding (LC1D12). The average receiver gain was 152, and for the tallest peak between 5 and 8 ppm, the average S/N was 89. See Experimental Section for additional details.

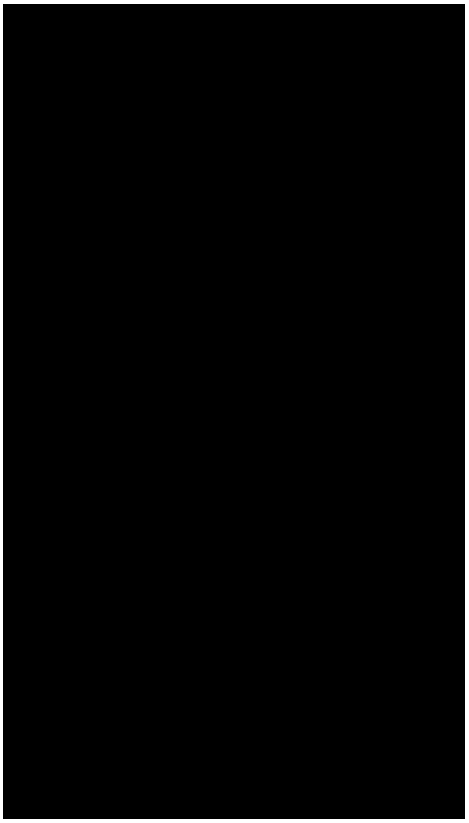

Figure 6.

Automatically acquired proton spectra of the same compounds as in Figure 5, but dissolved in protonated DMSO. 10 μL of 20 mM solutions was submitted in a low-volume, 384-well plate. Each spectrum was acquired with 128 scans and with a cycle time of 10.5 min between samples. The residual water and DMSO peaks at ~3.4 and 2.5 ppm, respectively, were automatically identified and suppressed using shaped pulses in a 1D NOESY pulse sequence and ^{13}C decoupling during acquisition. The average receiver gain was 119 and for the tallest peak between 5 and 8 ppm; the average S/N was 93. See Experimental Section for additional details.