[POSTER NOTE]

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METABOLOMIC WORKFLOW UTILISING RAPID MICROBORE METABOLIC PROCESSING (RAMMP) IN CONJUNCTION WITH A NOVEL SCANNING QUADRUPOLE DIA METHOD

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OVERVIEW

- **SONAR™ DIA is utilised with a rapid metabolic processing workflow (within 3 mins)**
- **The fast scanning capabilities of SONAR™ demonstrates quantitative precision for high throughput, large cohort experiments**
- **Optimisation of the quadrupole isolation window provides improved specificity**
- **Rapid metabolomic analysis of urine from a pregnant cohort over three trimesters shows applicability of the method with clear differentiation of each trimester**

INTRODUCTION

Previous studies utilizing rapid microbore metabolic profiling (RAMMP) have shown comparable group discrimination and improved selectivity over conventional UPLC chromatography.1 Here, we demonstrate further improvements of the workflow by coupling RAMMP with a novel DIA method (SONAR™), providing highly specific and unbiased two-dimensional metabolomic data. SONAR™ is an acquisition technique comprising of a low-resolution quadrupole mass filter, which is scanned repetitively and both precursor and MS-MS data are acquired at spectral rates approaching 2000 spectra/s. Sample sets consisting of urine collected from pregnant women over three trimesters were used to demonstrate SONAR™ for use with high throughput analyses. Data were analysed and interrogated using Progenesis QI, whilst targeted quantitation was provided using Skyline.

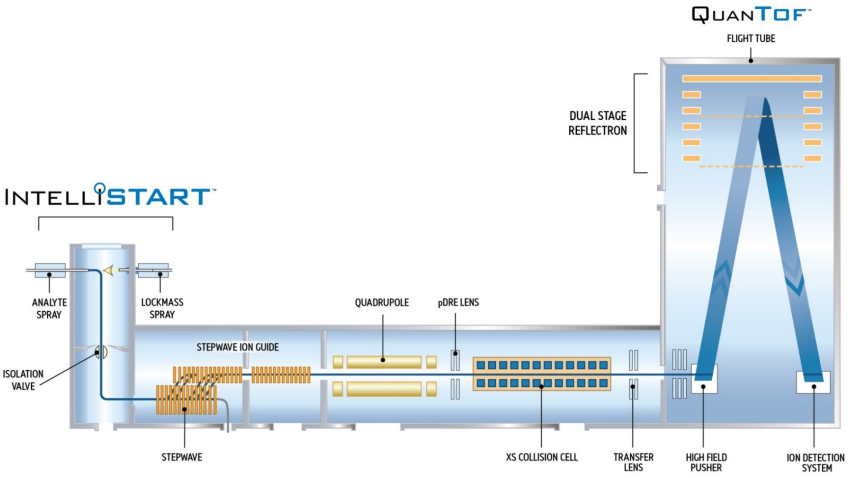


Figure 1. Schematic of the Xevo G2-XS mass spectrometer used for SONAR™ data acquisition.

METHODS

Sample preparation

Urine samples (Innovative Research Inc) were prepared as previously described.[2] Briefly, particulates and debris were removed by centrifuging at 10,000*g* for 10 min prior to diluting 2-fold with water. Samples were vortexed and transferred to glass vials in preparation for LC-MS analysis.

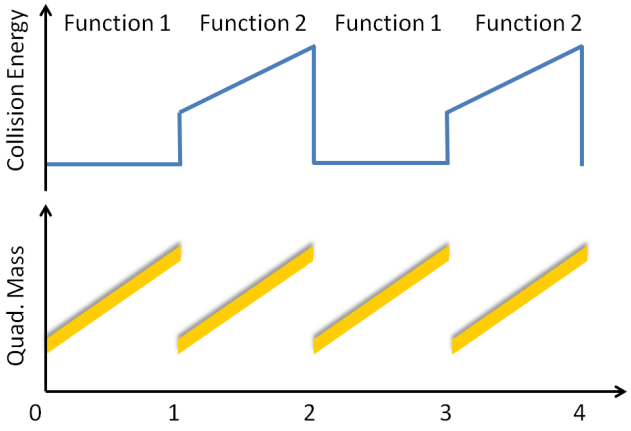
LC-MS conditions

Metabolites were chromatographically separated using an ACQUITY M-class configured with either a BEH 1.7 µm C18 reversed phase 1.0 x 100 mm or 300 µm x 100 mm LC column. Experiments were conducted over 12, 6 and 3 min using a gradient of 1 to 95% acetonitrile (0.1% formic acid) in all cases.

