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A Metabonomics Approach to the Study of Rat Urine After Dosing With the Hepatotoxin Hydrazine

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Abstract

Hydrazine is a model hepatotoxin commonly used in animal studies and the metabonomic analysis of ^{1}H NMR spectra has shown that the progression of toxicity and recovery can be monitored while also allowing identification of novel biomarkers of development and regression of the lesion.

The aim of this study was to determine whether similar data could be obtained using LC-MS. A rapid gradient on a Waters Alliance 2795 HT with a Waters Symmetry Column was employed to maximize throughput while maintaining chromatographic resolution and to help alleviate the problem of ion suppression that results from infusion of complex matrices. A Waters Micromass Q-Tof micro fitted with a LockSpray source ensured exact mass MS and MS/MS measurements to aid identification of any potential biomarkers highlighted.

To examine the wealth of data generated effectively, statistical data reduction and multivariate analysis techniques such as principal component analysis (PCA) need to be employed.

Benefits

LC-MS can provide complementary information to ^{1}H NMR in metabonomic applications and may be a viable alternative in the field of drug discovery and development

Introduction

Metabonomics is the identification and measurement of time-related metabolic profile changes as the result of exposure to a toxin or drug, to environmental effects or the onset of disease.[1] This is the study of endogenous metabolites rather than xenobiotics and their metabolites. The down-regulation and up-regulation of these endogenous metabolites are indicative of biological insults as a result of disease or toxins; knowledge of these metabolites can provide potential biomarkers that can be used in disease diagnosis or toxicity screening.[2] This is a rapidly growing area of scientific research which until now has primarily utilized ^{1}H NMR as the analytical method of choice.

Hydrazine is a model hepatotoxin commonly used in animal studies and the metabonomic analysis of ^{1}H NMR spectra has shown that the progression of toxicity and recovery can be monitored while also allowing identification of novel biomarkers of development and regression of the lesion.[3]

The aim of this study was to determine whether similar data could be obtained using LC-MS. A rapid gradient on a Waters Alliance 2795 HT with a Waters Symmetry Column was employed to maximize throughput while maintaining chromatographic resolution and to help alleviate the problem of ion suppression that results from infusion of complex matrices. A Waters Micromass Q-Tof micro fitted with a LockSpray source ensured exact mass MS and MS/MS measurements to aid identification of any potential biomarkers highlighted.

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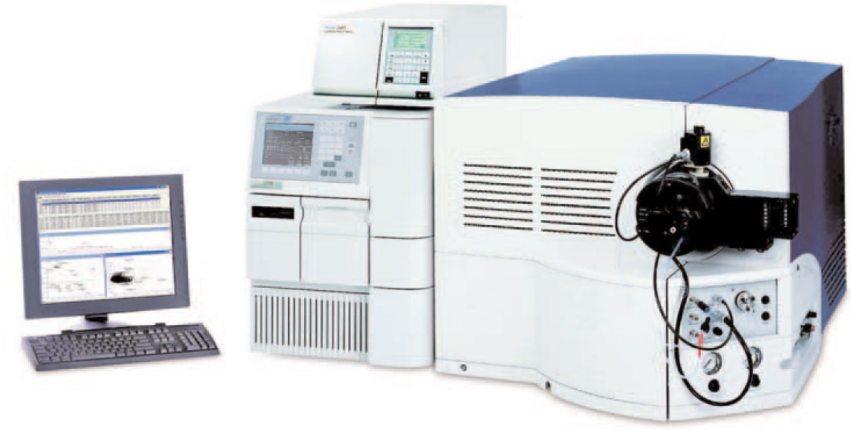

Figure 1. Waters Micromass Metabonomics System.

