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ARTICLE

The application of micro-coil NMR probe technology to metabolomics of urine and serum

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Abstract Increasing the sensitivity and throughput of NMR-based metabolomics is critical for the continued growth of this field. In this paper the application of micro-coil NMR probe technology was evaluated for this purpose. The most commonly used biofluids in metabolomics are urine and serum. In this study we examine different sample limited conditions and compare the detection sensitivity of the micro-coil with a standard 5 mm NMR probe. Sample concentration is evaluated as a means to leverage the greatly improved mass sensitivity of the micro-coil probes. With very small sample volumes, the sensitivity of the micro-coil probe does indeed provide a significant advantage over the standard probe. Concentrating the samples does improve the signal detection, but the benefits do not follow the expected linear increase and are both matrix and metabolite specific. Absolute quantitation will be affected by concentration, but an analysis of relative concentrations is still possible. The choice of the micro-coil probe over a standard tube based probe will depend upon a number of factors including number of samples and initial volume but this study demonstrates the feasibility of high-throughput metabolomics with the micro-probe platform.

Keywords Metabolomics · Micro-coil probe · High-throughput · Mass-sensitivity · Biofluids

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Introduction

Metabolomics involves the holistic profiling of metabolites to look for correlations between metabolite levels and disease or toxicity. Applications of metabolomics are experiencing exponential growth in diverse areas including drug development (Keun and Athersuch 2007; Schlotterbeck et al. 2006) and safety assessment (Griffin and Bollard 2004), disease diagnosis and monitoring (Gowda et al. 2008) and even human epidemiological phenotyping to assess disease risk (Bictash et al. 2010; Holmes et al. 2008). As the applications of metabolomics grow, so do the demands for sensitivity and throughput. NMR has been one of the most widely used analytical platforms in metabolomics since the field began. Features such as the long term stability, reproducibility, inherently quantitative signals and non-destructive nature make NMR ideal for metabolomics studies. The challenge for NMR has always come from the relatively low sensitivity as compared to other methods such as mass spectrometry.

The sensitivity issue is a formidable challenge when large studies require high-throughput and sample amounts may be quite limited. Metabolomics is widely used in rodent models of disease and toxicity but biofluid or tissue volumes from these studies can be quite small. In the case of a time-course study, serial blood draws from a mouse may yield only 50 µl of blood which gives only about 25 µl of serum for metabolomics analysis. Even with large scale human studies where the samples volumes collected from the subjects may be ample there are often large demands on these samples. Standard clinical chemistry tests as well as other omics

investigations such as transcriptomics and proteomics complete for the available samples. For NMR to continue as a front line analytical platform for large scale metabolomics studies the ability to generate high quality data from small sample volumes is critical.

The two main approaches to increasing sensitivity of NMR involve the use of higher field magnets and the development of more sensitive NMR probes. Ultra-high field magnet technology has finally broken the 1 GHz barrier (Bhattacharya 2010), but the cost of such a system prohibitive for most laboratories. The cost of purchasing and installing a 900 MHz NMR can be an order of magnitude higher than the cost of a 600 MHz NMR while the gain in sensitivity is slightly less than double. Cryogenically cooled probes have provided sensitivity increases of three to four-fold (Logan et al. 1999). This technology has been well applied to metabolomics (Griffin 2003), but the complexity of installation often dictates that an instrument be dedicated to this probe which can limit the versatility of the NMR platform. The other avenue of probe sensitivity improvement involves the development of micro-coil technology (Olson et al. 2004; Schroeder and Gronquist 2006). In these probes the sample volumes are down in the micro liter range. This small size enables the use of the more sensitive transverse solenoid coil design which yields a significant sensitivity increase (Hoult and Richards 1976; Webb 1997). Sensitivity gains are also enabled in these probes by having the receiver coil physically closer to the sample i.e. the coil is more magnetically coupled to the sample. This coil design increases the amount of the sample that is within or very near to the detection coil, called the observe factor. For a standard 5 mm NMR probe the observe factor is typically about 0.33 whereas for the 10 µl micro-coil probe used in this study the observe factor is 0.5. In total it has been determined that the mass sensitivity (sensitivity of the probe to receiving signals from a defined number of nuclei) is approximately tenfold greater than a conventional NMR probe (Olson et al. 2004).