Mass spectral data were acquired using a Xevo G2-XS QToF (Waters Corporation), Figure 1, operated in SONAR™ 2DMS mode with various quadrupole scanning and window settings used for optimisation purposes (Figure 2).

Bioinformatics

The LC-MS metabolite data were processed and searched with Progenesis QI (Non-Linear Dynamics, UK). Normalized label-free quantification was achieved with additional statistical analysis conducted using EZInfo (Umetrics, Sweden). Compound searches were conducted using HMDB. Quantitative analysis was performed with Skyline (University of Washington) using libraries derived from Progenesis QI compound searches.



Ionisation Mode	Quad Scan (Da)	Quad Window (Da)	ToF Scan (Da)	Function Integration Time (s)	Function 1 CE (V)	Function 2 CE (V)
+	250-800	12 & 22	50-1200	0.1	6	20-50
-	250-800	12 & 22	50-1200	0.1	6	25-55

Figure 2. SONAR™ acquisition method and DIA acquisition parameters used in the different experiments.

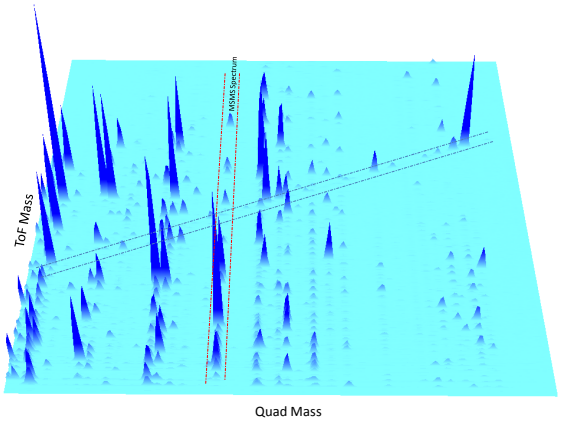


Figure 3. SONAR™ DIA ToF vs. quadrupole m/z data, showing product ions (vertical bands) from metabolites eluting over a 1 min window and the quadrupole sweep (diagonal line).

RESULTS

SONAR™ DIA acquisition provides multi-dimensional data sets, exhibiting improved specificity. Figure 3 represents typical SONAR™ data and demonstrates that the format is the same as other multi dimensional datasets, e.g. ion mobility; hence, exhibits improved specificity. Urine based data were acquired using either a 1 mm or a 300 µm i.d. column. Figure 4 provides example chromatographic urine profiles generated using RAMMP (3 min gradient) and conventional (12 min gradient) methods.

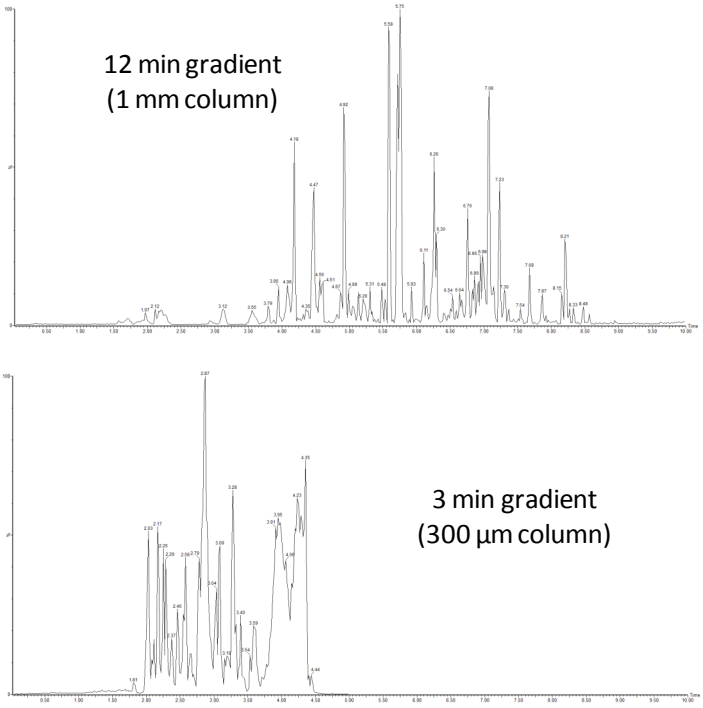


Figure 4. Example chromatograms of analysed urine based on 12 min gradient with 1 mm i.d. column and 3 min gradient with 300 µm i.d. column.

