Protasis
Magnetic Resonance Microsensors

Biofluids Analysis: Human Serum with High Throughput NMR

Analysis of biofluids can provide a detailed and specific view into pharmaceutically relevant processes and conditions; most importantly metabolism and disease state. One approach is to collect data on a large number of samples and treat these data statistically to tease out patterns that can lead to better understanding of the underlying biochemistry and, ultimately, better pharmaceuticals. MicroFlow NMR can speed this workflow on the front-end by requiring less sample for analysis and on the back-end with trouble-free high throughput analysis.

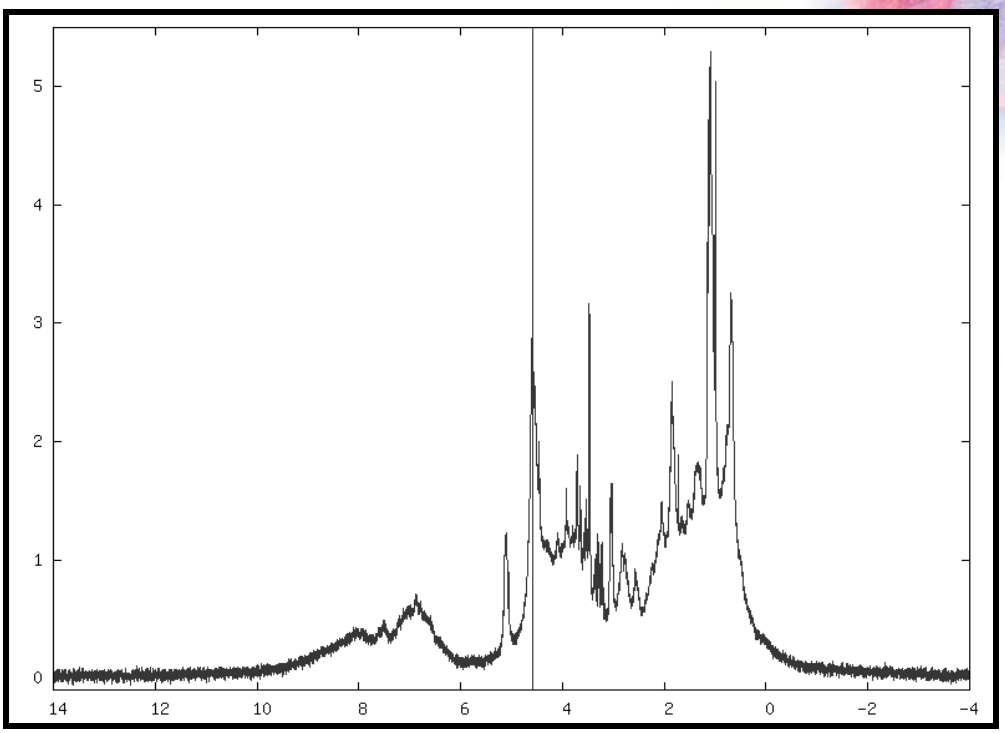

Figure 1. Typical Serum Spectra.

Representative human serum spectrum (straight serum with 10% D2O for spectrometer lock and a 11 minute acquisition at 600 MHz) showing good signal, baseline and resolution with MicroFlow NMR.

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Biofluid analysis provides rich information about changes in the concentrations and fluxes of endogenous metabolites involved in cellular pathways. The response of cells to different disease, drugs or toxins typically perturbs these processes and can be measured in biofluids. The patterns expressed in MS or NMR spectra can be used to identify specific disease states or external challenges to an organism. They can be used in research to provide clues leading the way into more detailed studies of the pathways involved.

Because urine and blood serum can be obtained easily, the volume of these samples in metabolic profiling applications is expected to increase dramatically as new instrumental methods are developed. Automating the routine analysis of complex biofluids is critical to fully exploiting the information available in the metabolome. MicroFlow NMR spectroscopy is especially suited for biofluid applications because it is fast, non-destructive, requires minimal sample preparation and can be performed with only a few microliters of fluid. As serum and urine analysis find increased usage in a broad range of medical research and diagnostic applications, MicroFlow NMR will help analysts keep up with this expanding workload.

