

## Metabolomic Workflow Utilizing Rapid Microbore Metabolic Processing (RAMMP) in Conjunction with SONAR

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### Abstract

This application note demonstrates improvements to the metabolic profiling workflow by coupling RAMMP with a novel DIA method (SONAR), providing highly specific and unbiased two-dimensional metabolomic data.

### Benefits

- SONAR DIA is utilized with a rapid metabolic processing workflow (within 3 minute gradients)
- The fast scanning capabilities of SONAR demonstrates quantitative precision for high throughput, large cohort experiments
- Optimization 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

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## Introduction

Previous studies utilizing rapid microbore metabolic profiling (RAMMP) have shown comparable group discrimination and improved selectivity over conventional UPLC chromatography.<sup>1</sup> Here, we demonstrate further improvements to 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 comprised 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 analyzed and interrogated using Progenesis Q1, while targeted quantitation was provided using Skyline.

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## Experimental

### Sample preparation

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

### LC conditions

LC system:	ACQUITY UPLC M-Class
Column:	BEH C <sub>18</sub> 1.7 $\mu$ m, 1.0 mm x 100 mm or 300 $\mu$ m x 100 mm Reversed Phase Analytical
Column temp.:	50 °C
Flow rate:	100 $\mu$ L/min (1 mm I.D.)/7 $\mu$ L/min (300 $\mu$ m I.D.)

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Mobile phase: (A) Water/0.1% formic acid; (B) Acetonitrile/0.1% formic acid

Gradient: 1% to 95% B over 12, 6, or 3 min

Injection volume: 5  $\mu$ L

## MS conditions

MS system: Xevo G2-XS

Ionization mode: ESI (+) at 2.2 kV; ESI (-) at 1.7 kV

Cone voltage: 30 V

Acquisition mode: SONAR 50 *m/z* to 1200 *m/z* both functions (low and elevated energy)

Quadrupole settings: 12 Da window operating over 250–800 Da

Acquisition rate: Low and elevated energy functions at 0.1 s

Collision energy: Low energy function at 5 eV and elevated energy function from 20 eV to 50 eV (positive ion) and 25 eV to 55 eV (negative ion)

Resolution: 30,000 FWHM

## Data management

Progenesis QI

EZInfo

MassLynx

Skyline

Spotfire

## Bioinformatics

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

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## Results and Discussion

SONAR DIA acquisition provides multi-dimensional data sets, exhibiting improved specificity. Figure 1 represents typical SONAR data and demonstrates that the format is the same as other multidimensional data sets, e.g. ion mobility; hence, exhibits improved specificity. Urine based data were acquired using either a 1 mm or a 300  $\mu$ m I.D. column. Figure 2 provides example chromatographic urine profiles generated using RAMMP (3 min gradient) and conventional (12 min gradient) methods.

