



Microflow NMR for Well Plate Analysis

Goal

A recent analysis was carried out at Protasis/MRM Corporation using samples provided by an industrial pharmaceutical company to assess the effectiveness of 96-well plate automated NMR analysis at the capillary scale. Conventional VAST is currently employed at this company for 96-well plate NMR using approximately 200 μg of sample per well. The goal was to obtain quality NMR spectra using 10-fold less sample mass in the same amount of time or less. Results validate the enhanced mass sensitivity of Protasis/MRM capillary NMR probes and effectiveness of capillary sample management techniques for this application*.

Experimental Conditions

A 96-well plate consisting of 29 samples was analyzed using a Protasis/Varian μVAST NMR system, consisting of a Protasis/MRM 600 MHz CapNMR™ probe, a Gilson 215 liquid handler equipped with a Protasis High Throughput Sample Loader (HTSL), and a Varian Inova NMR spectrometer with Varian-modified VAST software. A diagram of the experimental setup is shown in Figure 1. As shown in Figure 2, the HTSL is seated on the bed of the 215. A sample loop plumbed into valve 2 of the HTSL is loaded by the 215. A software “handshake” signal delivered to the HTSL by the 215 under μVAST control facilitates transfer of the sample under high pressure through the capillary to the NMR flowcell in the CapNMR probe. In the present configuration the Gilson needle picks up 4 μL of solvent followed by 10 μL of sample. The entire 14 μL is loaded into the transfer capillary and sample loop (see Figure 3), resulting in virtually 100% filling of the 9 μL sample loop with sample. The contents of the loop are subsequently put on-flow and transferred to the CapNMR probe by the HTSL. Optimization to minimize total sample volume in the well was not studied in this investigation.

Sample preparation involved the addition of 25 μL of DMSO- d_6 (Cambridge Isotopes, 99.9% D) solvent to dried sample in each well, resulting in analyte concentrations from 1.80 to 3.12 mM. Sample molecular weights ranged from 196 to 424 g/mol. 10 μL of sample was picked up from each well using the Gilson needle, and 9 μL was injected into the CapNMR probe from the HTSL sample loop as described above. Air gaps were not employed. A custom rinse station insert supplied by Protasis/MRM was employed for needle rinse. Rinse and flushing parameters and cycle times are summarized in Table 1 at the end of this note. A 7.5 min NMR acquisition (128 scans) was used for each sample, resulting in a total cycle time per well of 10.0 min. A zero ppm marker was included in each well. All ^1H spectra were processed with a line broadening of 0.7 Hz.

Experimental Results

Successful analysis of all 29 samples was accomplished in under 5 hours. Representative spectra from two of the 29 wells are shown in Figures 4 and 5. Analyte parking accuracy was confirmed by the presence of the zero ppm marker in each spectrum. The samples were prepared on the benchtop, without the benefit of a dry, inert gas environment. Spectra were acquired without presaturation of the HOD signal. No reshimming was performed between samples. No appreciable sample carryover was observed under these conditions. Similar experiments conducted at MRM using DMSO have yielded carryover of approximately 1%.

* Automated, 96-well plate analysis is currently supported by Varian NMR.

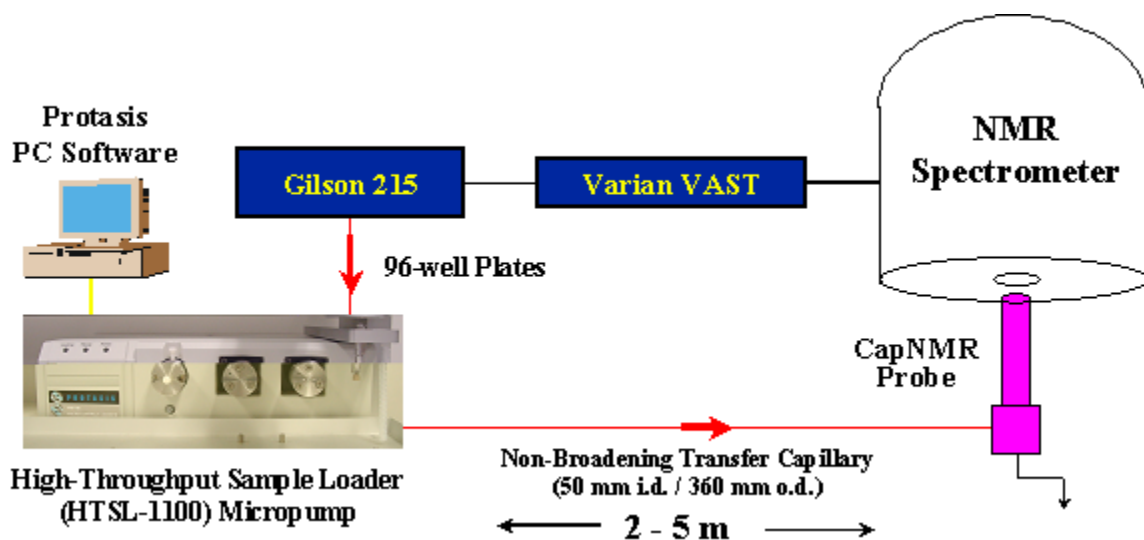


Figure 1. Automated Flow injection: 96-well plate μ VAST experimental setup consisting of Protasis/MRM CapNMR™ probe and High Throughput Sample Loader, Gilson 215 liquid handler, and a Varian 600 MHz Inova spectrometer with Varian μ VAST software.