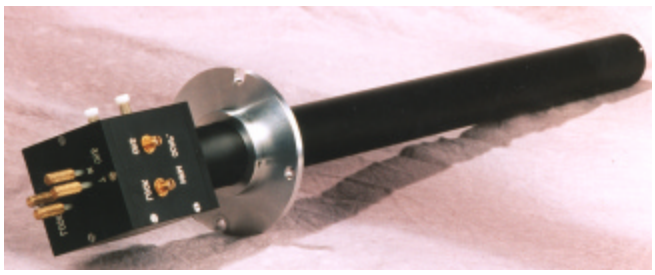
- ✓ Capillary-scale NMR shown effective for raw serum and urine analysis.
- ✓ Protasis OneMinuteNMR provides hands-free, “tubeless NMR” methods for efficient high throughput NMR automation.
- ✓ Carryover is virtually eliminated using prescribed automation protocols for flowpath rinsing.

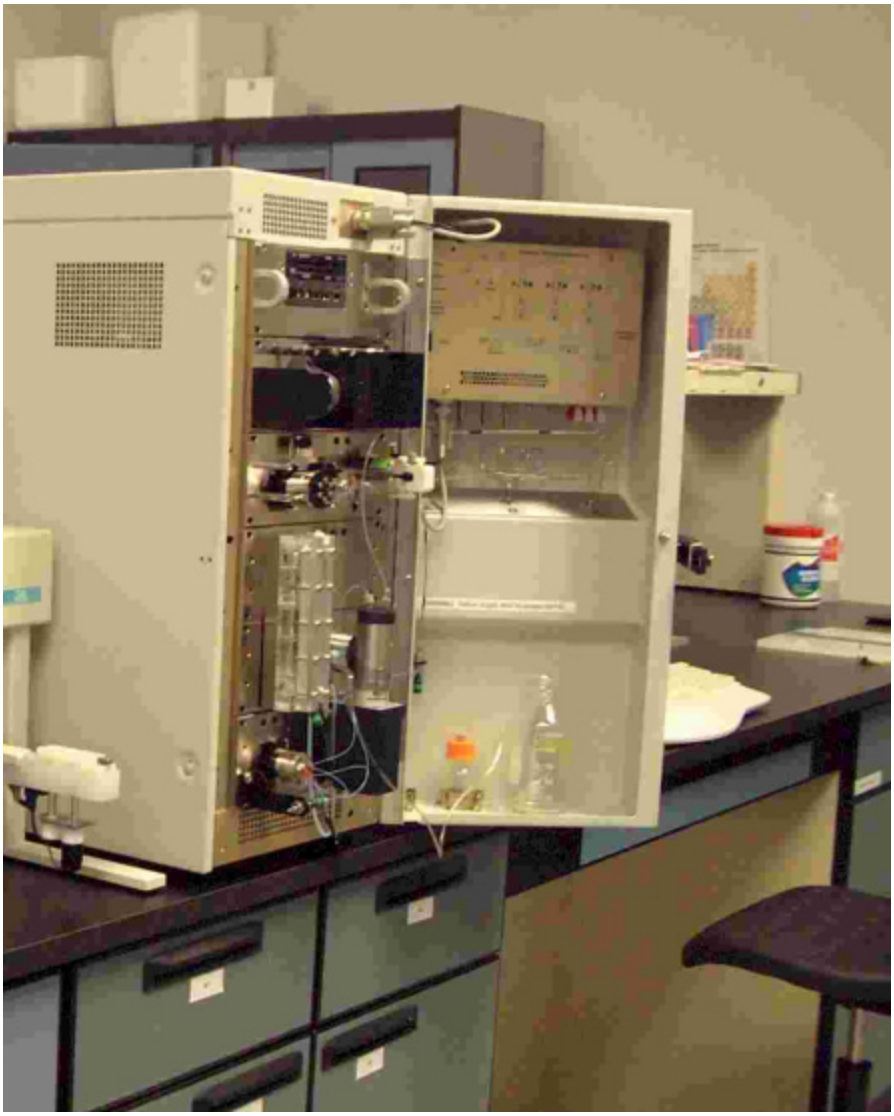
Introduction

Novatia, LLC has recently introduced contract NMR services, requiring only microliters and micrograms of sample in well-plate format for NMR analysis. Novatia is able to offer this progressive service due to the recent acquisition of a Protasis/MRM capillary NMR automation system. The system, illustrated in Figure 1, consists of a Protasis CapNMR probe, a Leap Technologies capillary-scale LC PAL liquid handler, Protasis Discovery Tower fluidic platform, and Protasis OneMinuteNMR ("OM-NMR") automation control software. One of the more popular requests involves analysis of human serum samples. Serum is considered to be one of the most challenging biological fluids for flow NMR analysis due to its high viscosity, molecular complexity, and protein content. High-resolution ^{1}H-NMR spectra are collected from a series of 10 uL injections of 90%/10% mixtures of serum/D_2O. Sample-to-sample carryover is maintained at approximately 1% using a simple, pre-programmed injector rinse protocol. Further reduction in carryover is readily achieved using an optional flowpath rinse, the details of which are described below. Carryover concerns can be effectively eliminated using this combined approach.

Figure 1. Protasis MicroFlow NMR Automation system consisting of Protasis CapNMR probe, a CTC/Leap Technologies capillary-scale LC PAL liquid handler, Protasis Discovery Tower fluidic platform, and Protasis OneMinuteNMR