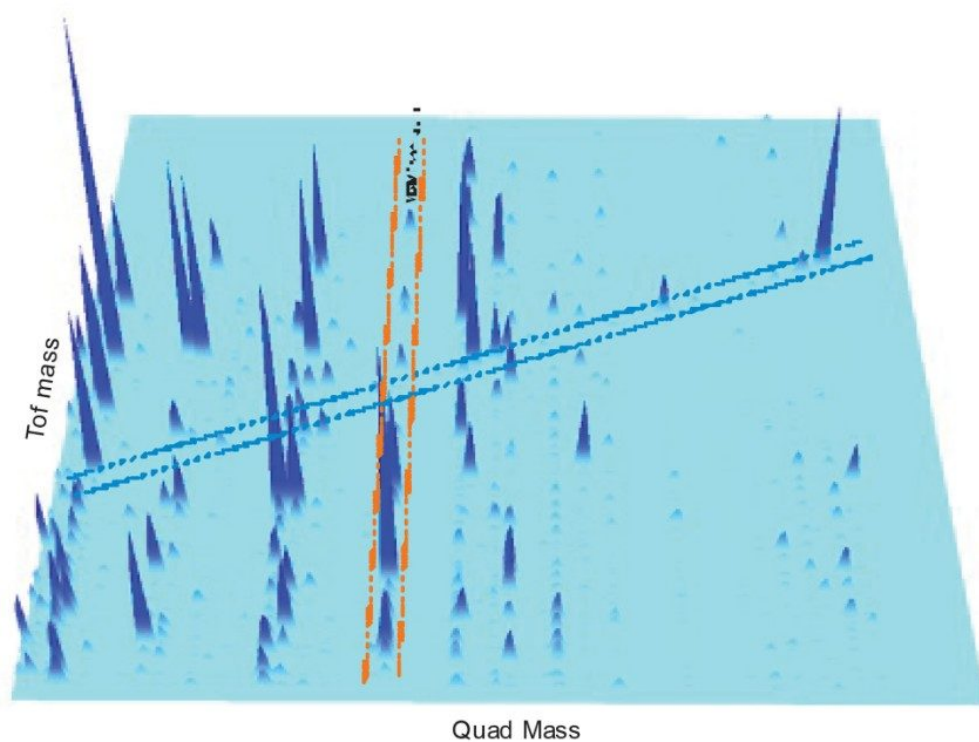
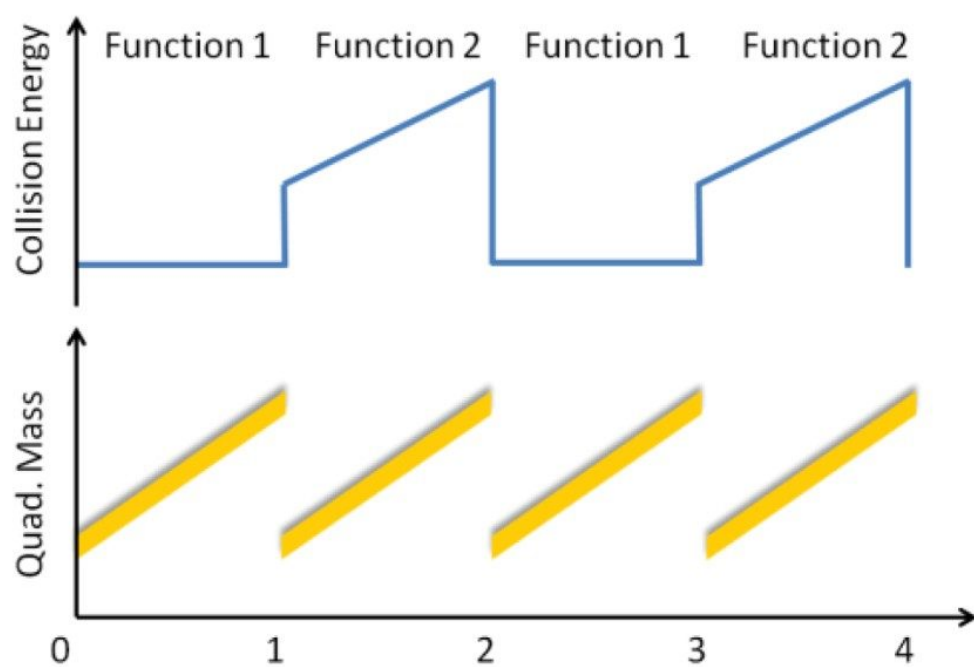