In this study an evaluation of the performance of a 10 µl micro-coil NMR probe for metabolomics studies of urine and serum with limited sample volumes is made. This micro-coil probe is integrated with a high-throughput fluidics automation system which takes samples from either vial or well plate formats. We examine the signal to noise and quantitation of metabolites in routine samples and with varying degrees of concentration in order to increase the amount of sample in the receiver coil, thereby leveraging the improved mass sensitivity. Theoretically the increase in sensitivity that is achieved by concentrating the samples should go linearly with concentration, but factors such as metabolite solubility, high salt concentrations and other matrix effects must be considered. This study aims to determine the practical details of maximizing the efficient use of a high-throughput micro-coil automation system for sample limited metabolomics studies.

Materials and methods

Sample collection and preparation

Urine and serum samples were collected at the University of North Carolina General Clinical Research Center. The subjects in this study were part of the placebo group in a clinical study of acetaminophen. Full details of this study are reported by Harrill et al. (2009) Fasted blood samples were drawn at 8:00 am and 24 h urine samples were collected and frozen at −80°C. Frozen serum and urine samples were thawed and mixed with a deuterated solution containing 100 mM phosphate buffer with 5 mM formate and 5 mM trimethylsilylpropionate-d_4 (TSP) as a chemical shift and quantitation standard. All of the serum was passed through a 10 KDa molecular weight centrifugal filter to remove proteins. The tube based samples were prepared by diluting the samples to 600 µl with the deuterated buffer solution and placing the solution in a 5 mm NMR tube. Sample concentration was carried out by taking a 200 µl aliquot of the biofluid and bringing it to dryness in a vacuum centrifuge. The extent of concentration was determined by the amount of buffer used to resuspend the dried material.

^{1}H NMR spectroscopy

All NMR spectroscopy was performed on a Varian INOVA spectrometer (Agilent (formerly Varian Inc.), Palo Alto, CA) operating at 599.64 MHz (^{1}H frequency) and 25°C. The 5 mm probe is a Varian pulsed field gradient, inverse detection probe. The micro-coil probe is a Protasis/MRM 10 µl capillary NMR probe (Magnetic Resonance Microsensors, Savoy, IN). Samples were introduced into the micro-coil using a Protasis High-throughput Sample Automation System (Protasis, Marlboro, MA). A simple presaturation pulse sequence was used which included a 1 s solvent presaturation, followed by an Ernst angle optimized read pulse and a 2.3 s acquisition delay. The Ernst angle was based upon an assumed average metabolite T1 of three seconds. A sweep width of 7,195.5 Hz was digitized with 16,384 complex points. For the serum spectra shown in Fig. 1a 100 ms CPMG sequence was added between the presaturation delay and the read pulse. A total of 256 transients were collected for all serum and urine samples requiring a total of approximately 20 min.

Data processing and analysis

All spectra were processed with the ACD 1D NMR Manager software version 12, (Advanced Chemistry Development, Toronto, Canada). Linear back prediction of the first two points was carried out with 32 coefficients and