The OneMinuteNMR automation system readily facilitates easy implementation of fluidic protocols and sample-specific procedures by the user while simultaneously providing complete control of NMR acquisition conditions and NMR data management in a centralized manner from the OneMinuteNMR graphical user interface screen. The results of this study show the successful application of the Protasis capillary NMR automation to human serum samples, and support a new means of streamlined, tubeless NMR for biofluid analysis. Novatia's confidence in the Protasis microflow automation system and capillary NMR analysis has enabled Novatia, at the time of creation of this application note, to enter into a new contract involving capillary NMR analysis of thousands of serum samples. This area of service work has rapidly become a profitable component of Novatia's core business.



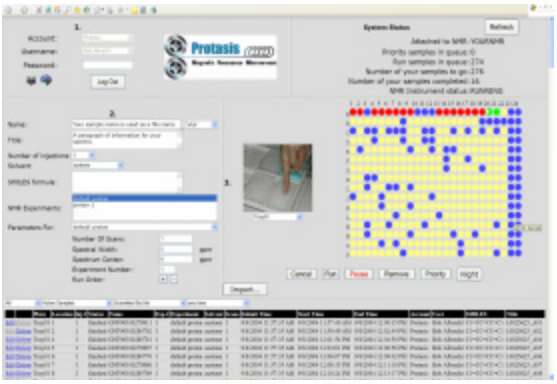


Experimental

Samples. Twenty-four (24) raw human serum samples (12 from male participants, 12 from female participants) were received and divided into 90 uL aliquots in a series of 300 uL snapcap polypropylene vials. To each vial, 10 uL of D_2O was added for NMR spectrometer frequency lock.

NMR Automation. All data were acquired at 14.1 T using a Protasis capillary automation system and a Varian Inova NMR spectrometer. The CapNMR probe was a triple resonance ($^1H/^{13}C/^{15}N$) fused silica capillary probe with z-gradient and 5 uL enhanced NMR flowcell. NMR spectra were collected to correspond to the first increment of a presat pulse sequence (nt = 256, satdly = 1s) with sample temperature at 25C. The LEAP LC PAL was equipped with a 6 port stainless steel injection valve coupled to a Protasis HTSL fluidic delivery system for sample transport.

The fluidic configuration is shown in Figure 2. Approximately 3 meters of 75/360 (ID/OD, mm) FEP (Teflon) transit tubing was employed between the liquid handler and the probe. OneMinuteNMR software was configured as outlined in Table 1. A sample pickup volume of 10 uL was injected into an 8 uL sample loop plumbed into the stream select valve of the Protasis HTSL. Flowrates of 15 uL/min were employed for both sample delivery and refill. A Protasis reverse rinse injector cleaning protocol was employed to maintain filter longevity.