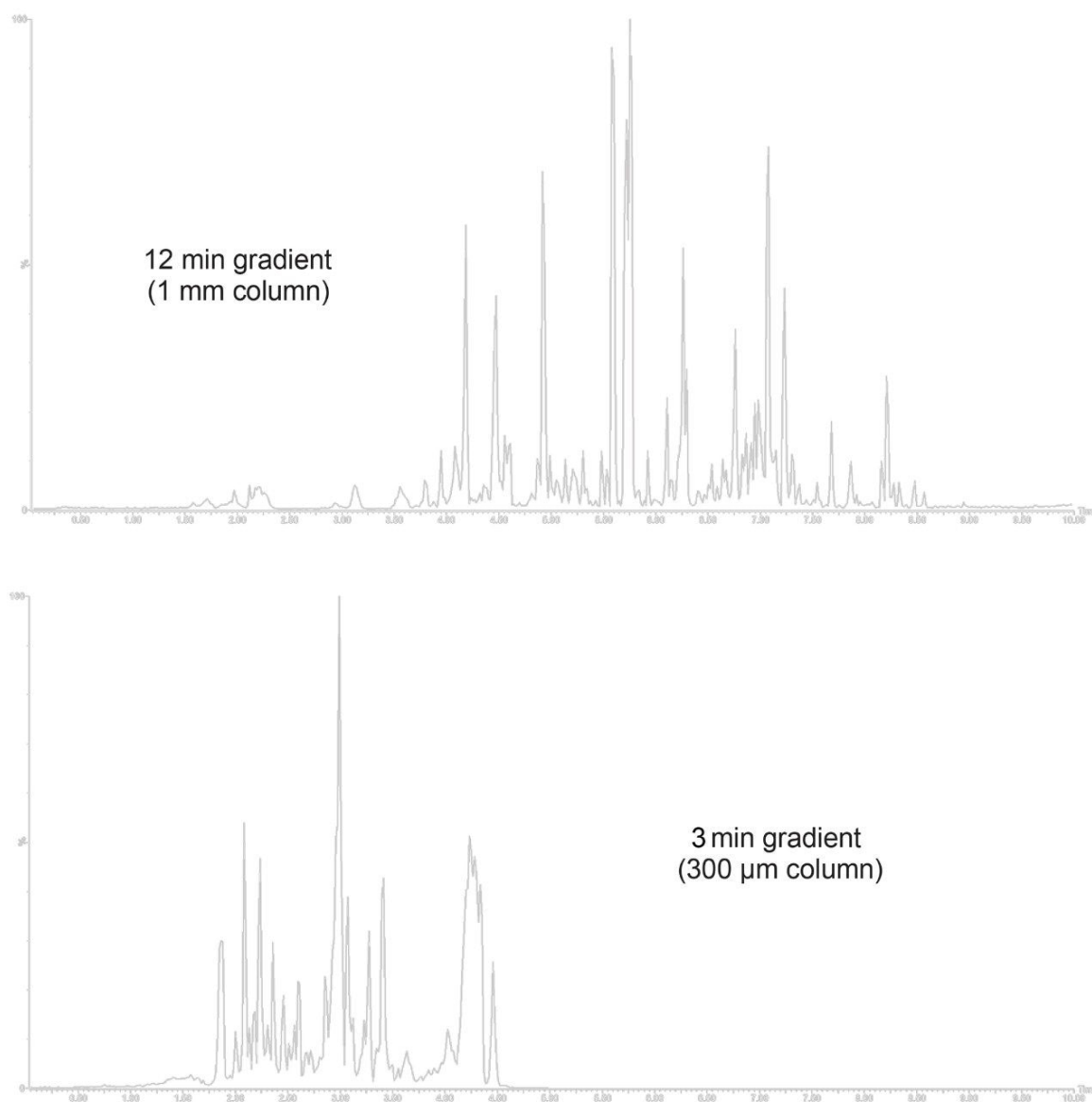