Experimental

Animal Studies

Male Han Wistar rats (8 per group) were either given a single oral dose of hydrazine (30 or 90 mg/kg) formulated in saline or used as a control group. The animals were housed in metabolism cages, and urine collected into refrigerated urine racks. Urine samples were collected from all animals over the periods -24 to -12 hr and -12 to 0 hr (pre-dose samples), and 0–12 hr, 12–24 hr, 24–48 hr, and 48–72 hr post-dose. At 72 hr four animals from each group were sacrificed for histopathological examination. Urine samples were collected from the remaining animals for the periods 72–96 hr, 96–120 hr, 120–144 hr and 144–168 hr after dosing. Each animal was sacrificed after completion of the final collection and all urine samples were frozen at -20 °C until analysis.

Conditions

LC-MS was performed on a Waters Alliance 2795 HT coupled to a Waters Micromass Q-Tof micro equipped with a LockSpray source operating in either electrospray positive ion or negative ion mode.

A 10 µL aliquot of rat urine was injected onto a 2.1 x 100 mm Symmetry C_{18} 3.5 µm Column held at 40 °C. The initial condition of 100% mobile phase A was held for 0.5 min at a flow rate of 600 µL/min. The column was then

eluted with a linear gradient of 0-20% B over 0.5–4 minutes then 20-95% B over 4–8 min, held at 95% B for 1 minute then returned to 100% A, where mobile phase A = 0.1% aqueous formic acid and B = 0.1% formic acid in acetonitrile. The column eluent was split such that approximately 120 µL/min were directed to the mass spectrometer.

The source temperature was set at 120 °C with a cone gas flow of 50 L/h, a desolvation gas temperature of 250 °C and a desolvation gas flow of 500 L/h. The capillary voltage was set at 3.2 kV for positive ion mode and 2.6 kV in negative ion mode and the cone voltage to 30 V. An accumulation time of 0.4 sec with an inter-scan delay of 0.1 sec was used. A collision energy of 4 eV and a collision gas pressure of ~2.8 x 10^{-3} mbar argon was employed throughout. A lock mass of leucine enkephalin at a concentration of 0.5 ng/µL, in 50:50 acetonitrile: water + 0.1% formic acid for positive ion mode and 1 ng/µL in 50:50 acetonitrile: water for negative ion mode, was employed at a flow rate of 30 µL/min via a LockSpray interface. Data were collected in centroid mode, the LockSpray frequency was set at 5 seconds and data were averaged over 10 spectra.

The mass spectrometric data were collected from 50 to 1500 *m/z* over 0–10 minutes, in both positive and negative ion modes.

Data Processing - MarkerLynx

Due to the wealth of data produced, statistical data reduction and multivariate analysis techniques such as principal component analysis (PCA) need to be employed. MarkerLynx is an application manager for MassLynx v4.0 Software incorporating a peak deconvolution package which allows detection and retention time alignment of the peaks eluting in each chromatogram. The data is then combined into a single matrix by aligning peaks with the same mass/retention time pair together from each data file in the dataset, along with their associated intensities. The mass and retention time windows employed to do this are set by the user. An example of the resulting MarkerLynx report is shown in Figure 2.

