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A Metabonomics Approach to Determining Strain, Gender, and Diurnal Variations in Mouse Urine by LC-MS

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Abstract

Metabonomics is defined as a "systems approach to investigating the metabolic consequences of (patho) physiological or genetic modification in a multivariate and dynamic manner".[1] Furthermore, it is a rapidly emerging science that allows the natural clustering of sample groups to be highlighted after analysis of biofluids (e.g. urine and plasma). In the past, these clustering variations have been elucidated by sample analysis via ^{1}H-NMR and GC-MS. Recently, an LC-MS alternative to the aforementioned techniques has been developed and is readily accomplished by using a Waters exact mass LC-MS system with a dedicated MassLynx v4.0 Application Manager to allow the seamless acquisition and analysis of metabonomics data.

Benefits

With this analysis strategy, variations are easily identified amongst groups of female and male mice, and more impressively, amongst black, white, and nude mice.

Introduction

One of the challenges of research in the post-genomic era has been the quest for a more complete set of information to provide an understanding of the entire organism as opposed to its components (e.g. cellular macromolecules). Metabonomics, which embraces a whole-organism strategy, can be used to evaluate the biological response to some type of stimuli by determining the variation in low molecular mass metabolites. Several significant contributions to this approach have been made by using ^{1}H-NMR and multivariate data analysis techniques (e.g. principal component analysis (PCA), and partial least squares discriminant analysis (PLS-DA)) to investigate strain-related differences,[2] gender and diurnal variations in mice,[3] and metabolic variations in rats.[4] Although an extremely effective analytical tool for metabonomic studies, high field NMR spectrometers are not always a feasible approach for research due to their cost and limited availability. A viable alternative to a NMR-based metabonomics approach is found in LC-MS where one has the capacity to separate analytes in complicated sample matrices, determine the exact masses of key components, and elucidate the chemical structure of the potential biomarkers via MS/MS. Preliminary studies of the use of LC-MS with data analysis by PCA to determine differences in the composition of urine from control and dosed rats have successfully placed LC-MS as an invaluable resource in the metabonomics toolbox.[5]

In this study, we use an exact mass LC-MS strategy with PCA data analysis to investigate variations in the endogenous urinary metabolite profile for mice from three distinct strains (white, black, and nude) as well as those resulting from gender differences and diurnal variations. The ability to determine the natural clustering amongst such sample groups lays the foundation for distinguishing between animals of varying health status due to disease states or toxic insult.

Technology

Inherent to the creation of a complete and successful metabonomics LC-MS solution is the marriage of three key system components: a chromatographic system with excellent reproducibility, a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer with a lock mass source for exact mass determination, and processing software for PCA. In order to achieve this, the Waters Metabonomics MS System is comprised of a Waters 2795 XC Separations Module and a Waters Micromass Q-Tof micro with a LockSpray interface, both operating under MassLynx v4.0 Software control with the MarkerLynx Application Manager.

The analysis of large numbers of complex biological samples without rigorous sample preparation (in order to

avoid a biased loss of analytes) can be a challenging prospect. For the analysis of such biofluids as urine, a robust chromatography-based approach was selected over direct infusion due to the diverse polar nature of the sample components. Additionally, we use a chromatographic strategy that employs the use of a short linear gradient to ensure high throughput.

In addition to a reliable chromatographic system and method, there are several merits to biomarker identification using a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer. First, the instrument is more sensitive in full scan mode and has a good linear dynamic range. Furthermore, the use of a Q-Tof Mass Spectrometer with a LockSpray lockmass interface allows the determination of the exact mass of a compound through MS/MS experiments.

Currently, the analysis of metabonomic data involves exporting the raw data to an external statistical package. In most instances, this inter-software transfer process can be tedious and time-consuming since it doesn't allow dynamic interaction with the raw data. For these reasons a new MassLynx Applications Manager, MarkerLynx, has been developed. MarkerLynx is a single software solution that detects peaks in the data set, creates a list of the detected peaks and their corresponding intensities, allows for removal of xenobiotics, and performs PCA. The results are displayed in a scores and loadings plot, from which ion identification can be performed.