Figure 1. SONAR acquisition method and DIA acquisition parameters used in the different experiments. DIA ToF

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vs. quadrupole  $m/z$  data (lower figure), shows product ions (vertical bands) from metabolites eluting over a 1 min window and the quadrupole sweep (diagonal line).



*Figure 2. Example chromatograms of analyzed urine based on 12 min gradient with 1 mm I.D. column and 3 min gradient with 300 µm I.D. column.*

An evaluation of the number of peak-detected features achieved for the different column configurations, gradient

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lengths, and quadrupole window was conducted (Figure 3). 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 the gradient selected (Figure 4). 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 5).

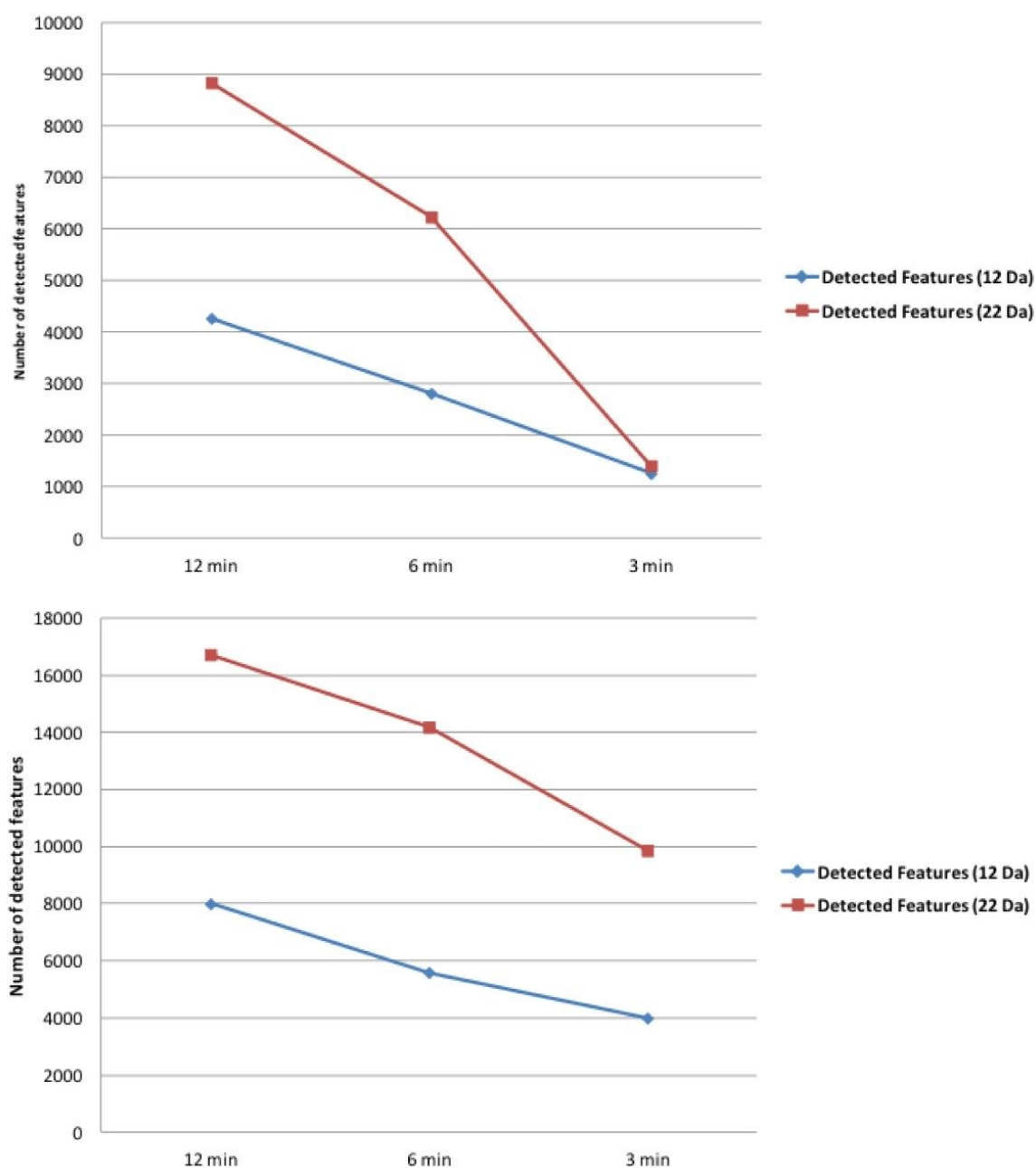