Evaluating the number of peak detected features achieved for the different column configurations, gradient lengths and quadrupole window was conducted (Figure 5). An increase of approximately 50% is observed with decreasing column diameter, when comparing against the same gradient and quadrupole window.

Unsupervised principal component analysis (PCA) highlights differentiation of the three trimesters regardless of gradient selected (Figure 6). To ensure robustness and consistency of the results when switching between conventional and RAMMP based methods, the discriminating features responsible for the PCA based separation were assessed for both scenarios (Figure 7).

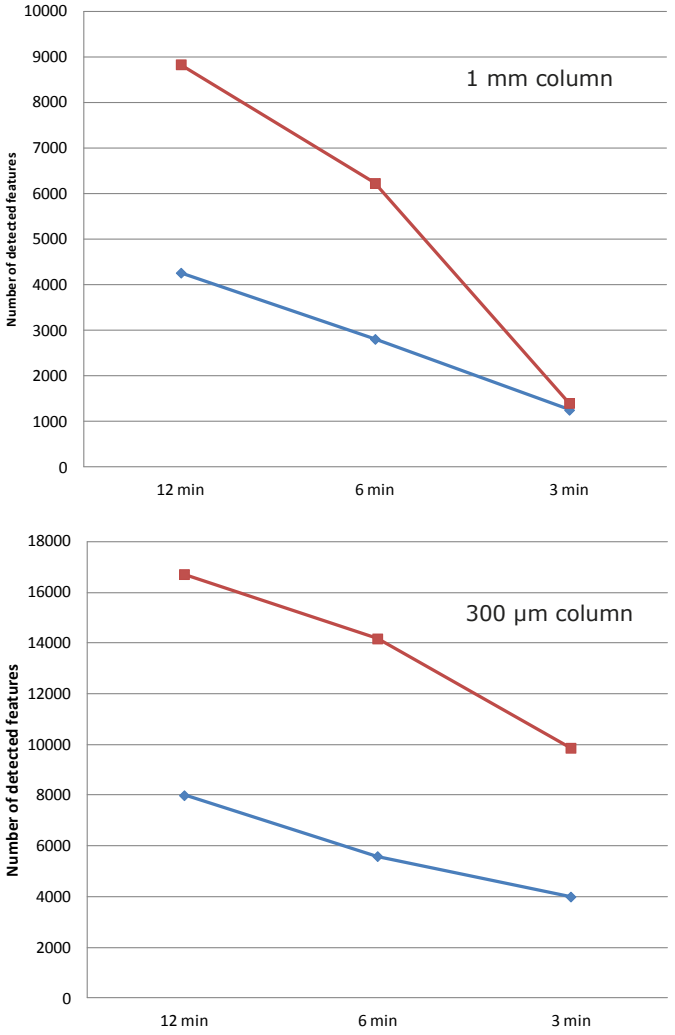


Figure 5. Comparison of the features detected for 1 mm and 300 micron chromatography over different gradients (12, 6 and 3 mins) and quadrupole windows (12 (blue) and 22 (red) Da). The number of detected features increases with decreasing column diameter. RAMMP based methods (3 min gradient; 12 Da window) utilizing a 300 micron column provides a comparable number of detected features the 12 min gradient based on a 1 mm i.d. configuration.

The high specificity provided by SONAR™ reduces the potential of interference effects thereby increasing quantitative confidence. A number of metabolites based on the RAMMP method (3 min) were selected for targeted analysis using open source Skyline informatics (Figure 8). Precursor/product ions list were provided to the software, along with quadrupole (precursor) *m/z* extraction information.