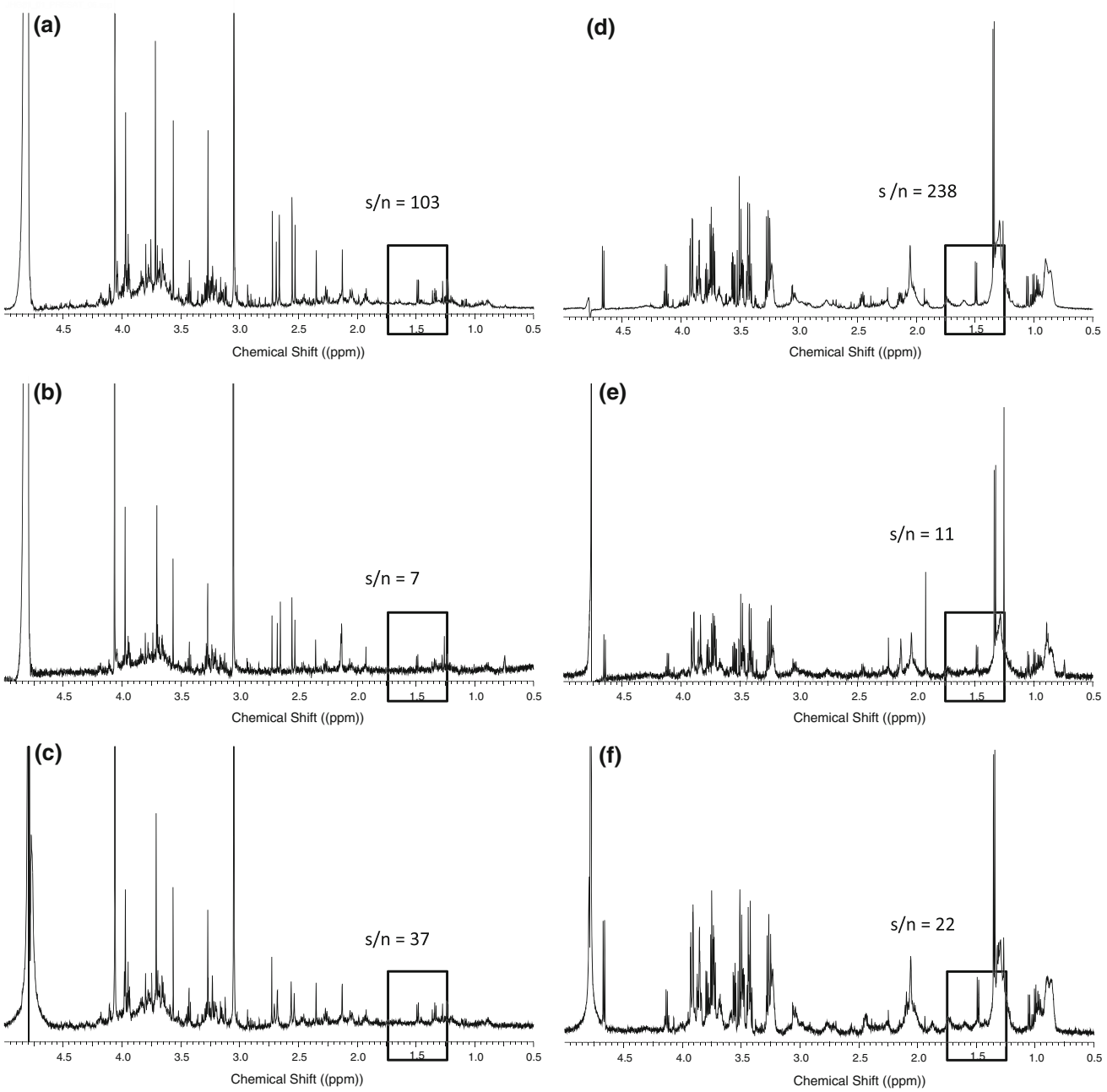

Fig. 1 Comparison of urine and serum spectra with different starting volumes using a 5 mm probe versus a 10 µl probe. Starting with 200 µl of urine (**a**) and serum (**d**) the sample was diluted to 600 µl with buffer and the spectrum acquired with a 5 mm NMR probe. Starting with only 10 µl of urine (**b**) and serum (**e**), spectra were acquired after dilution to 600 µl and data collection with the 5 mm probe. Using the micro-coil probe, 10 µl was dilute to 15 µl and the urine (**c**) and serum (**f**) spectra were acquired. The signal to noise was calculated using the alanine peaks for all spectra as indicated. Signal to noise calculations using other peaks are listed in supplemental Table 1

base points from 3 to 512. An exponential line broadening of 0.3 Hz and zero filling to 32,768 points were applied to each spectrum After Fourier transformation, each spectrum was manually phased and an automated 6th order polynomial baseline correction algorithm was applied over the entire spectrum. The chemical shifts in both urine and serum were referenced to the formate peak at 8.47 ppm. Signal to noise calculations were carried out with the ACD Calculate RMS Noise function with automatic detection of noise region. Metabolite quantitation was carried out using the Chenomx NMR Profiler software version 5.1 (Chenomx, Edmonton, Canada). Metabolite identifications

were confirmed using TOCSY experiments as well as literature tables (Holmes et al. 1997; Nicholson and Foxall 1995).

Results and discussion

Comparison of standard versus micro-coil probe for biofluids

The increased mass sensitivity of the micro-coil NMR probe is a significant advantage with volume limited samples, but the question needs to be asked, at what point is a sample really defined as volume limited. In Fig. 1, spectra for both urine and serum are shown for three different sample limited situations. These are human samples and each biofluid is from a single subject so the composition of the metabolites in the spectra should be the same. In Fig. 1a, a 200 µl sample of urine has been diluted to 600 µl and the spectrum acquired with a 5 mm NMR probe. The signal to noise ratio of the spectrum, based on the alanine peaks is determined to be 103. In this case the 5 mm probe has a concentration sensitivity advantage. Concentration sensitivity is defined as the sensitivity of the probe to detecting a defined number of nuclei per unit volume. Figure 1b shows the spectrum of a sample of 10 µl of urine, diluted to 600 µl. With the initial sample volume going down by a factor of 20 a linear decrease in s/n would be expected. The s/n for alanine was determined to be 7 which is roughly the expected value. Figure 1c shows the spectrum from a 10 µl urine sample, diluted with 5 µl of buffer and injected into the micro-coil probe. The s/n ratio for alanine has increased by approximately fivefold to 37 by using the micro-coil probe. A table with the s/n values for other selected metabolites is given in the supplementary information. The average sensitivity increase is 2.9-fold. This advantage could be increased if a smaller volume of buffer was added, but for practical reasons a 15 µl volume has been found to be robust for sample injection with the fluidics system. The serum samples show a similar pattern of sensitivity with the sensitivity advantage of the micro-coil versus the 5 mm probe for a 10 µl starting volume being a factor of two.