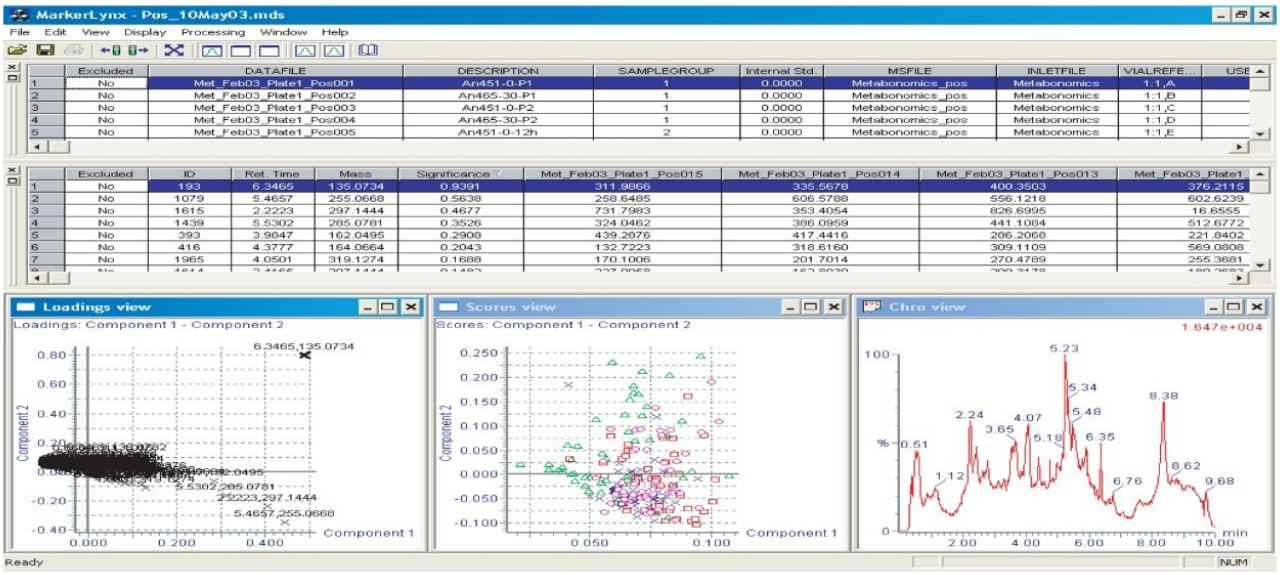

Figure 2. MarkerLynx report for positive ion hydrazine data.

This includes a Sample List with all the information associated with each sample a Marker List which lists all the components detected and a chromatogram window. The final part of the report is associated with the principal components analysis (PCA) which allows the simple visualization of separation or clustering between samples as a 2-dimensional plot of the principal components (scores). There is an option to turn the annotation off to allow clearer visualization of any separation between groups.

The associated loadings plot indicates which variables contribute to the formation of the scores and allows for the identification of those variables (*m/z* values) of greatest influence to the separation/clustering. These data provide insight into the biochemical process or mechanism of toxicity.

Export of Data

PCA can give useful information about separation between classes but is an unsupervised technique *i.e.* no prior knowledge of the samples is assumed. The use of supervised techniques such as PLSDA (partial least squares discriminant analysis) makes use of any knowledge of the sample class and maximizes the separation between the classes.

In addition to the ability to undertake PCA analysis within MarkerLynx the facility to export data to third party

packages such as SIMCA-P (Umetrics, Sweden) and Pirouette (Infometrix, USA) has also been made easy through customized style sheets. As an example, the results were exported to SIMCA multivariate software and the data processed by PLS-DA.

Results and Discussion

Positive Ion Results

The positive ion data was exported from MarkerLynx into SIMCA-P and the PLS-DA scores plot for all the positive ion data is shown in Figure 3. It is apparent that only the high dose samples show separation from the pre-dose and the controls although the separation is slightly improved compared with the PCA. Examination of the weights plot, Figure 4, indicates that the principal ions responsible for the separation in positive ion are *m/z* 135, 311, 217, and 132 increasing after dosing and *m/z* 297, 162, 260, 319, and 285 decreasing after dosing.