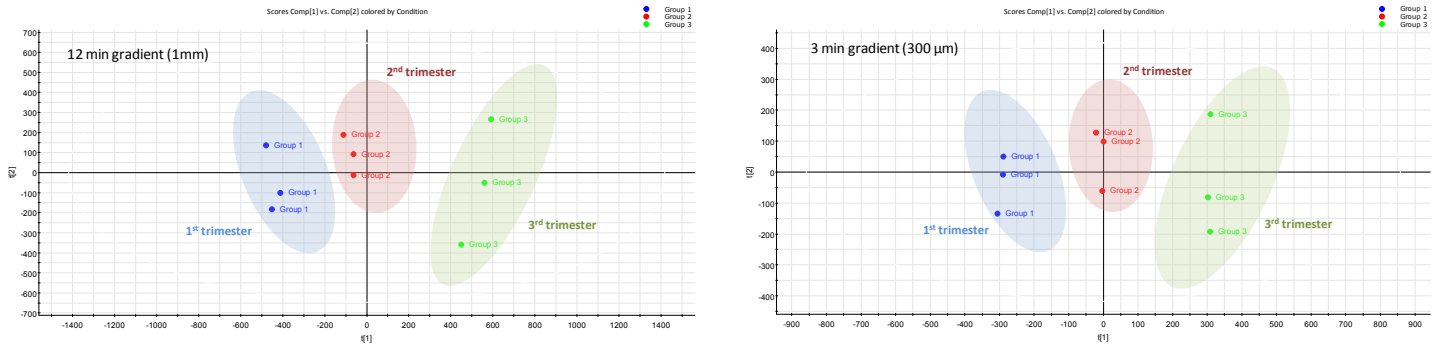
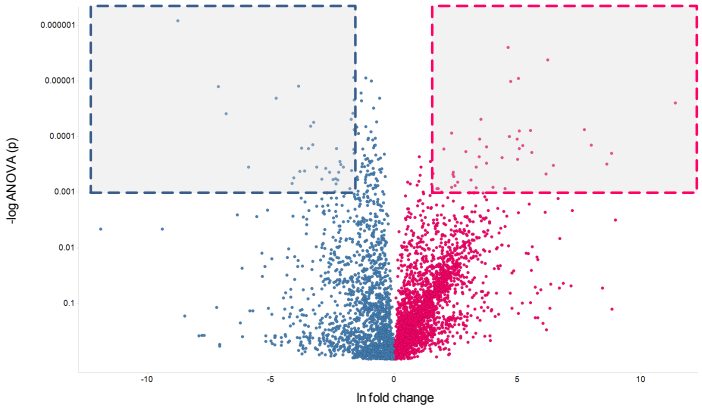


Figure 6. Representative PCA plots for 1 mm (12 min) and 300 µm (3 min) i.d. chromatography. In both cases, clear separation is observed between the three trimesters. Separation characteristics are based on PC1 versus PC2 and is maintained when transferring from conventional to RAMMP.



m/z	Compound Identification	Fold Change	ANOVA (p)	Highest Mean	%CV
192.1015	Hydrocinnamic acid	3.2	0.00103	First Trimester	26.8
231.0982	Glutarylglycine	6.6	0.00129	First Trimester	32.6
777.2413	Hyaluronan	2.8	0.00104	Third Trimester	20.5
597.3629	L-Urobilinogen	2.5	8.28E-05	Third Trimester	4.8
447.2002	Estrone gluconoride	2.5	3.17E-06	First Trimester	11.33
310.1505	Hydroxyprolyl-Histidine	4.5	8.69E-04	First Trimester	39.9

Figure 7. Assessing consistency of the discriminating features for conventional and RAMMP based methods responsible for driving the separation of the unsupervised PCA. Data are visualized as a volcano plot (-log ANOVA (p) vs. ln fold change) comparing first (pink) and third trimester (blue) urine samples acquired using the 300 µm i.d. RAMMP method. Only features adhering to a fold change ≥ 2 and ANOVA (p) ≤ 0.001 were considered for comparison as highlighted by the shaded areas of the volcano plot. Example discriminating features common between the two methods are displayed in the accompanying table.

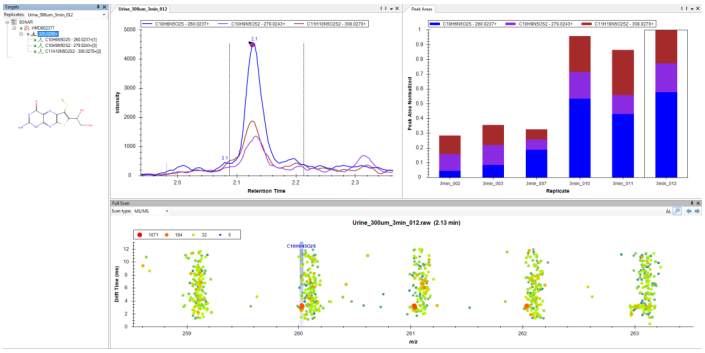


Figure 8. Targeted analysis of potential metabolite markers of interest related to the stage of pregnancy. This example is representative of RAMMP, showing urothion (t_r = 2.1 min) quantified over the first and third trimester.