Push solvent:	D_2O
Sample pickup volume:	10 uL
Sample loop:	8 uL
Delivery flow rate:	15 uL/min
Refill flow rate:	15 uL/min

Table 1. Fluidic Parameters programmed into the OneMinuteNMR software.

Rinse Protocol

A rinse protocol for the injector assembly (syringe, needle, valve, and port-in-valve filter) of the instrument configuration was programmed into the OneMinuteNMR automation software. This 5-step protocol (Table 2) involved a combination of aqueous and organic reagents resident in the wash station of the Leap PAL autosampler. Wells of D_2O were employed as sample "blanks" for quantitative measurement of carryover. Each blank was programmed as a sample in the OneMinuteNMR injection protocol, i.e., injector rinse was performed after injection of the blank in a manner analogous to sample injection. In addition, flowpath rinsing was investigated for further reduction in carryover. The rinse agents were treated as samples in the OneMinuteNMR injection protocol. Three rinse agents were investigated, shown in Table 3.

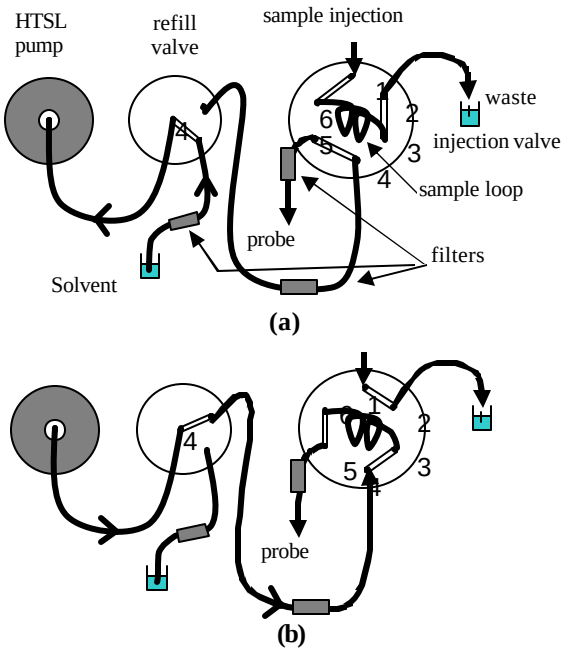


Figure 2. Fluidic configuration employed in this study. Components of Figure 2 are packaged in the instrument modules shown in Figure 1. (a) Configuration employed for sample injection, where sample is injected into the sample loop. Solvent refill from solvent reservoir to pump is accommodated during this phase. The probe is sealed during this phase so that sample previously positioned in the probe can undergo NMR data acquisition. (b) Configuration employed for sample load, where sample is transferred from the injection loop to the NMR flowcell in the CapNMR probe. The injection loop is flushed with push solvent used to transfer the sample to the probe. The injector assembly (syringe, needle, valve, and port-in-valve filter) is rinsed during this phase to prepare for subsequent sample injection

Carryover

A single serum sample was selected and injected multiple times for quantitative investigation of sample-to-sample carryover. In each case, the sample was injected using a standard OneMinuteNMR injection protocol. Blank (D_2O) samples were employed as described previously for quantitative measurement of carryover. All spectra were processed with 0.7 Hz line broadening and drift correction. Spectral integration was employed from 0 to 2.5 PPM for the serum samples, and from 0 to 2.5 PPM and from 5 PPM to 7.5 PPM for the blanks.

Step	Action
1	30 uL syringe rinse, D_2O/d-ACN
2	30 uL syringe rinse, D_2O
3	100 uL injection of D_2O/d-ACN from wash station to waste
4	100 uL injection of D_2O from wash station to waste
5	Reverse rinse of 5 uL to backflush injection port-in-valve filter

Table 2. Rinse protocol executed between sample injections to ensure clean syringe, needle, injection valve, and injection loop

A series of differing flowpath rinse agent conditions were employed (see Table 4, results) to assess the effect of flowpath rinse on carryover. The mean and standard deviation of the spectral integrals were calculated for each run.