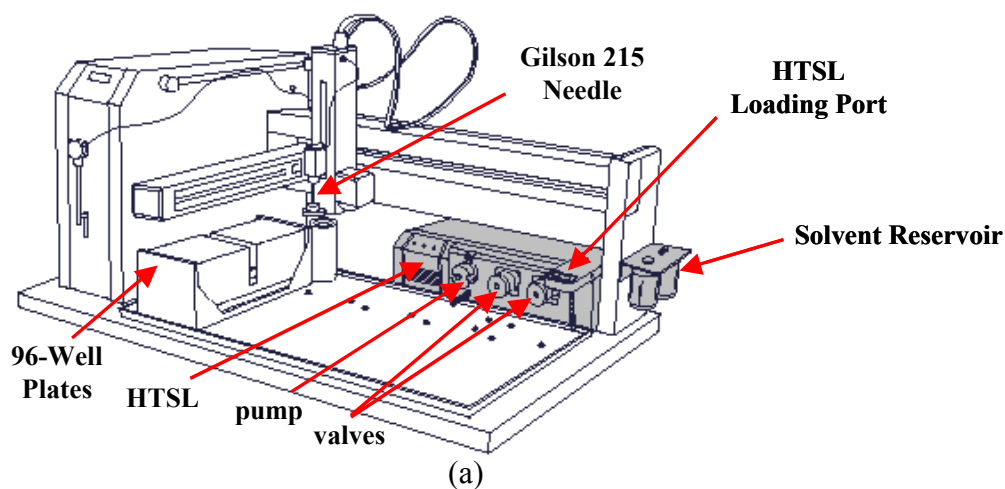


Figure 2. (a) schematic & (b) pictorial illustrations of the Gilson 215/Protasis HTSL platform for μ VAST/ μ BEST NMR.

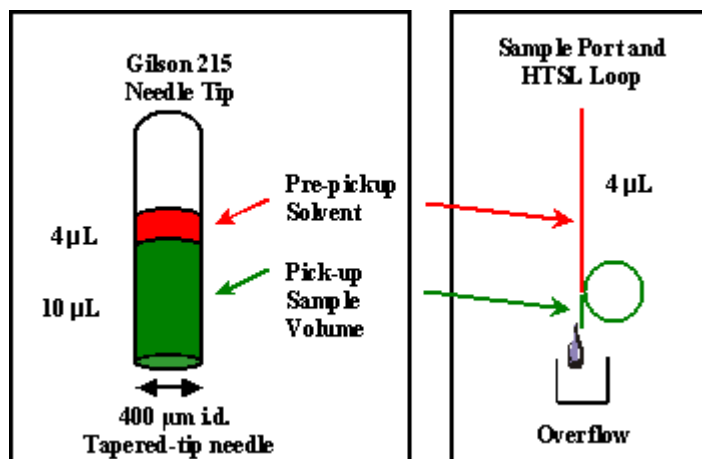


Figure 3. Illustration of the sample injection methods used for this study. 4 μL of push solvent is first picked up by the Gilson needle, followed by 10 μL of sample (from a 25 μL total sample volume in the well.) The contents of the needle are pushed through a short length of feed capillary to a 9 μL injection loop that resides on valve #2 of the HTSL (see Figure 2). The push solvent fills the feed line, and the 9 μL loop is filled with sample. At injection, the loop is placed on-flow and the sample is transferred to the NMR flowcell in the CapNMR probe for NMR detection.

