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A Comparison of UPLC™/MS(TOF) and ^{1}H-NMR Metabonomics Strategies For the Analysis of Urine From Zucker Rats

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Introduction

Metabonomics is a rapidly expanding area of scientific research, which has the potential to improve the drug discovery process by providing an insight into the mechanisms of toxicity and efficacy. The approach relies on comparing the endogenous metabolic profiles of mammalian systems following exposure to an external stimuli, *e.g.* a candidate pharmaceutical or environmental stress using chemometric data analysis strategies. Much recent interest has been focused on LC/MS analysis methods as an alternative to ^{1}H-NMR. An additional resource for LC/MS based metabonomics has been found in Ultra Peformance Liquid Chromatography (UPLC). UPLC offers several advantages, namely improved chromatographic resolution, increased sensitivity and reduced analysis times. This is achieved through the use of small particle sizes (< 2um) and pumping-injection systems capable of operating under such exacting pressure regimes (up to 15,000 psi). When coupled with time of flight mass spectrometry, UPLC presents a means to achieve high sample throughput with reduced spectral overlap, increased sensitivity and exact mass detection capabilities. This aspect of UPLC/MS (TOF) is particularly attractive for metabonomics applications where the rapid and accurate detection and identification of potential biomarkers by exact mass is beneficial. Herein, we compare results obtained from PCA of both ^{1}H-NMR and UPLC/MS(TOF) data generated from the analysis of urine samples from prediabetic male and female Zucker rats. Diurnal and gender variations will be considered as well as a comparison of the Zucker rats to control rats. The data generated in this study shows that UPLC/

Methods

Urine samples were collected from male and female obese (fa/fa) Zucker rats (n=10 each) at two time periods, morning and evening for a total of 40 samples plus 3 control rat urine samples from male Alderley Park (AP) (Wistar derived). A 100 µL aliquot of deionized water was added to each sample of rat urine and was vortex mixed. All samples were then centrifuged at 13,000 rpm for 5 minutes at 10 °C and the supernatant liquid removed. A 50 µL aliquot of the supernatant was diluted with 150 µL of distilled water and vortex mixed; the resulting solutions were transferred to an autosampler vial for analysis. The chromatography was performed on a Waters Acquity UPLC™ system which was coupled to a Waters Micromass® LCT Premier™ equipped with an electrospray source operating in either positive or negative ion mode and an integrated LockSpray™ interface for exact mass measurements.

UPLC-MS Conditions

Column	(2.1 x 100)mm 1.7 µm ACQUITY UPLC™ C_{18}
Flow Rate	500 µL/min
Injection Volume	5 µL
Gradient	Linear, 0-20%B over 0.5-5 min, 20-95%B over 5-8 min, hold at 95%B for 1 min
Mobile Phase	A = 0.1% Formic Acid in Water; B= 0.1% Formic Acid in Acetonitrile
Lock Mass	25 fmol/µL Leucine Enkephalin in 50:50 H_2O:ACN (0.1% Formic Acid) at 30 µL/min
Cone Voltage	80 V
Desolvation Temp.	250 °C
Source Temp.	120 °C
Acquisition Range	100—850 m/z
Dwell	0.1 s

^{1}H-NMR Conditions

Spectra were acquired using a Bruker DRX 500 Spectrometer (operating at 500.13MHz for proton) employing the 'Noesypresat' pulse sequence (Bruker Spectrospin Ltd.) for water suppression. Spectra were acquired at 30°C with 64K data points and typically 64 scans. All spectra were referenced to the internal reference standard TSP (δ_{1H} = 0.0) and corrected for phase and baseline distortions.

Results & Discussion

The collected UPLC/MS data was analysed using MarkerLynx™ Application Manager for MassLynx 4.0 software to discern ions of interest for further analysis by MS/MS with exact mass. MarkerLynx integrates the detected peaks from each sample to construct a comprehensive list of all components in the analyzed samples in an interactive browser format. The processed data list is then analyzed by Principal Components Analysis (PCA) within the MarkerLynx program. An example of the MarkerLynx browser for male and female Zucker rat urine samples is presented in Figure 1.

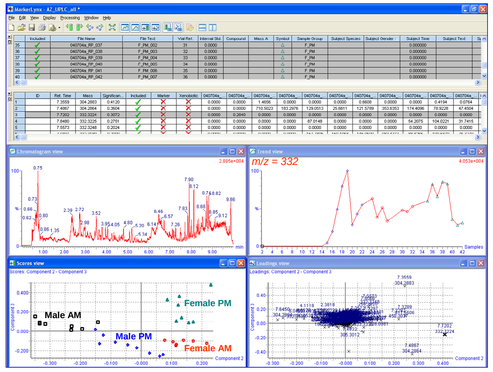

Figure 1. *MarkerLynx browser screen capture for the UPLC-MS analysis of male and female Zucker rats. The browser window shown here displays the scores and loadings plots from PCA as well the trend line for the m/z = 332 ion across all samples.*

Distinct clustering of male and female rats is observed in Figure 1. In comparison, both PCA (not shown) and PLS-DA of ^{1}H-NMR data does not show significant clustering based on gender as illustrated in Figure 2.

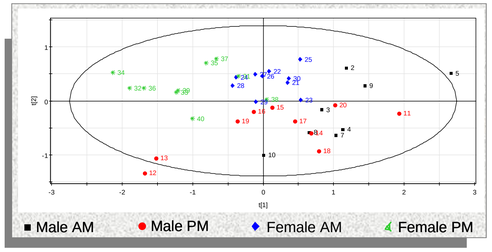

Figure 2. *Scores plot from PLS-DA of ^{1}H-NMR data for male and female Zucker rats. Both AM and PM time collection points have been included in the processing of this dataset. The scores plot was generated using Simca-P+ software from Umetrics.*

When considering diurnal variations, PCA of the UPLC/MS data alone is not sufficient to view clustering in the scores plot as illustrated in Figure 3a. However, using a supervised method, such as PLS-DA, results in some discernable clustering (Figure 3b).

