Ultra Performance Liquid Chromatography-MS(TOF): New Separations Technology for High Throughput Metabonomics

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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 Performance 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. UPLC/MS(TOF) has also been applied to the metabonomics analysis and elucidation of endogenous biomarkers of gender, genetic state and diurnal variations in black, white and nude mice and obese (fa/fa) Zucker rats. The results presented here will demonstrate the positive impact that high-pressure separations have on resolution and sensitivity. Furthermore, use of UPLC results in the detection of more peaks, which implies that an increased number of possible metabolites and biomarkers may be found. Several biomarkers of gender, genetic state and diurnal variation have been detected using this approach, and will be discussed in terms of biological relevance.

Methods

Sample Set I: Rat 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).
Sample Set II: Mouse urine samples were collected from black (C57BL19J), white (Alpk:ApfCD), and nude male and female mice (n=10) at two time periods, morning and evening for a total of 120 samples.

All samples were 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 and an integrated LockSpray™ interface for exact mass measurements. Data was collected in positive ion mode.

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, 20-95%B in either 10, 5, or 1 min as noted
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
Scan Rate/Interscan	0.4s/0.1s

Results & Discussion

One of the most valuable attributes of UPLC is the dramatic improvement in resolution over a conventional LC approach. Figure 1 compares two total ion current chromatograms (TICs) for male Zucker rat urine analyzed using LC/MS and UPLC/MS.

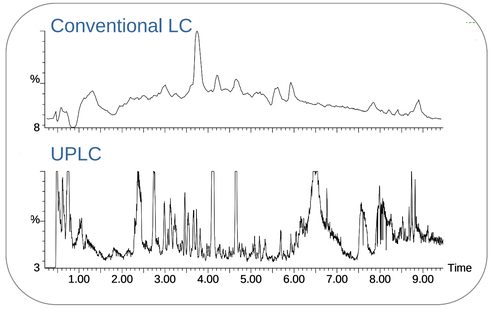

Figure 1. *Comparison between conventional LC (upper) and UPLC (lower) TIC over a ten minute gradient time or the same sample of male Zucker rat urine.*

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 sample set. The processed data list is then analyzed by Principal Components Analysis (PCA) within the MarkerLynx program. An example of the MarkerLynx browser for diurnal variation in male Zucker rat urine samples is presented in Figure 2.

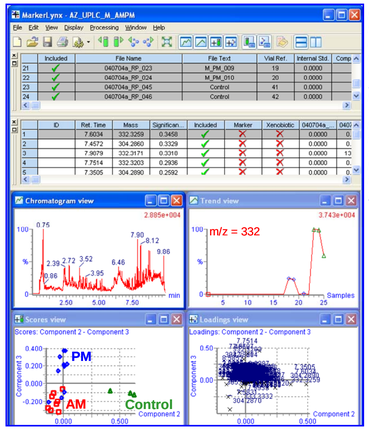

Figure 2. *MarkerLynx browser screen capture for the UPLC-MS analysis of diurnal variation in male Zucker rats. The browser window shown here displays the scores and loadings plots from PCA as well as the trend line for the m/z = 332 ion across all samples.*

It is also possible to investigate the entire sample set using a more sophisticated chemometric algorithm such as partial least squares discriminant analysis (PLS-DA) after data export from MarkerLynx. Figure 3 displays a three-dimensional scores plot from PLS-DA of UPLC/MS(TOF) data from male and female Zucker rat urine and control samples.

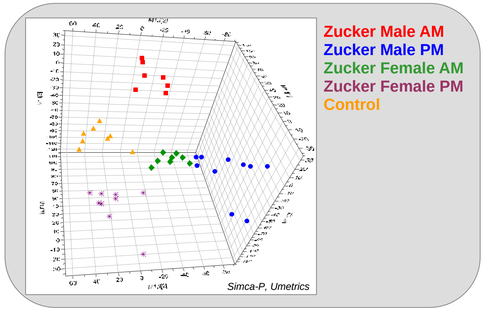

Figure 3. *Resulting Scores plot from PLS-DA of UPLC/MS data from analysis of male and female Zucker rat urine in comparison to control samples.*

In addition to Zucker rat urine, a second, and larger, set of samples was investigated by UPLC/MS(TOF) to determine the effect of rapid gradients on chemometric results. This sample set is comprised of urine from male and female black, white and nude mice at two time collection points for a total of 120 samples. Data was acquired using a ten, five, and one minute gradient. Figures 4-6 show the resulting scores and loadings plots for PCA of the UPLC/MS data.