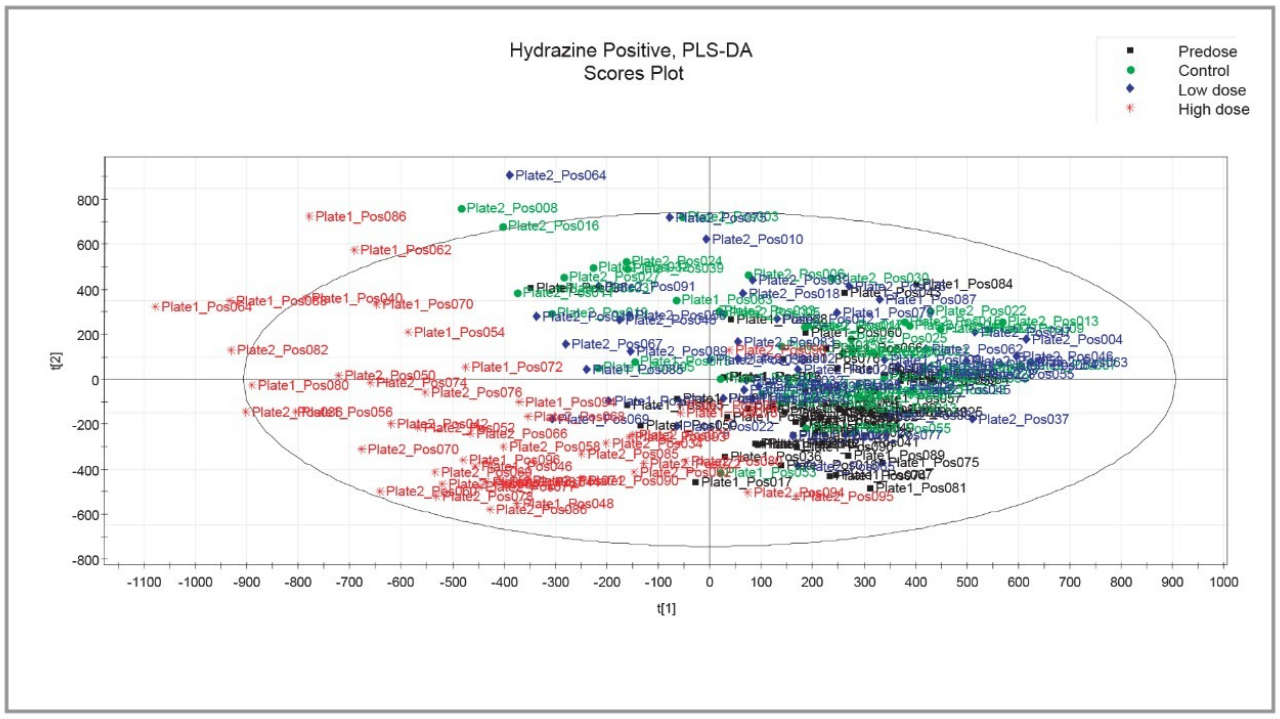

Figure 3. PLS-DA scores plot for all the positive ion data processed using SIMCA-P Software.