Flow Rinse Agent #1	D_2O
Flow Rinse Agent #2	10% H_2O_2
Flow Rinse Agent #3	70% Formic Acid, 30% ACN[1]

Table 3. Rinse agents employed for flowpath rinse in this study.

Multiple rinses or combination of different types of rinses could be have been scheduled using OneMinuteNMR, if more study had been warranted.

Results

A picture of the experimental configuration owned by Novatia and used for NMR sample analysis service is provided in Figure 3. Novatia is undergoing a rapid business growth in the new area of NMR sample analysis due primarily to the acquisition of the Protasis automation instrumentation previously described.

Figure 4 illustrates a representative spectrum of five injections of human serum obtained in this study. This data demonstrate excellent water suppression performance with good baseline and resolution. The protocols built into the OneMinuteNMR automation software readily enabled the integrity of the flowpath to be maintained; not a single occurrence of blockage or restriction was observed in this study that involved over 40 serum injections.

Figure 3. Dave Detlefsen, and the Protasis Capillary Automation system employed for sample analysis services offered by Novatia, LLC

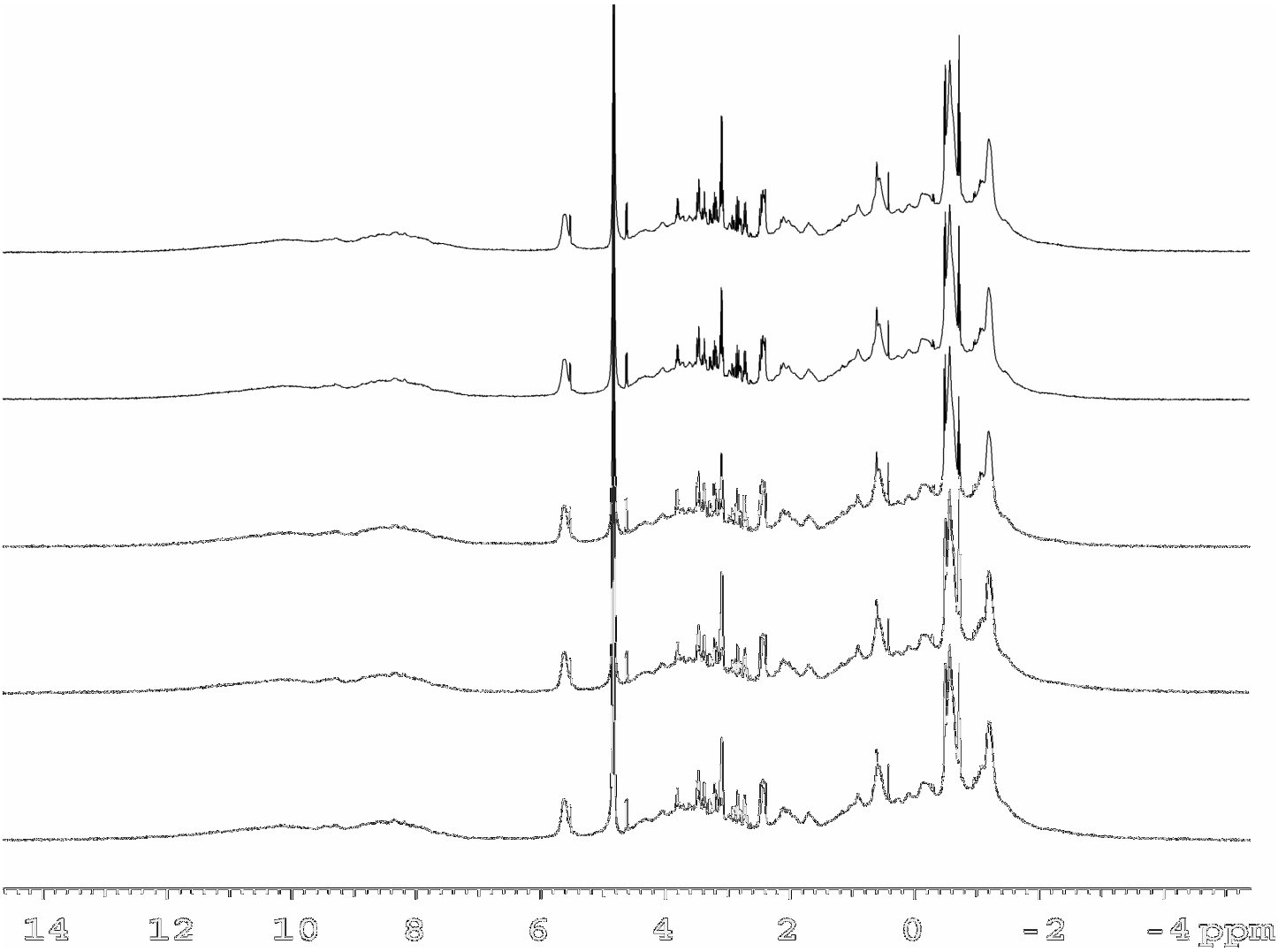


Figure 4. Stacked ^{1}H-NMR spectrum of human serum showing repeatability. Five samples prepared identically from the same stock serum and injected separately illustrate the good water suppression, excellent baselines and high resolution of MicroFlow NMR.