At what point does diluting the sample to the volume required for a 5 mm NMR probe become more advantageous than gaining the sensitivity advantage of the micro-coil NMR probe? To address this, the following theoretical calculation is proposed. Consider a sample containing 100 nuclei in a 50 µl volume. In a 5 mm probe with an observe factor of 0.33, only 33 nuclei would be in the detection region of the coil. If it is assumed that each nucleus leads to 1 unit of signal then the final detected signal in this sample would be 33. Next consider taking 10 µl of this same sample and injecting it into the micro-coil NMR probe. In this case only one fifth of the nuclei would make it into the probe and only half of those would be in the detection region leading to only 10 nuclei being detected. But, with the tenfold sensitivity advantage of the micro-coil the final detected signal would be 100 compared with 33 from the 5 mm probe. This same calculation has been carried out over a range of volumes starting at 20 µl and going through 300 µl with the results shown in Fig. 2. This calculation finds that the break even volume when dilution for a 5 mm probe and the sensitivity increase in the 10 µl probe would yield the same signal to noise is 150 µl. As shown in Fig. 1, the full theoretical advantage of the micro-coil may not be realized due to details such as dilution with deuterated buffer and practical sample handing, but this calculation provides a rough guide for when the micro-coil platform may be advantageous.

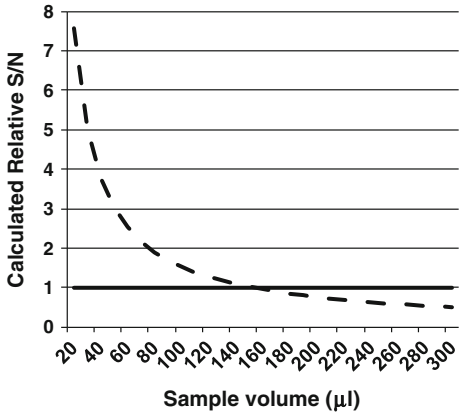

Fig. 2 Theoretical signal to noise calculation starting with increasing volumes of sample. This plot shows that based on theoretical calculations of probe performance, a sample containing 150 µl would give the same signal to noise when the sample is diluted to 600 µl and data collected with a 5 mm NMR probe versus taking a 10 µl aliquot of that same sample and injecting it into the micro-coil

Effects of sample concentration on metabolite profiles

For high-throughput studies it is important to be able to acquire large numbers of spectra very quickly. One way to leverage the increased mass sensitivity of the micro-coil platform is to concentrate biofluids to get a greater number of nuclei into the observe volume of the probe. The biochemical constitution of the samples must be considered when thinking about concentrating samples. Some metabolites with limited aqueous solubility could precipitate from highly concentrated samples. The typical salt concentration of serum is approximately 150 mM and for urine it can range from around 100 to 300 mM. Although it has been observed that the micro-coil probe is less susceptible to effects from high dielectric samples (Olson et al. 2004), very high concentrations of salt could affect the solubility of some metabolites. Protein concentrations have to be

considered for both urine and serum samples. Non-specific binding of metabolites can alter their observed concentrations in NMR spectra. For serum the protein concentrations are so high that the samples need to be filtered or undergo some type of protein precipitation before concentration can be carried out. Without this step the samples simply become too viscous to operate with the micro-capillary tubing in the fluidics system. Although healthy urine should have very low concentrations of protein (<20 μg/ml) (Marshal 2000) concentrating the samples could lead to issues with metabolite binding that are not observed with standard samples.