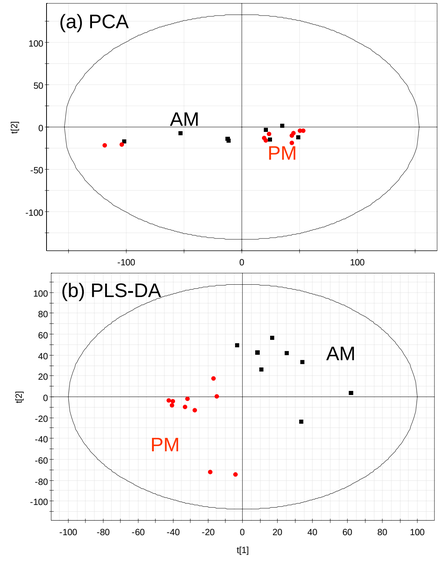

Figure 3. *(a) Scores plot from PCA of LC/MS data for male Zucker rats during the AM and PM time collection points. (b) Scores plot from PLS-DA of UPLC/MS data for the same sample set in (a). Both of the scores plots shown here were generated in Simca-P+ (Umetrics) from an exported MarkerLynx markers table.*

Comparatively, the Scores plot from PLS-DA of the same dataset analyzed by ^{1}H-NMR shows well-defined clusters of AM and PM samples (Figure 4).

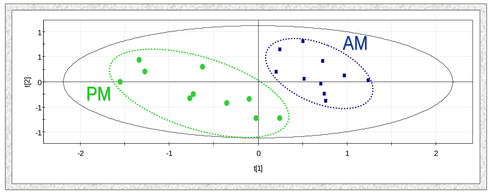

Figure 4. *Scores plot from PLS-DA of ^{1}H-NMR data showing diurnal variation amongst Zucker rats.*

In addition to samples from Zucker rats, control urine samples were also analyzed using the same approach and can be incorporated into the chemometric analysis. Visual inspection of the UPLC/MS data shows observable differ-

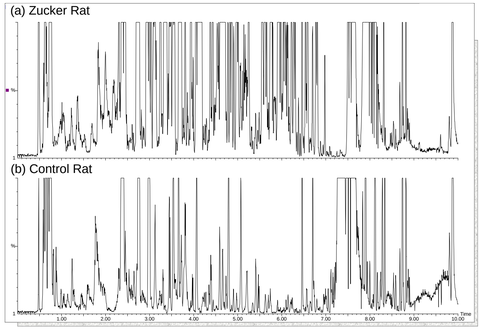

Figure 5. *Representative total ion chromatograms from UPLC/MS analysis of urine from*

Visual inspection of representative ^{1}H-NMR spectra of the same samples also results in observable differences between control and Zucker urine sam-

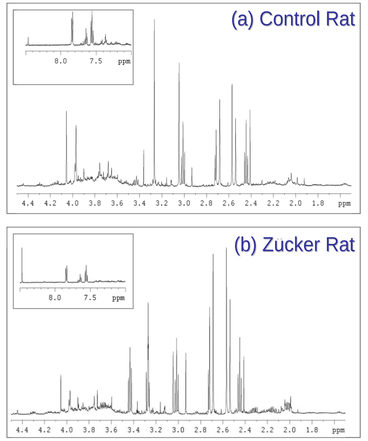

Figure 6. *^{1}H-NMR spectra of (a) control rat urine and (b) urine from a male Zucker rat. In both cases, the inset shows the spectral region from 7.0-8.5 ppm.*

Chemometric treatment of the entire dataset by PCA generates the scores plots in Figure 7. The same group clusterings are seen for both the UPLC/MS and ^{1}H-NMR data illustrating the complimentary nature of the two analytical approaches.

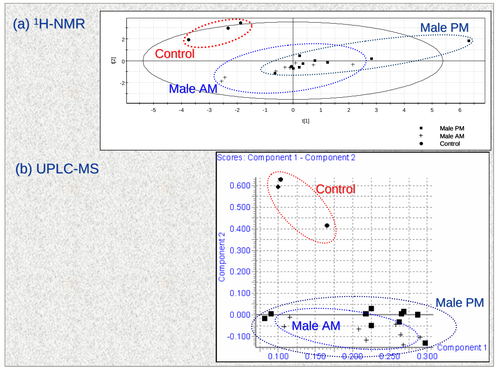

Figure 7. *Scores plots from PCA of (a) ^{1}H-NMR data and (b) UPLC/MS data comparing control rat urine to urine samples from male Zucker rats. The same group clustering is ob-*

The separation shown in Figure 7a for PCA of the ^{1}H-NMR data is based on a higher level of taurine, hippurate and formic acid in the Zucker urine compared to the control animals. Experiments to confirm the identify of the ions responsible for the corresponding separation observed in the scores plot for the UPLC/MS data are currently underway.

Conclusions

- Rapid gradient UPLC-MS, ^{1}H-NMR and multivariate analysis have been successfully employed to identify diurnal and gender differences amongst prediabetic Zucker rats.
- Observable clustering due to diurnal variation amongst Zucker rats is more pronounced in Scores plots generated from ^{1}H-NMR data whereas chemometric analysis of UPLC/MS data shows excellent clustering for gender variation.
- Both UPLC/MS and ^{1}H-NMR data generate Scores plots that show clustering between Zucker rat and control rat urine samples.
- UPLC/MS and ^{1}H-NMR are complementary approaches for metabonomics applications.