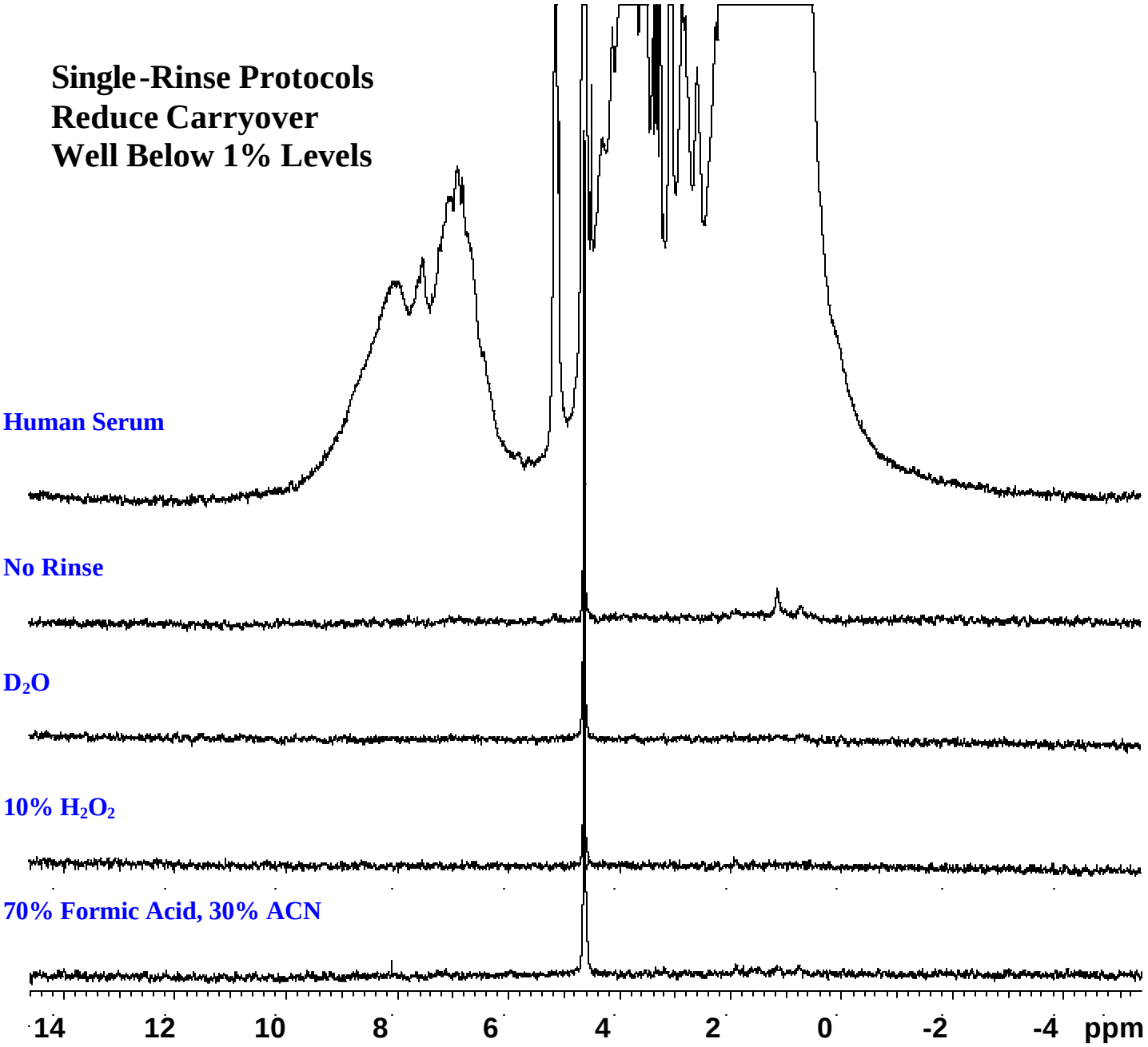


Figure 5. **^{1}H-NMR spectrum of Human Serum (top spectrum) and subsequent injections of "blank" D_2O samples for quantification of sample-to-sample carryover. All injections included the injector rinse protocol of Table 2. In addition, flowpath rinse agents (Table 3) were optionally employed as shown in the figure to further reduce carryover. Carryover data is provided in Tables 4 and 5.**

Figure 5 provides a visual assessment of sample-to-sample carryover. The top spectrum in Figure 5 represents a human serum sample. Lower spectra shown in Figure 5 represent subsequent "blank" (D_2O, described previously) injections after flowpath rinse employing the rinse agent(s) shown for each spectrum in the figure.

Sample	Spectral integral 0 - 2.5 PPM
Serum #1	35.335
Serum #2	37.104
Serum #3	34.720
Serum #4	36.364
Serum #5	34.700
Serum #6	35.652
Serum #7	34.984
Serum #8	33.922
Serum #9	33.702
Serum #10	33.755
Mean	**35.024**
St. Dev.	**1.125**

Table 4. Spectral integration from 0 to 2.5 PPM for 10 randomly selected serum samples injected into a previously cleaned flowpath.