Figure 3. Comparison of the features detected for 1 mm (upper) and 300 micron (lower) chromatography over different gradients (12, 6, and 3 mins) and quadrupole windows (12 (blue) and 22 (red) Da). The number of detected features increases with decreasing column diameter. RAMMP based methods (3 min gradient;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



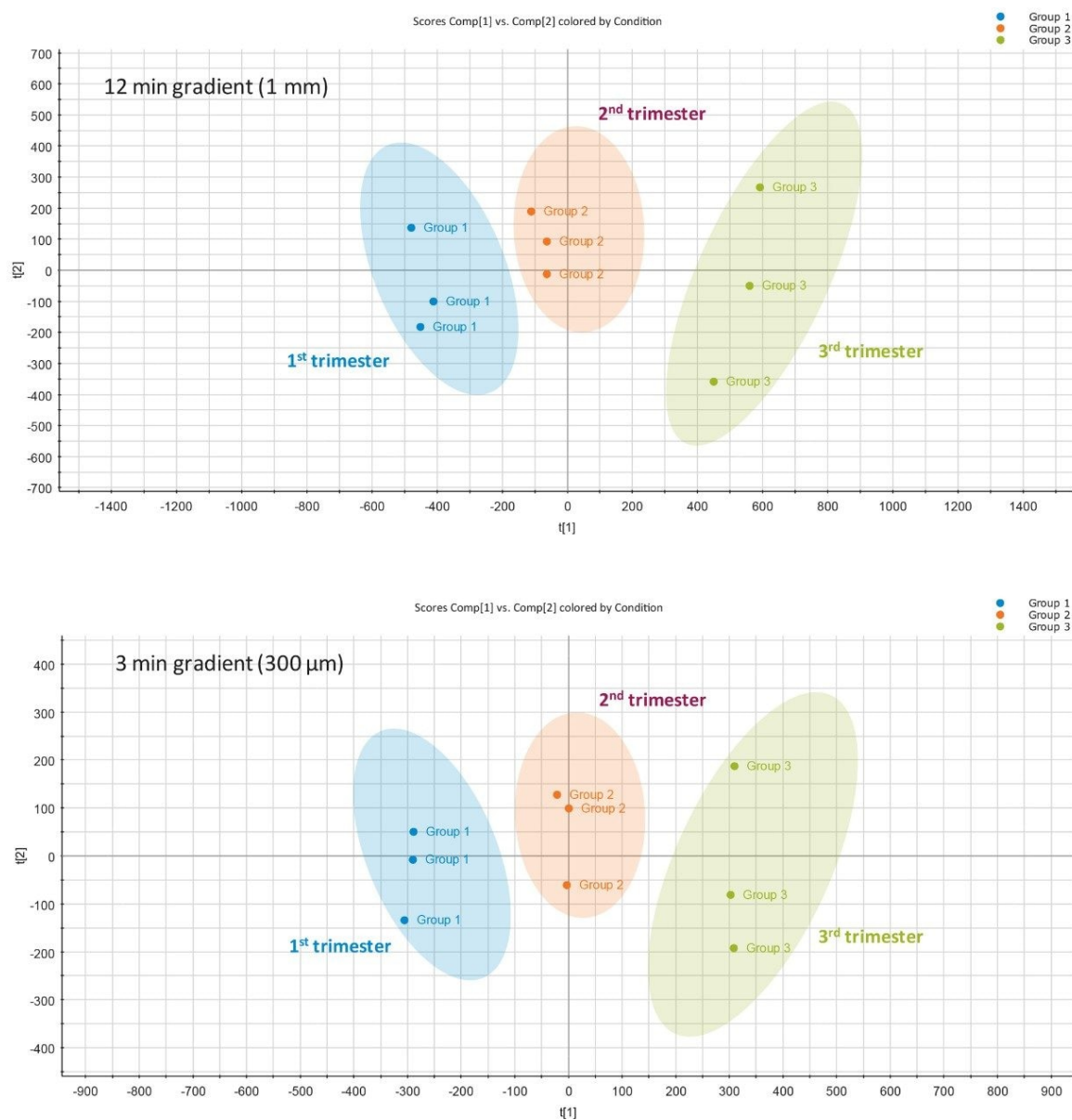
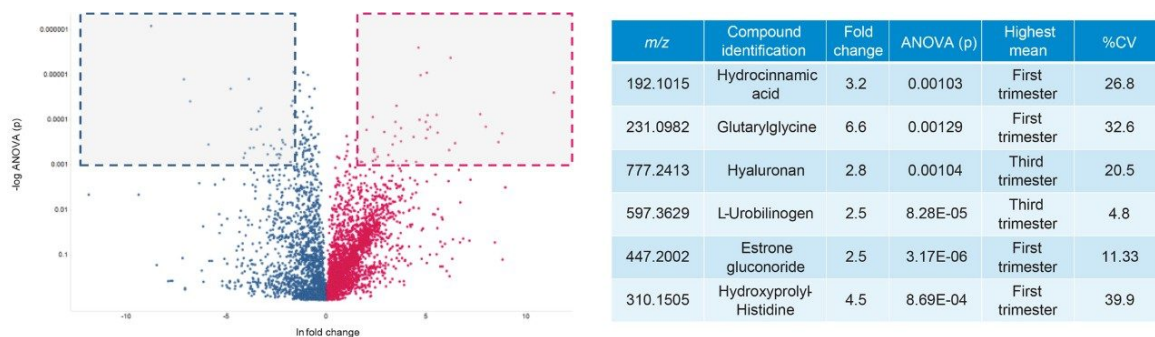