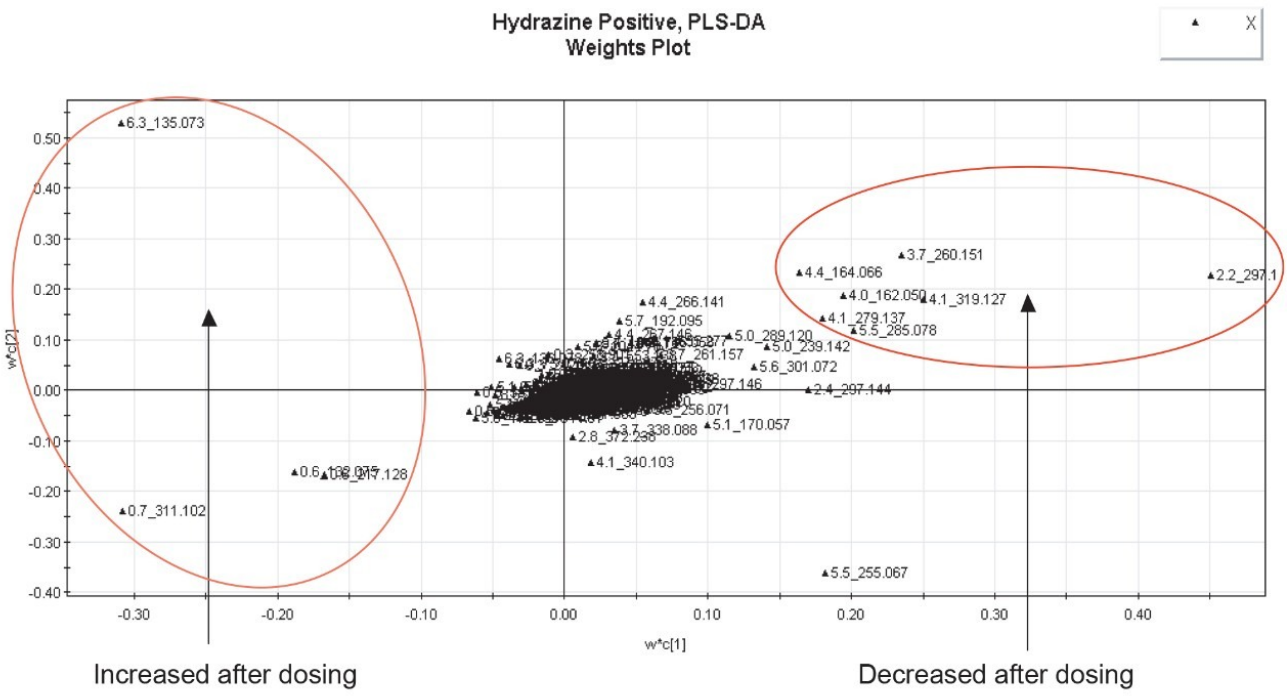

Figure 4. PLS-DA weights plot for all positive ion data processed using SIMCA-P.

The exact mass measurement of the ion at *m/z* 132, shown to increase after dosing, was consistent with creatine, $C_4H_9N_3O_2$, with a calculated mass of 132.0773 for the $(M+H)^+$ ion, see Figure 5.