Experimental

Samples were collected from black (C57BL19J) white (Alpk:ApfCD) and nude male and female mice (n=10) by minimal manipulation of the lower abdomen as described elsewhere. Samples were stored at -20°C prior to analysis. Prior to the study, animals were housed in polycarbonate solid bottom cartridges, according to strain. Animals were allowed free access to water and food (pelleted irradiated rat and mouse diet 1) (Special Diet Services, Witham, Essex, UK), from weaning until the end of the experiment. Animals were maintained at room temperature with artificial 12 hr. dark/12 hr. light cycles.

A 100 µL aliquot of deionized water was added to each sample of mouse 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.

All analytical work was accomplished using the Waters Metabonomics MS System. The chromatography was performed on a Waters 2795 XC Separations Module equipped with a column heater. The HPLC system was coupled with a Waters Micromass Q-Tof micro equipped with an electrospray source operating in either positive ion or negative ion mode.

LC Conditions

LC system: Waters 2795 XC Separations Module

Column: Waters Symmetry C_{18} 3.5 µm 2.1 x 100 mm

Flow rate: 600 µL/min. split to 150 µL/min. into MS

Injection volume: 20 µL

Gradient: Linear, 0-20% B over 0.5-4 min., 20-95% B over 4-
                                          8 min., hold at 95% B for 1 min. then return to 0%
                                          B at 9 min. where A = 0.1% Formic Acid in Water,
                                          and B = 0.1% Formic Acid in Acetonitrile

MS Conditions

MS system:	Waters Micromass Q-Tof micro with a LockSpray interface
Ion mode:	Positive and Negative Electrospray
Cone voltage:	40 V
Desolvation temp.:	150 °C
Source temp.:	100 °C
Collision energy:	10 eV
Detection mode:	Full Scan (100–1400 *m/z*)
Dwell:	0.1 sec.
Collision gas:	Argon, 5×10^{-3} mbar
Lock mass:	50 fmol/µL Leucine Enkephalin in 50:50 Water:Acetonitrile (0.1% Formic Acid) at 200 µL/min.

The resulting collected LC-MS data was analyzed using the MarkerLynx Application Manager on a MassLynx v4.0 workstation. The raw data was integrated and the detected peaks from each sample were used to construct a comprehensive list of all components in the analyzed samples.

Results and Discussion

A set of 120 urine samples from male and female (n=20 each) white, black, and nude mice were analyzed using both positive and negative ionization. A representative total ion chromatogram for the morning urine collection
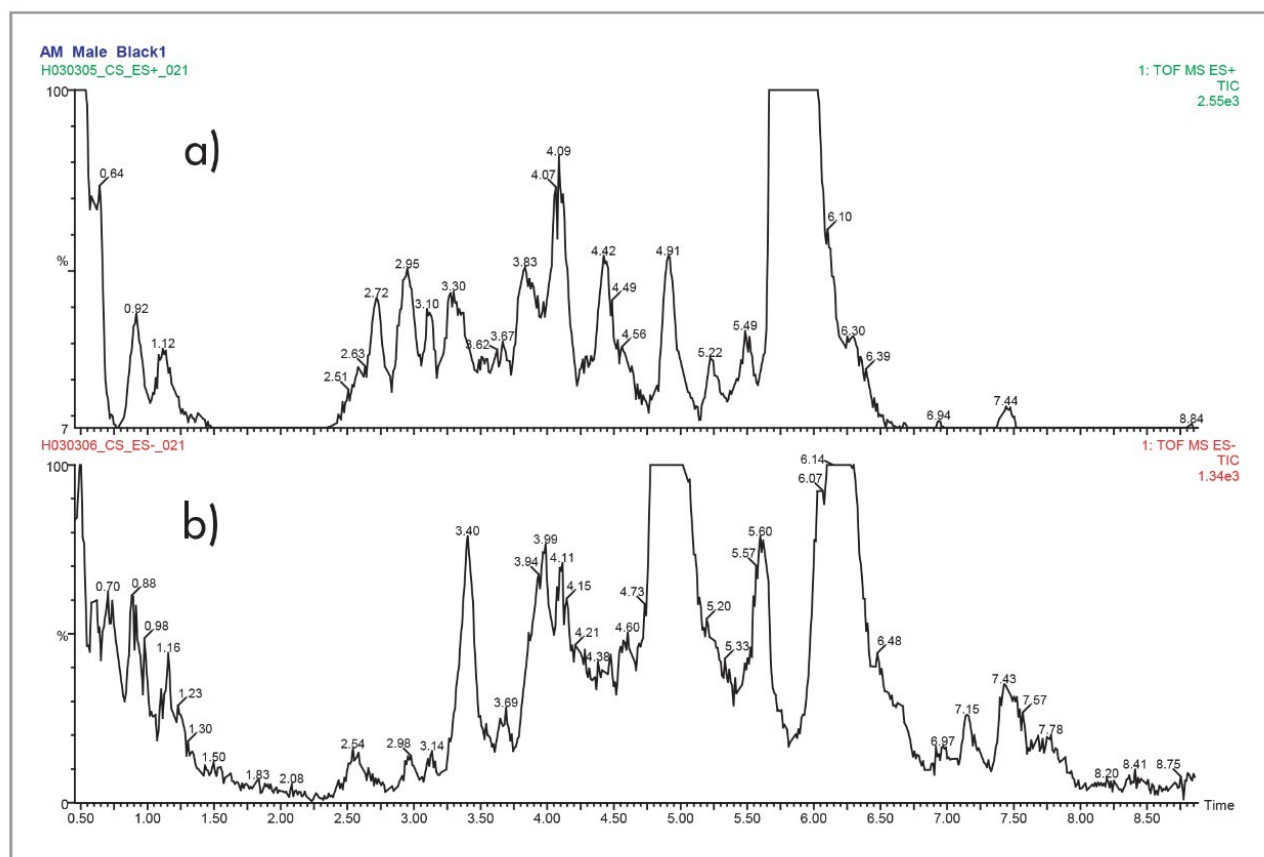
from a male black mouse is presented in Figure 1.