To evaluate the potential for sample concentration with biofluids a set of samples from five subjects were analyzed with zero, twofold, fourfold and sixfold concentration. Example spectra are shown for urine in Fig. 3 and for serum in Fig. 4. Concentration past sixfold was not carried out as it was anticipated that the salt concentration would become too high. Assuming a staring salt concentration of 150 mM the sixfold concentration would bring that level to 900 mM. The effects of this high salt were observed in the ninety degree pulse width for the sixfold concentrated samples which increased by nearly 50% from 11 to 16 ms. Figures 5 and 6 show the absolute concentrations of some of the readily quantifiable metabolites. For both urine and serum all of the metabolites show an increase in s/n with increasing concentration, but close inspection shows that the increase is not the same across all metabolites. The concentration data is also shown in Table 1. In theory, the concentration would lead to a consistent linear increase in all observed metabolite concentrations. In order to compare the expected linear signal increase to the observed values, the fold change in concentration across the concentration ranges was determined. The slopes of the fold changes should be 1, but for both urine and serum the values were much different. The values of the slopes are also given in Table 1. For urine the range went from a high value of 1.26 for valine. The value greater than 1 is likely due to inaccuracy in quantitation at the low concentration samples and some potentially confounding spectral overlap. At the other end of the range is tyrosine with a slope of 0.69. For serum the closest to a linear

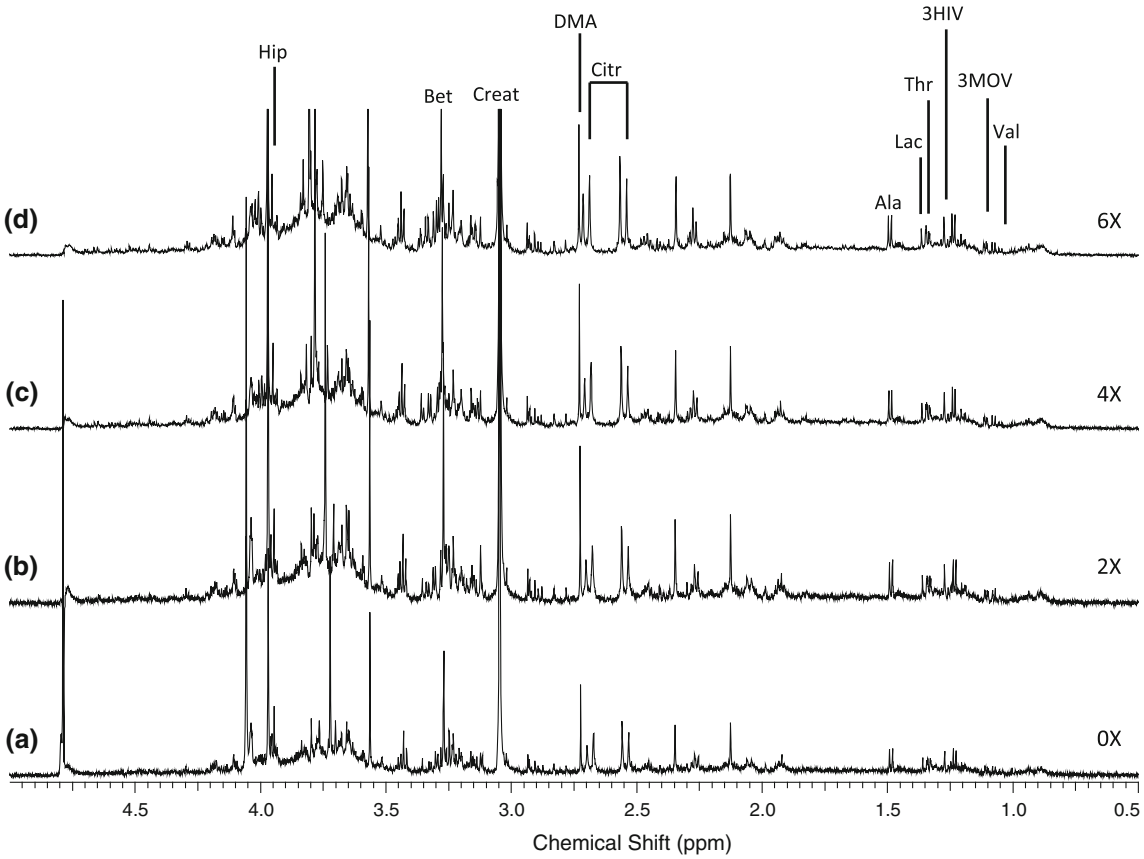

Fig. 3 Expansions of the urine spectra over a range of sample concentrations. The samples were dried down and resuspended in a reduced volume to yield samples that were 2X, 4X and 6X concentrated. The top spectrum has some of the more readily quantifiable metabolites annotated. *Val* valine; *3MOV* 3-methyl-2-oxovalerate; *3HIV* 3-hydroxyisovalerate; *Thr* threonine; *Lac* lactate; *Ala* alanine; *Citr* citrate; *DMA* dimethylamine; *Creat* creatinine; *Bet* betaine; *Hip* hippurate