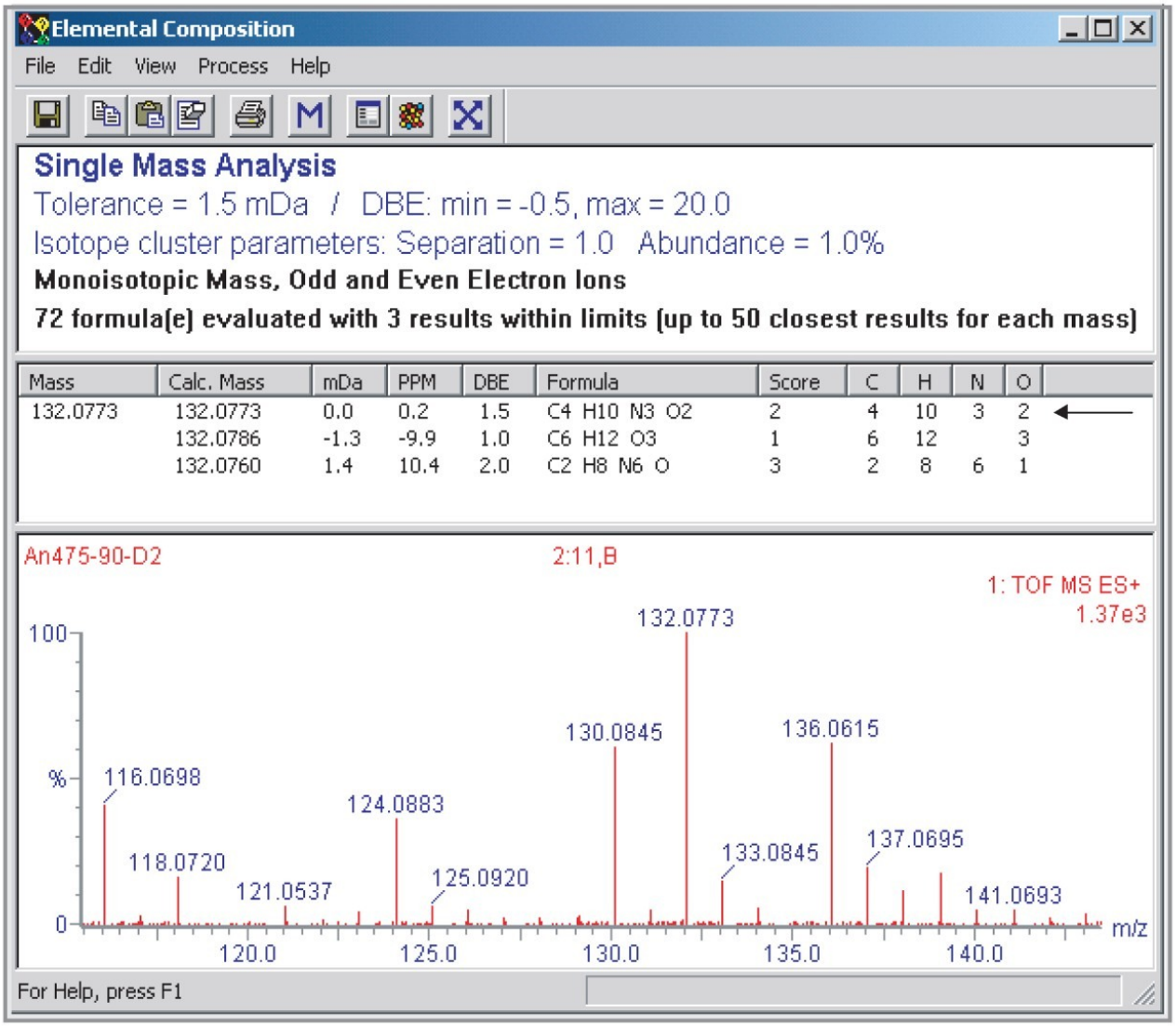

Figure 5. Exact mass measurement and elemental composition report for ion at m/z 132 shown to increase after dosing with hydrazine.

Exact mass MS/MS product ion analysis confirmed the ion as being from creatine, see Figure 6.