Statistical data are provided in Tables 4 and 5 for quantitative analysis of carryover. Shown in Table 4, the value for spectral integration from 0 to 2.5 PPM is provided for 10 randomly selected serum samples injected into a previously cleaned flowpath. Also shown are the statistical mean and standard deviation for these data. Provided in Table 5 are similar data representing the injection of D_2O sample "blanks." The first series of blanks were injected without the prior use of any flowpath rinse agent beyond normal solvent push, whereas the second through fourth groups of blanks correspond to the rinse agents of Table 3, i.e. 100% D_2O, 10% H_2O_2, and 70%/30% Formic Acid/ACN, respectively.

Sample	Flowpath rinse prior to sample injection	Spectral integral 0 - 2.5 PPM
Blank #1	No Flowpath Rinse	0.522
Blank #2	No Flowpath Rinse	0.418
Blank #3	No Flowpath Rinse	0.403
Blank #4	No Flowpath Rinse	0.359
Blank #5	No Flowpath Rinse	0.326
Blank #6	No Flowpath Rinse	0.390
Blank #7	No Flowpath Rinse	0.392
Mean		**0.401**
St. Dev.		**0.062**
Blank #8	D20	0.133
Blank #9	D20	0.202
Blank #10	D20	0.284
Blank #11	D20	0.194
Blank #12	D20	0.110
Mean		**0.185**
St. Dev.		**0.068**
Blank #13	10% H2O2	0.127
Mean		**0.127**
St. Dev.		-
Blank #14	70% Formic acid/30% ACN	0.135
Blank #15	70% Formic acid/30% ACN	0.136
Mean		**0.136**
St. Dev.		**0.001**

Table 5. Spectral integration from 0 to 2.5 PPM for 15 injected blanks. Prior flowpath rinse conditions are provided.