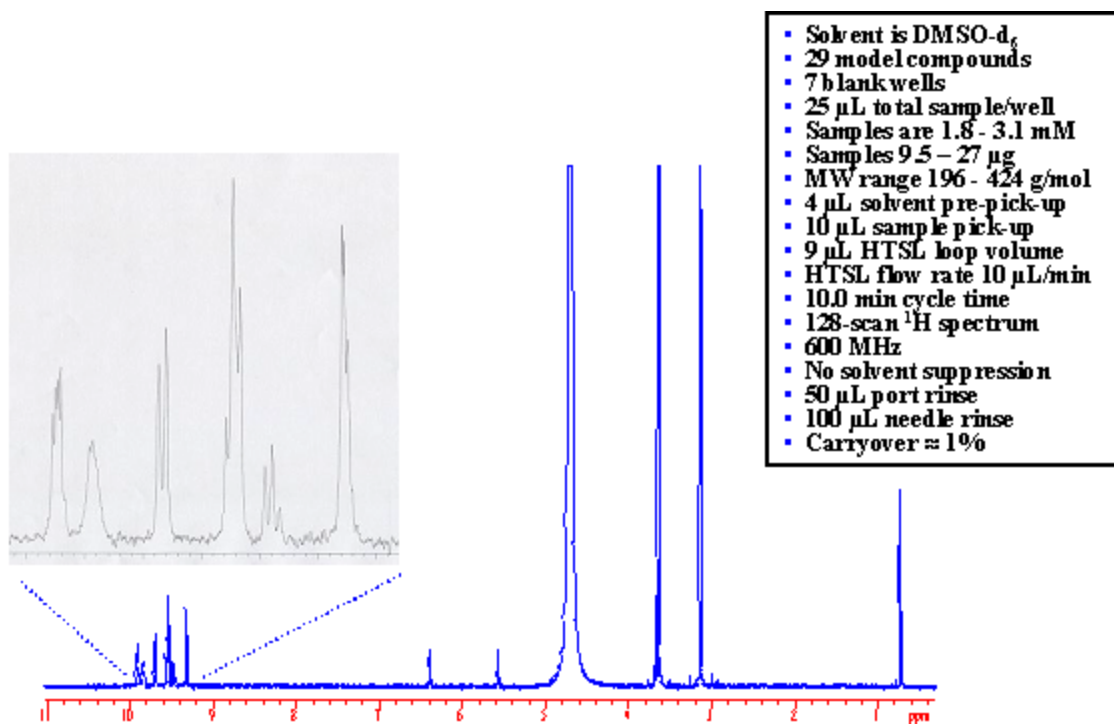


Figure 4. ^1H -NMR μVAST spectrum (well #5) corresponds to 2.56 mM trans-2,3-diphenyl-1-phthalimidoazirdine (340 g/mol). A total sample mass of 22 μg (64 nmol) was provided in the well. Experimental conditions are provided in the figure.

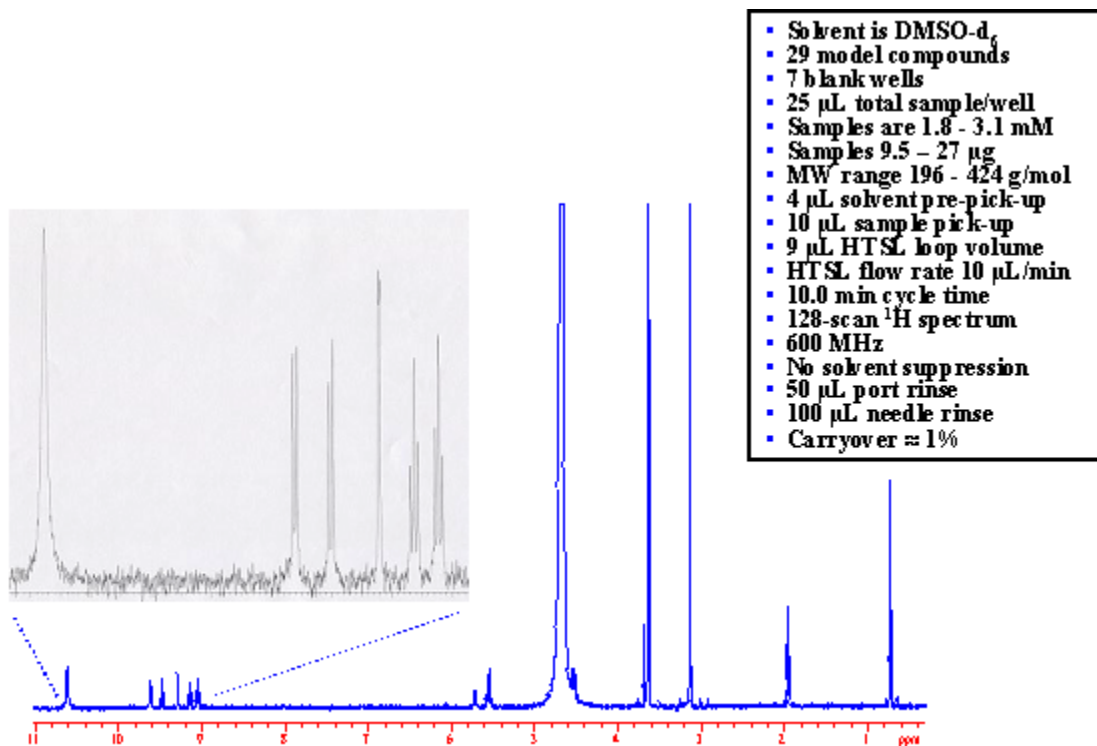


Figure 5. ¹H-NMR μ VAST spectrum (well #16) corresponds to 2.3 mM L-tryptophan ethylester HCl (364 g/mol). A total sample mass of 15.4 μ g (57 nmol) was provided in the well. Experimental conditions are provided in the figure.