Figure 1. Typical total ion chromatograms for (a) positive and (b) negative ionization modes for the morning urine collection from a male black mouse. Plots have been enlarged to show fine structure.

After data collection, the raw data was analyzed by PCA using MarkerLynx. The resulting scores plots correspond to the data set by showing clustering according to intersample variation. The loadings plots indicate the most influential ions that are responsible for separation between sample classes, and these ions are then selected for further MS/MS experimentation. Figures 2, 3, and 4 display representative MarkerLynx screen shots of scores and loadings plots that illustrate clustering based on diurnal variation, gender, and strain.
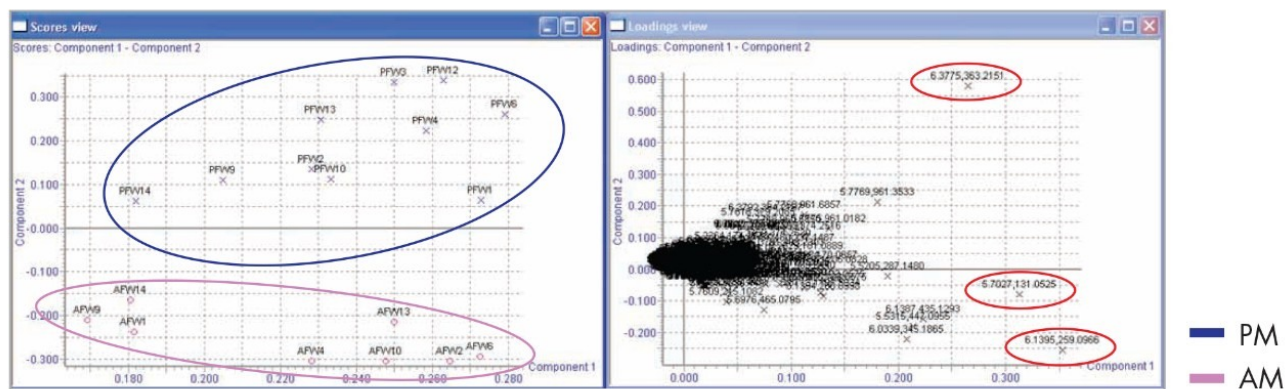
Figure 2. Scores and loadings plots for urine collected in the morning and evening from female white mice. The scores plot is shown on the left, and the corresponding loadings plot is displayed on the right.
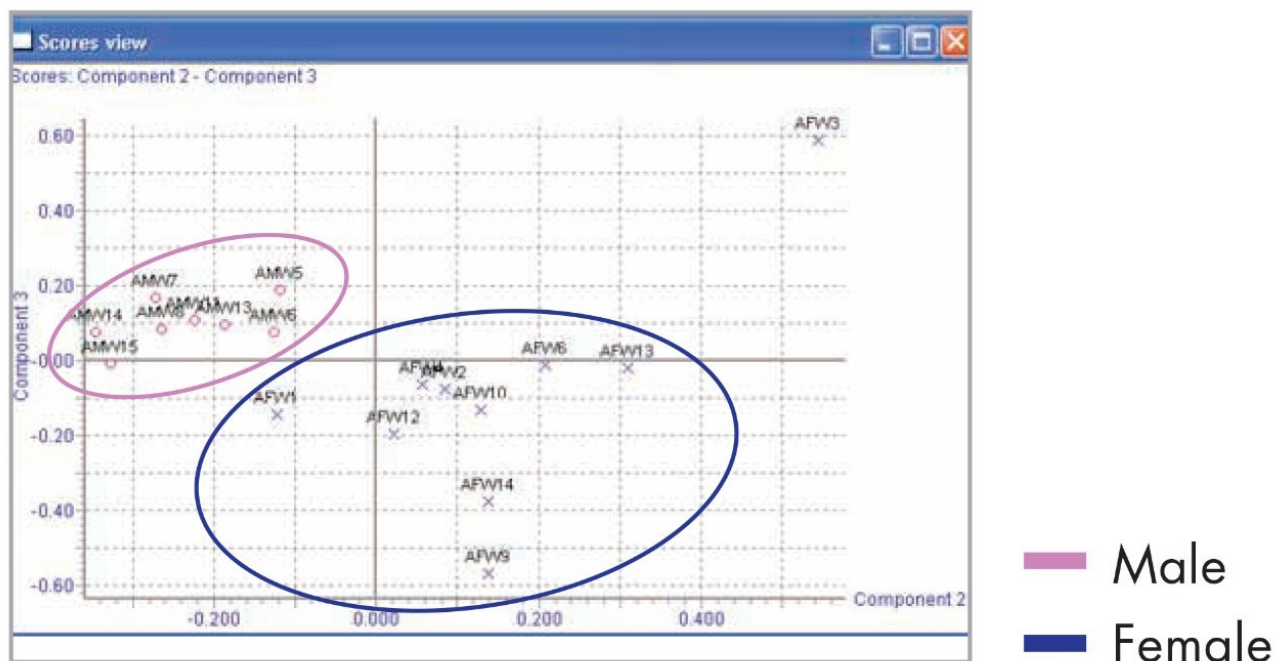

Figure 3. Scores plot for urine collected in the morning from female (blue crosses) and male (pink circles) white mice.
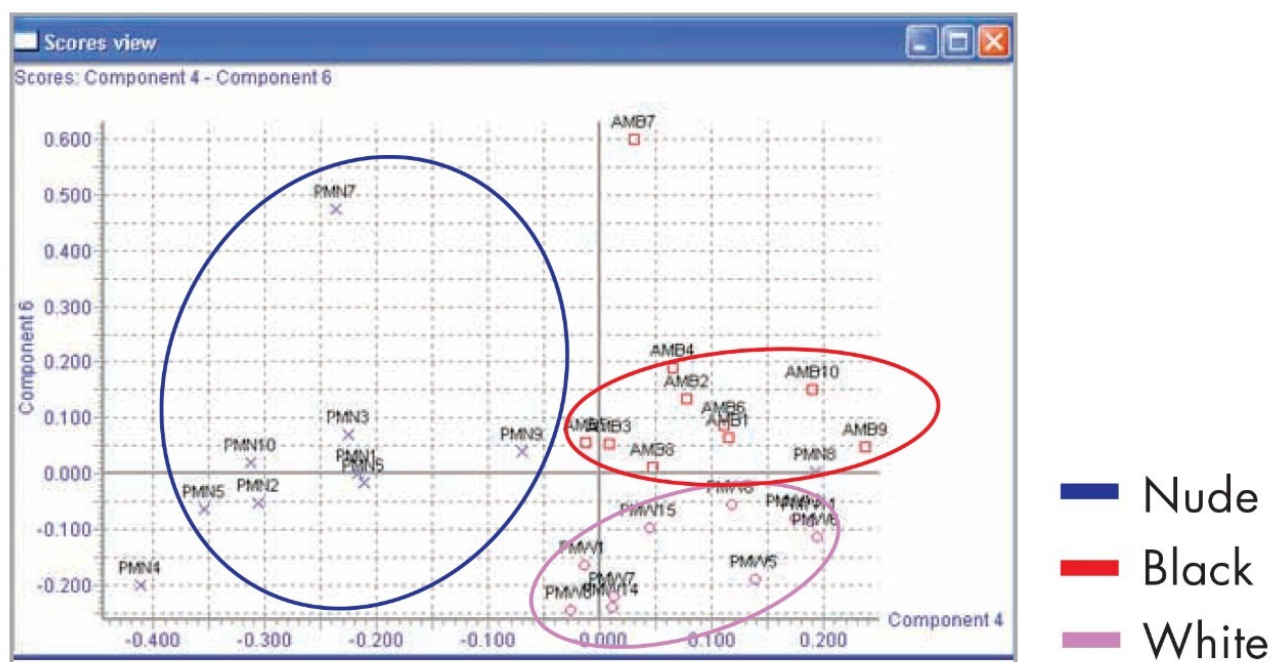
Figure 4. Scores plot for urine collected in the morning from male black (red squares), white (pink circles) and nude (blue crosses) mice.