Figure 4. Representative PCA plots for 1 mm (12 min) and 300 μm (3 min) I.D. chromatography. In both cases, clear separation is observed between the three trimesters. Separation characteristics are based on PC1 versus PC2 and is maintained when transferring from conventional to RAMMP.



*Figure 5. 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 ( $-\log \text{ANOVA } (p)$  vs.  $\ln \text{fold change}$ ) comparing first (pink) and third trimester (blue) urine samples acquired using the 300  $\mu\text{m}$  I.D. RAMMP method. Only features adhering to a fold change  $\geq 2$  and ANOVA ( $p$ )  $\leq 0.001$  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.*

High specificity provided by SONAR reduces potential interference effects and thereby increases quantitative confidence. A number of metabolites based on the RAMMP method (3 min) were selected for targeted analysis using open source Skyline informatics (Figure 6). Precursor/product ions list were provided to the software, along with quadrupole (precursor)  $m/z$  extraction information.

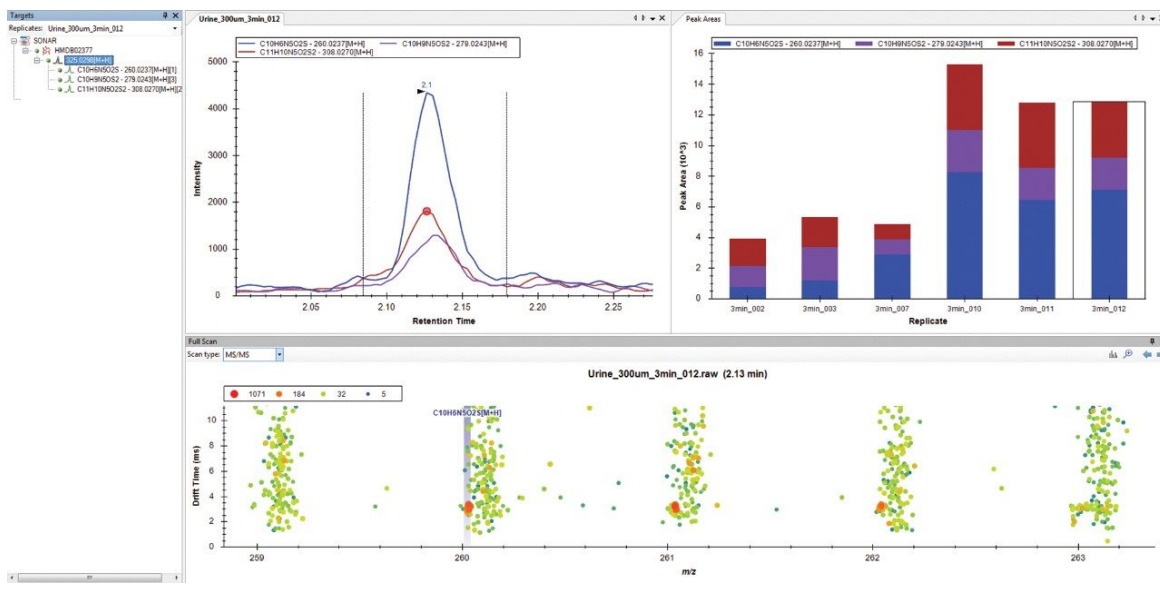
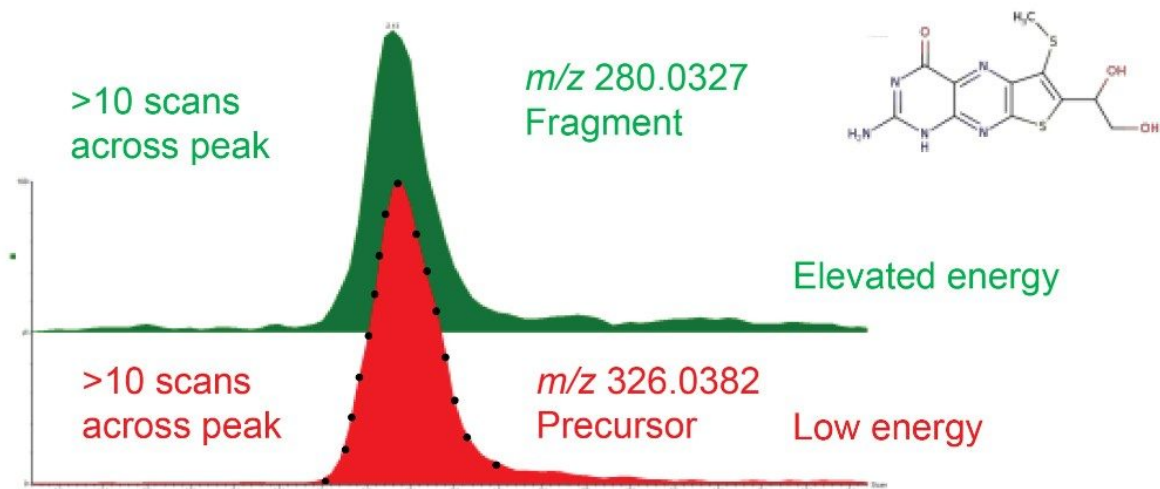


Figure 6. Targeted analysis of potential metabolite markers of interest related to the stage of pregnancy. This example is representative of RAMMP, showing urothion ( $t_r = 2.1$  min) quantified over the first and third trimester.

Achieving an adequate number of scans is imperative for maintaining quantitative precision. Figure 7 demonstrates the fast scanning capabilities of SONAR for both precursor and product ions. This 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 chromatographic peak (1.7 sec FWHM) and generates the expected 2.5-fold ratio when comparing transitions for first and third trimester cohorts from Figure 6. Comparing three representative transitions shows consistency with scan rate providing additional confidence and increased quantitative precision.