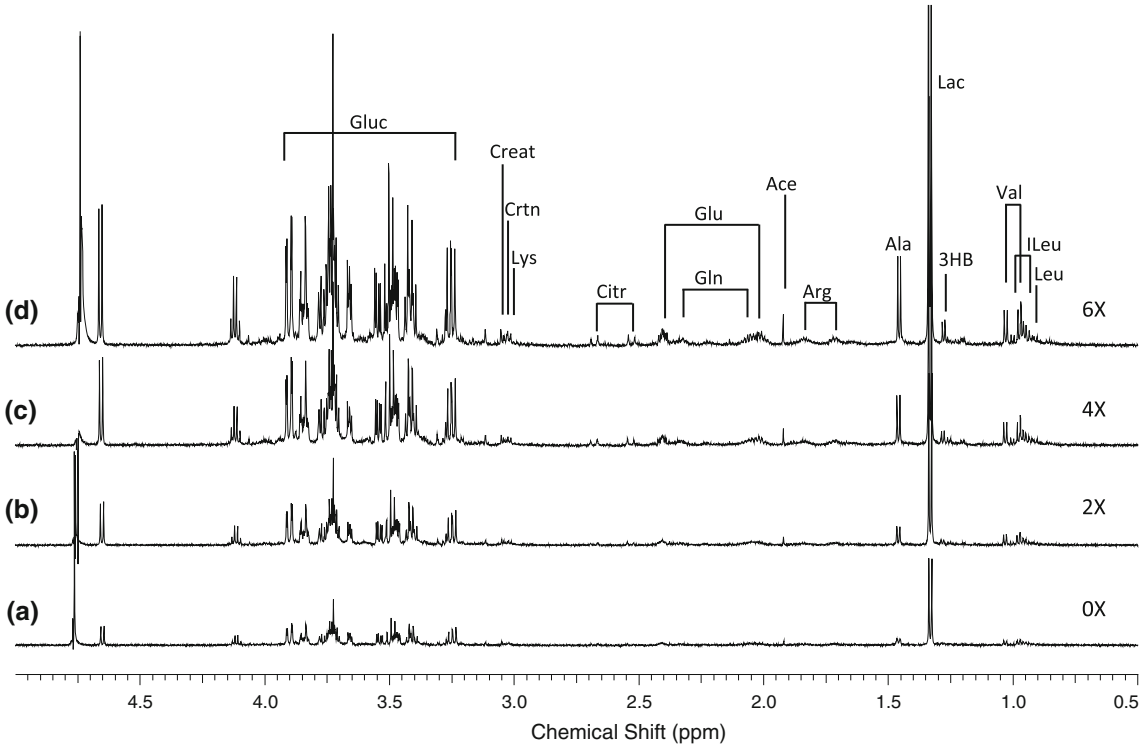

Fig. 4 Expansions of the serum spectra over a range of sample concentrations. The samples were filtered through a 10 KDal MW cutoff filter to remove the proteins, and then dried down. The samples were then resuspended in a volume to yield samples that were 2X, 4X and 6X concentrated. The top spectrum has some of the more readily quantifiable metabolites annotated. *Leu* leucine, *ILeu* isoleucine, *Val* valine, *3HB* 3-hydroxybutyrate, *Lac* lactate, *Ala* alanine, *Arg* arginine, *Ace* acetate, *Glu* glutamate, *Gln* glutamine, *Citr* citrate, *Lys* lysine, *Crtn* creatine, *Creat* creatinine, *Gluc* glucose