MarkerLynx processes LC-MS data for analysis and visualization by PCA with no export required. PCA is a multivariate analysis technique that provides a visual medium (*i.e.* the scores plot) to view clustering of samples that are similar. As an example of this, Figure 2 presents the scores plot for mouse urine collected from female white mice during two time periods, morning and evening. It is evident from this plot that the morning and evening samples cluster separately in discrete regions. Furthermore, important information regarding the ions responsible for the observed clustering can be discovered in the loadings plot. In a loadings plot, ions which are furthest away from the origin are the most significant contributors to the clustering. In turn, these ions might be likely candidates for biomarker identification via MS/MS. In the aforementioned case of the male and female mice (Figure 2), one might select m/s = 259.0966 as such a targeted ion. Figures 3 and 4 illustrate further investigations using PCA. With this analysis strategy, variations are easily identified amongst groups of female and male mice, and more impressively, amongst black, white, and nude mice.

Conclusion

The successful use of an exact mass LC-MS-based strategy for metabonomics applications has been demonstrated by the analysis of urine samples from three different strains of mice using the Waters Metabonomics MS System. Data analysis is facilitated by implementation of the MarkerLynx Applications Manager, which performs PCA and allows the experimenter to elucidate sample clustering via a scores plot. Further biomarker identification can then be accomplished by identifying key ions for MS/MS from the loadings plot.

References

1. E. Holmes and H. Antti, *The Analyst*, 2002, 127, 1549.

2. C.L. Gavaghan, E. Holmes, E. Lenz, I.D. Wilson and J.K. Nicholson, *FEBS Lett*., 2000, 484, 169.

3. C.L. Gavaghan, I.D. Wilson and J.K. Nicholson, *FEBS Lett*., 2002, 530, 191.

4. C.L. Gavaghan, J.K. Nicholson, S.C. Connor, I.D. Wilson, B. Wright and E. Holmes, *Anal. Biochem*., 2001, 291, 245.

5. R.S. Plumb, C.L. Stumpf, M.V. Gorenstein, J.M. Castro-Perez, G.J. Dear, M. Anthony, B.C. Sweatman, S.C. Connor, and J.N. Haselden, *Rapid Commun. Mass Spectrom*., 2002, 16, 1991.

6. J.H. Granger, R.S. Plumb, C.L. Stumpf, I.D. Wilson, J. Castro-Perez, and H. Major, "Identification of Novel Biomarkers of Genetic State in Mice by NMR, LC-MS, and Metabonomics", *ASMS* 2003, Montreal, Canada.

7. R. Plumb, J. Granger, C. Stumpf, H. Major, J.M. Castro-Perez, I.D. Wilson, J.A.E. Evans, and E.M. Lenz, *The Analyst*, in press.

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