100 msec SONAR scan      1.7 sec wide UPLC peak  
 $m/z$  400–850

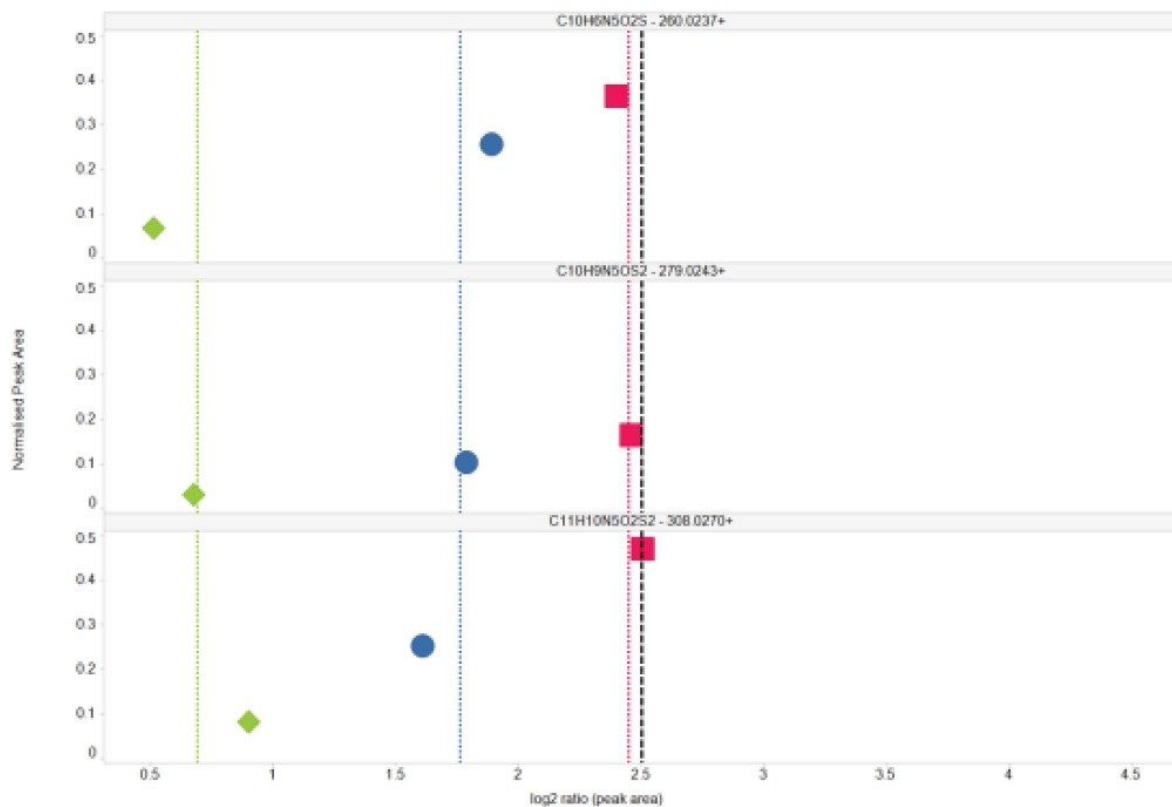


Figure 7. Increased quantitative precision of urothion demonstrated with faster scan rates. (A) Precursor (low

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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). Normalized peak area versus log2 peak area ratio (trimester1/trimester3) 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.

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## 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  $\mu$ m I.D. column provides equivalent numbers of identified features for a 3 min gradient when compared with 12 min gradient using a 1 mm i.d. column.
- Quantitative precision is demonstrated through utilizing 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.

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## 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.
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2. Want et al. Global Metabolic Profiling Procedures for Urine Using UPLC-MS. *Nature Protocols*. 2010;5;1005–18.

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