Conclusions

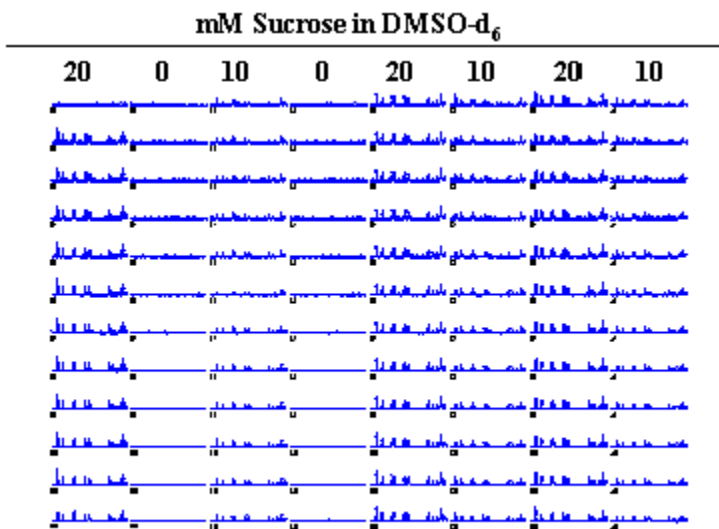
Time saved in running each well in 10 min is 23% compared to 13 min on conventional scale VAST. The amount of sample used is estimated at 90% less than conventional conditions. Solvent consumption is approximately 88% less than conventional conditions. Resultant S/N appears to be reasonably comparable to data provided by a customer from a conventional-scale VAST run. A comparative summary of solvent and sample consumption for both conventional and capillary-scale flow injection applications is provided in Table 1.

Reproducibility and Carry-over

Separately from the experiment described above, an assessment of reproducibility and carryover was performed at MRM using a 96-well plate loaded with patterned alternation of blanks and sucrose samples of 20 mM and 10 mM concentrations, show in Figure 6. The experimental conditions are shown in the figure. Quantitative reproducibility is \pm 5 %, and carryover is consist at a level of approximately 1 %. Attempts to minimize carryover to less than 1 % were not undertaken in this study.

Acknowledgements.

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- Solvent is DMSO-d₆
- 200 µL total sample/well
- Sucrose of 10 or 20 mM
- 4 µL solvent pre-pick-up
- 10 µL sample pick-up
- 9 µL HTSL loop volume
- HTSL flow rate 10 µL/min
- 4 min cycle time per well
- Single-scan ¹H spectrum
- 1 x 50 µL port rinse
- 1 x 100 µL needle rinse
- Carryover about 1%
- Sampling pattern:

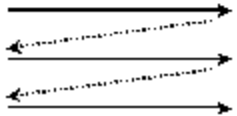


Figure 6. Assessment of well-to-well reproducibility and carryover using solvent blanks and sucrose samples of 20 and 10 mM in DMSO-d₆. As indicated in the figure, sample concentration was maintained consistent for each column in the well plate.

	Capillary		Conventional (typical)
Gilson Conditions			
Pre-pick-up solvent Volume	4 µL		N/A
Sample pick-up volume	10 µL		25 µL
Load volume (to fill injection loop)	14 µL		N/A
Port and needle rinse volume	150 µL		500 µL
Flow rate	100 µL/min		≈ 1 mL/min
Air gaps?	None		Yes
Injection Conditions			
Sample loop dead volume	4 µL		N/A
Sample loop volume	9 µL		N/A
Flow rate	10 µL/min		≈ 1 mL/min
Injection time	1.8 min		variable
Total cycle time per well	10.0 min		13 min
Total time per 29-wells	4.83 hr		6.28 hr
Total time per 96 wells	16.00 hr		20.8 hr
Solvent Use			
	29 wells	96 wells	96 wells
Sample wells	0.73 mL	2.40 mL	48 mL
Pre-pick-up solvent volume	0.116 mL	0.384 mL	N/A
Injection volume (required to deliver sample to NMR flowcell)	0.52 mL	1.73 mL	35.5 mL
NMR probe rinse volume	0 mL	0 mL	24 mL
Needle & port rinse volume	4.35 mL	14.4 mL	48 mL
<i>Solvent consumed/plate (total)</i>	<i>5.72 mL</i>	<i>18.91 mL</i>	<i>155.5 mL</i>

Table 1. Representative comparison of conventional and capillary-scale flow injection conditions.