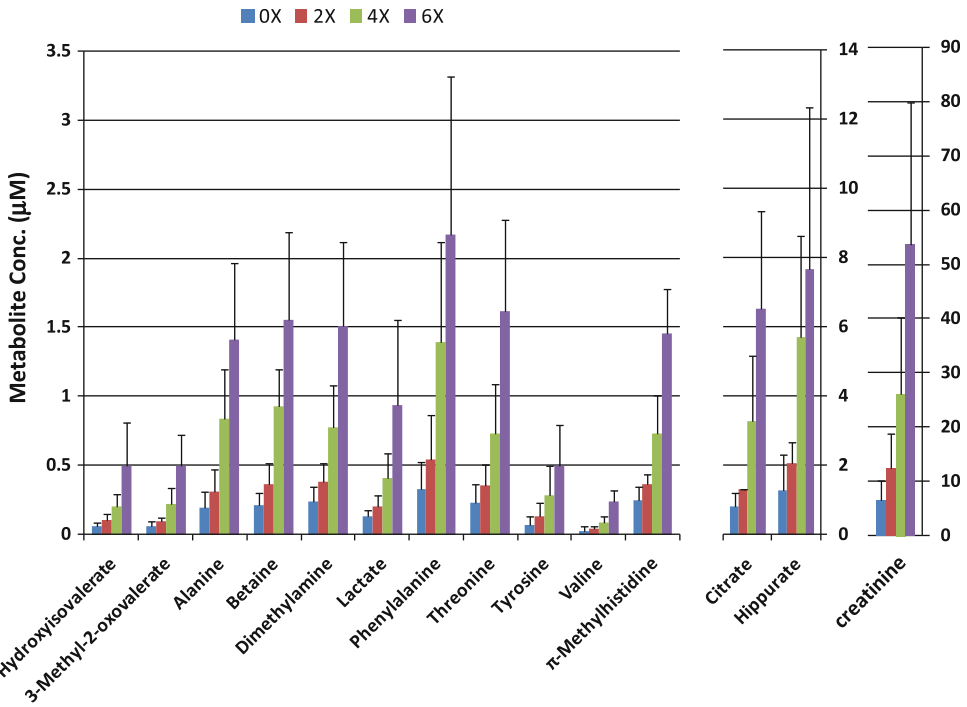

Fig. 5 Targeted profiling of metabolite concentrations of urine samples over the concentration ranges. Each bar represent the average of urine samples from 5 individuals at the various concentrations. Error bars indicate the standard deviations

increase came for alanine with a slope of 0.62 and the weakest effect was for glutamate with a slope of 0.23. It appears that the concentration effects are consistently less beneficial in serum. For example the slope for alanine in urine is 40% higher with a value of 0.88. This comparison indicates that it is not simply a matter of solubility, but

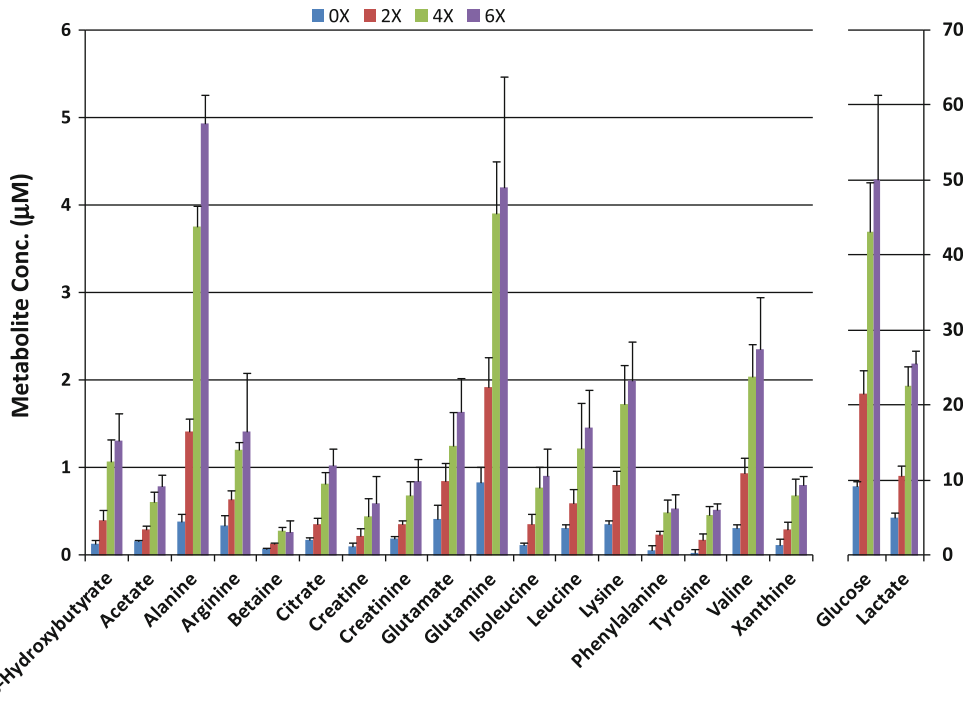

Fig. 6 Targeted profiling of metabolite concentrations of serum samples over the concentration ranges. Each bar represent the average of urine samples from 5 individuals at the various concentrations. Error bars indicate the standard deviations

Table 1 Average metabolites concentrations and slope of detection increase with concentration