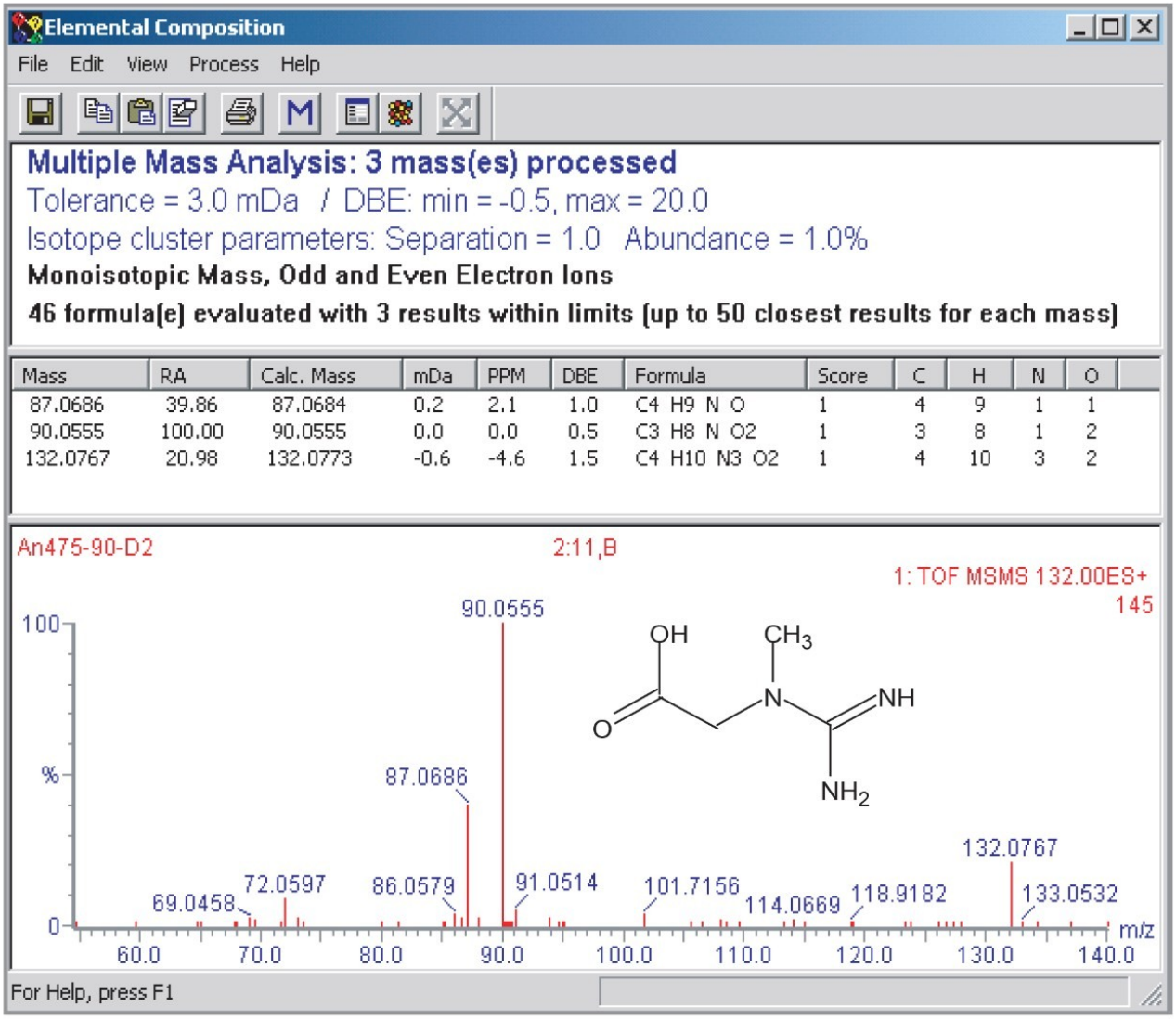

Figure 6. Exact mass MS/MS product ion spectrum for m/z 132 from creatine.

Negative Ion Results

The MarkerLynx negative ion results for all 192 samples are shown in Figure 7.