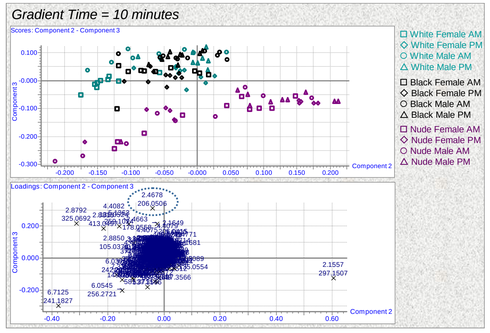

Figure 4. *Scores (upper) and loadings (lower) plots for strain variation in male mice (evening time collection). Distinct groups are observed for black, white and nude mice. The loadings plot reveals several key ions, one of which is m/z = 206, encircled in blue. This ion is attributed to xanthurenic acid (4,8-dihydroxy-quinoline-2-carboxylic acid), a known metabolite of tryptophan catabolism (Figure 7).*

Since the m/z = 206 ion is a typical marker for strain variation, one can use this ion, as well as the scores and loadings plots from PCA, to determine if any critical information is lost when moving to faster gradient times. Figures 5 and 6 present the scores and loadings plots generated from PCA of UPLC/MS(TOF) data where a five minute and one minute gradient time was employed.

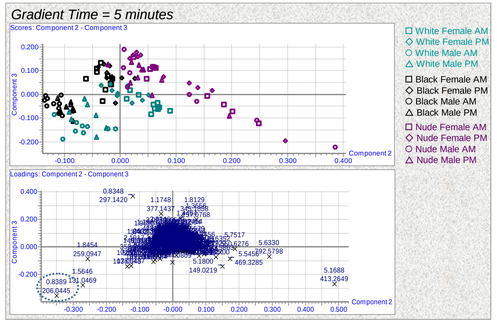

Figure 5. *Scores (upper) and loadings (lower) plots for strain variation in male mice (evening time collection). In this instance the black and white mice group closely together and apart from the nude mice. The m/z = 206 (encircled in blue) in the loadings plot remains a significant contributor to the observed clustering in the scores plot.*

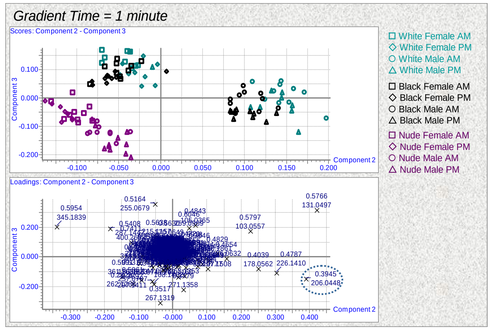

Figure 6. *Scores (upper) and loadings (lower) plots for strain variation in male mice (evening time collection). In this instance the black and white mice group closely together and apart from the nude mice. The m/z = 206 (encircled in blue) in the loadings plot remains a significant contributor to the observed clustering in the scores plot. The total analysis time for the entire sample set was 220 minutes.*

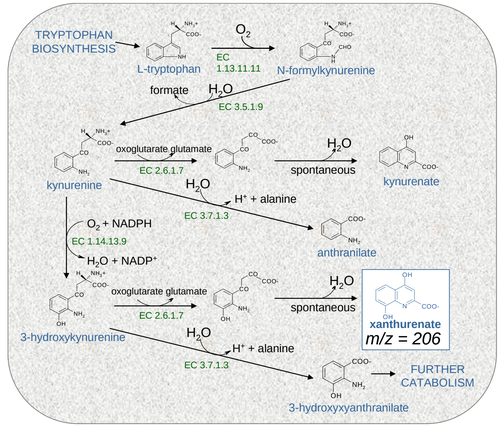

Figure 7. *Metabolic pathway for tryptophan catabolism. Xanthurenic acid has been identified as a marker of strain variation in black, white and nude mice.*

The trend plot for the m/z = 206 ion also does not vary with the reduction in gradient times, as illustrated in Figure 8.

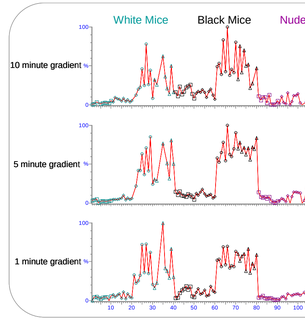

Figure 8. *Ion intensity trend lines for the m/z = 206 ion for a ten, five and one minute gradient time.*

Conclusions

- UPLC-MS and multivariate analysis have been successfully employed to identify diurnal, gender and strain differences amongst Zucker rats.
- High-throughput metabonomic analysis using one minute gradient times in UPLC-MS is possible with key markers still identifiable in the loadings plot.