Urine metabolites	Average conc. (mM)				
	0X	2X	4X	6X	Slope
3-Hydroxyisovalerate	0.056	0.101	0.204	0.493	0.971
3-Methyl-2-oxovalerate	0.061	0.098	0.224	0.494	1.009
Alanine	0.197	0.311	0.835	1.411	0.884
Betaine	0.211	0.360	0.924	1.551	0.826
Citrate	0.806	1.305	3.273	6.524	1.000
Creatine	0.455	0.842	1.912	3.587	0.815
Dimethylamine	0.237	0.378	0.775	1.510	0.750
Hippurate	1.292	2.079	5.701	7.658	0.671
Lactate	0.128	0.206	0.406	0.931	0.881
Phenylalanine	0.324	0.546	1.394	2.170	0.743
Threonine	0.224	0.355	0.732	1.616	0.888
Tyrosine	0.067	0.133	0.285	0.501	0.689
Valine	0.024	0.039	0.085	0.234	1.259
π-Methylhistidine	0.245	0.361	0.731	1.452	0.757
Creatinine	6.549	12.351	26.076	53.678	0.837
Serum metabolites	0X	2X	4X	6X	Slope
3-Hydroxybutyrate	0.122	0.401	1.070	1.301	0.561
Acetate	0.152	0.288	0.601	0.779	0.425
Alanine	0.379	1.414	3.757	4.935	0.623
Arginine	0.341	0.634	1.200	1.408	0.305
Betaine	0.068	0.126	0.277	0.257	0.258
Citrate	0.170	0.358	0.821	1.027	0.468
Creatine	0.101	0.211	0.445	0.598	0.459
Creatinine	0.187	0.347	0.683	0.846	0.360

Table 1 continued

Serum metabolites	0X	2X	4X	6X	Slope
Glutamate	0.416	0.839	1.249	1.631	0.236
Glutamine	0.824	1.922	3.901	4.196	0.296
Isoleucine	0.117	0.348	0.768	0.902	0.398
Leucine	0.306	0.589	1.223	1.454	0.367
Lysine	0.346	0.803	1.726	1.999	0.372
Phenylalanine	0.056	0.233	0.485	0.528	0.317
Tyrosine	0.022	0.173	0.459	0.522	0.504
Valine	0.306	0.930	2.036	2.353	0.383
Xanthine	0.117	0.296	0.679	0.803	0.429
Glucose	9.184	21.437	43.124	49.983	0.333
Lactate	4.970	10.579	22.493	25.493	0.352

The concentrations were determined using the Chenomx NMR database. The slope was calculated from the fold changes in concentration. Three points were used, starting with the 2X concentration and going to 4X and 6X values

there are effects from the matrix that make concentration less beneficial for serum.

These results indicate that concentrating urine and serum samples alters the absolute concentrations of the metabolite levels differently. For example, the data above indicates that the absolute concentration of valine in a sixfold concentrated sample of urine would be approximately sixfold greater than the unconcentrated sample, but for the tyrosine concentration is about 30% reduced. It could be expected that any sample processing could lead to selective changes in metabolites concentrations. It is well known that protein precipitation methods can lead to selective decreases in lactate concentration as lactate has a high propensity for protein binding (Bell et al. 1988). This result suggests that the calculation of the breakeven point between the benefits of concentration sensitivity with the 5 mm probe versus the mass sensitivity of the micro-coil probe will vary depending upon the matrix.

The absolute concentrations of metabolites are affected by concentration, but it appears that concentration does not increase the noise of the data. To evaluate this, the coefficient of variance of each of the metabolites at the concentration levels was calculated. These data are shown in supplementary Table 2 for both urine and serum metabolites. There is no significant increase in the variability of the data at the higher concentration levels. Therefore, if concentration will provide a benefit in metabolite detection, the relative concentrations across samples can be compared with no anticipated increase in variance.

Concluding remarks

The data presented here demonstrates that the micro-coil probe can provide a valuable increase in sensitivity for metabolomics studies with very limited sample volumes. Sample concentration has been demonstrated as a viable option to increase the sensitivity and throughput of NMR metabolomics, but the data shows that the increase is not linear and changes with both the matrix and specific metabolites. The relative benefits for urine appear to be greater than for serum, but in essentially all cases the detected concentration of metabolites is reduced. The use of the micro-coil NMR system comes with other advantages such as higher magnetic field homogeneity as the sample's volume is so small compared with a standard tube based probe. This issue of cross-contamination is also minimized by the micro-coil platform. The amount of solvent used to push the sample from the vial into the coil is typically at least several coil volumes which very effectively cleans the coil between samples. Recently a set of standard operating procedures were published for metabolomics of biofluids in order to minimize random and systemic variation in the data collection (Sukumaran et al. 2009). The preparation of samples in vials or 96 well plates is also easier and less expensive than the tube format. All of these factors need to be taken into account when embarking on a large scale metabolomics study involving hundreds or even thousands of samples.

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