An adequate number of data points/scans over a chromatographic peak is imperative for maintaining quantitative accuracy precision. Figure 9 demonstrates the fast scanning capabilities of SONAR™ for both precursor and product ions. This particular example shows urothion being acquired over various scan rates (0.5, 0.3 and 0.1 sec). Applying a 0.1 sec scan provides more than 10 points over the peak (1.7 sec FWHM) and generates the expected 2.5-fold ratio when comparing transitions for first and third trimester cohorts from Figure 8. Comparing three representative transitions shows consistency with scan rate providing additional confidence and increased quantitative precision.

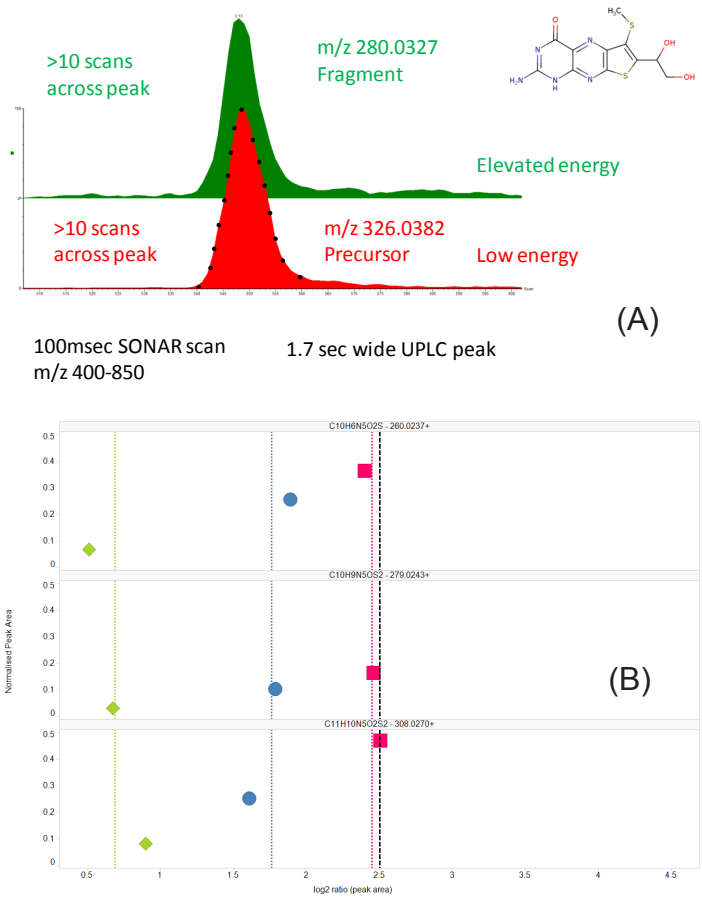


Figure 9. Increased quantitative precision of urothion demonstrated with faster scan rates. (A) Precursor (low energy) and product ion (elevated energy) scans at 100 msec provide >10 scans across a peak of 1.7 sec (FWHM); (B) Fragment ion transitions acquired with corresponding scan rates 0.5 (green), 0.3 (blue) and 0.1 sec (red). Normalised peak area vs. log2 peak area ratio (trimester 1/trimester 3) show consistent ratios over all scan rates with the average for each scan rate represented as dashed lines. The expected ratio between 1st and 3rd trimester is estimated to be 2.5-fold (black dashed line) based on the previous Skyline analysis. Implementing the faster scan rates of 0.1 sec offered by SONAR™ DIA is shown to be necessary to avoid under sampling.

CONCLUSION

- **SONAR™ DIA acquisition provides multi dimensional data sets exhibiting improved specificity and over other DIA methods**
- **Rapid profiling of urine using a RAMMP based approach with a 300 µm i.d. column provides equivalent numbers of identified features for a 3 min gradient when compared with 12 min gradients using a 1 mm i.d. column**
- **Quantitative precision is demonstrated through utilising the fast scanning capabilities of SONAR with both precursor and product ions.**
- **Multi-variate analysis shows clear separation between the three trimesters using conventional or RAMMP methods. Features contributing the greatest variance are shown to be the same in both cases**
- **A variety of metabolites using this qualitative/ quantitative workflow combined with a RAMMP profile have identified a number of potential markers to distinguish between pregnancy trimesters**

References

1. Gray *et al*. Development of a Rapid Microbore Metabolic Profiling Ultraperformance Liquid Chromatography-Mass Spectrometry Approach for high-Throughput Phenotyping Studies. Anal. Chem. 2016; 88:5742-51.
2. Want *et al*. Global metabolic profiling procedures for urine using UPLC-MS. Nature Protocols. 2010;5;1005-18.