Using the mean integral values of the blanks of Table 5, and comparing with the mean integral value of the sample in Table 4, the carryover is determined to be 1.1%, 0.53%, 0.36%, and 0.39% for the conditions of no flowpath rinse (beyond that inherent to the push solvent required for sample positioning in the NMR flowcell), D_2O rinse, 10% H_2O_2 rinse, and 70% Formic acid/30% ACN rinse respectively.

Summary

An analysis of biofluids (blood, urine, cell extracts, etc.) provides a snapshot of the biological processes of an individual that can be used in applications from diagnostics to drug efficacy and toxicity. This report detailed Novatia's experiences with human serum during initial setup for contract analysis of human serum where the carryover features of the capillary NMR system was of critical importance to the customer. The results of this study show the system performs extremely well, providing high quality data in an automated and robust manner with low (1% or less) carryover. In addition, we have collected urine data for other customers with similarly excellent results. The Protasis capillary NMR automation system is an essential component of Novatia's biofluid NMR analysis package and allows us to provide a unique service to the research community.

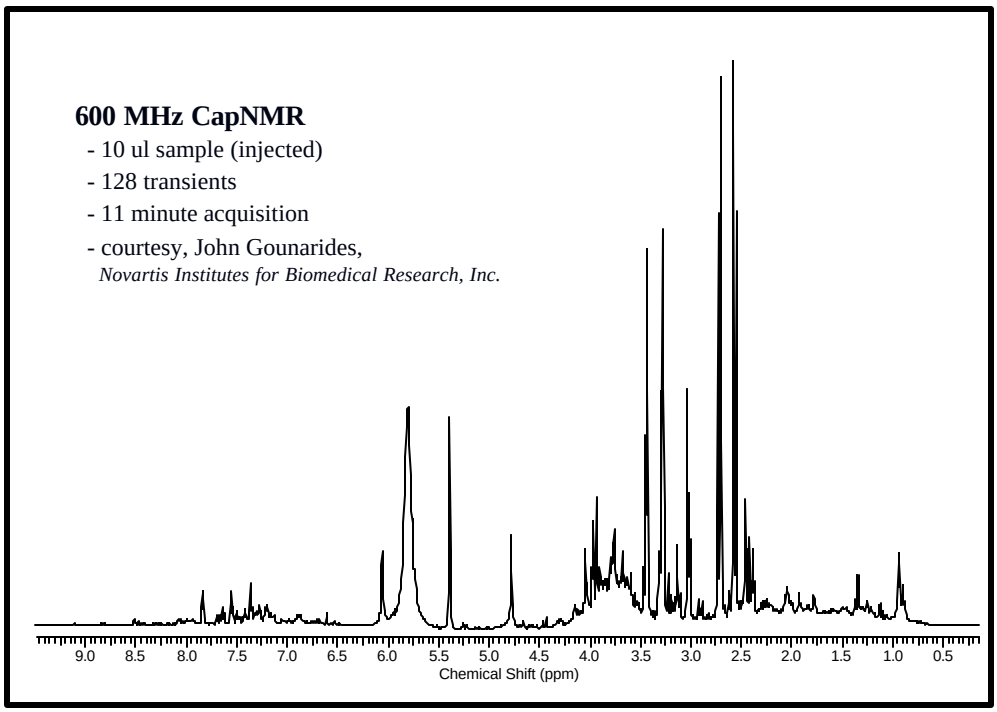


Figure 6. ^{1}H-NMR Mouse urine sample collected by D. Detlefsen for client.

Novatia

To contact Novatia about running your NMR samples,

Call Dave Detlefsen at (609) 951-0181

References

1. Hail, M. E., Nedved, M.L., Warrack, B. M., Thompson, W.L., Lee, M.S., 44th ASMS Conference on Mass Spectrometry and Allied Topics, 1996.

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