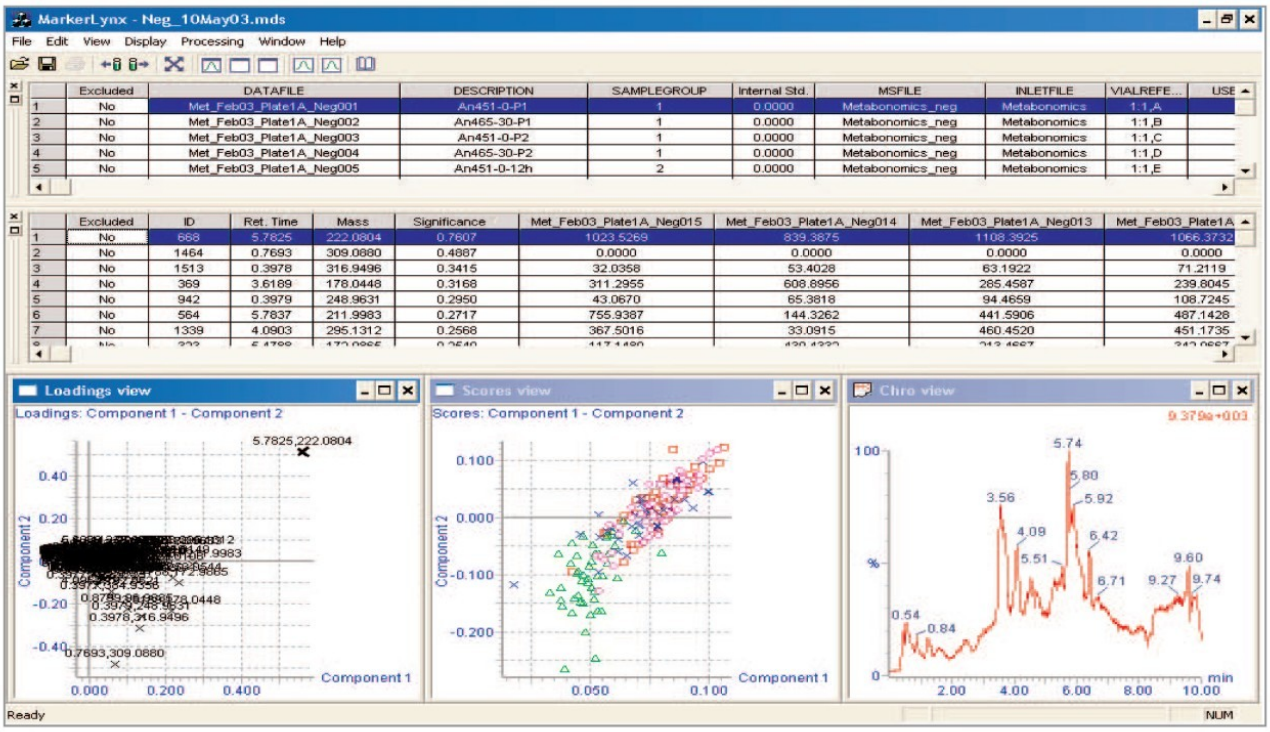

Figure 7. MarkerLynx report for negative ion hydrazine data.

The PLS-DA scores and weights plot for all the negative ion data are shown in Figures 8 and 9 respectively. These indicate that the *m/z* values responsible for the separation are *m/z* 309, 317, and 192 increasing after dosing and *m/z* 222, 295, and 178 decreasing after dosing.

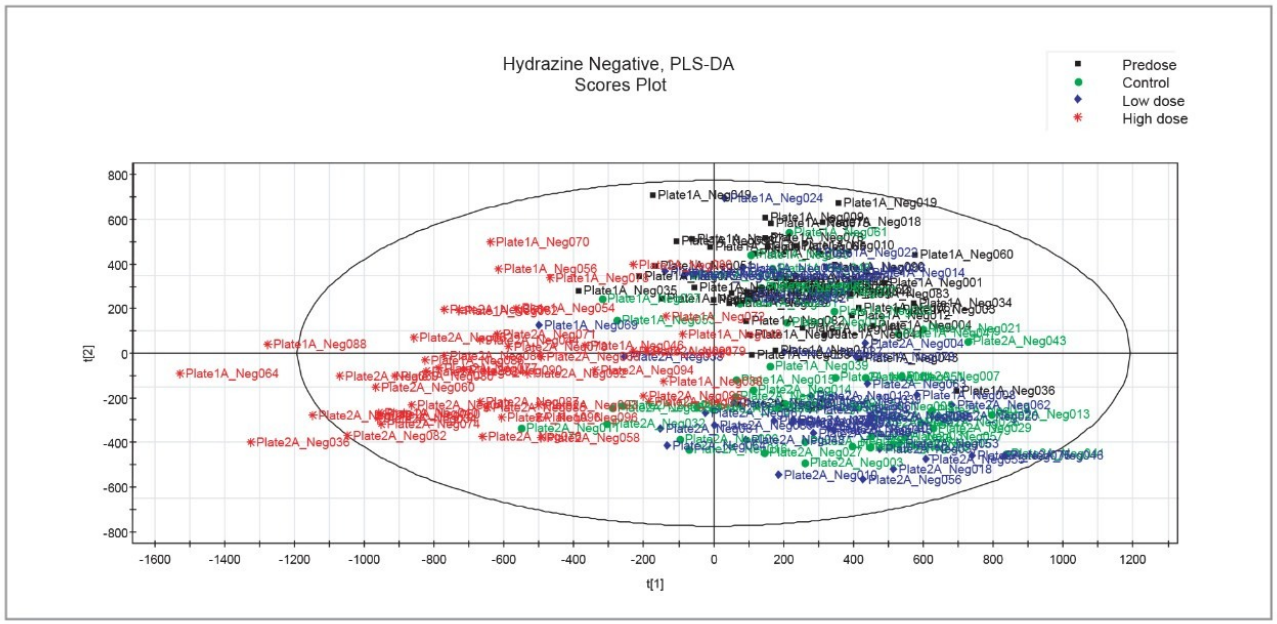

Figure 8. PLS-DA scores plot of all negative ion data.

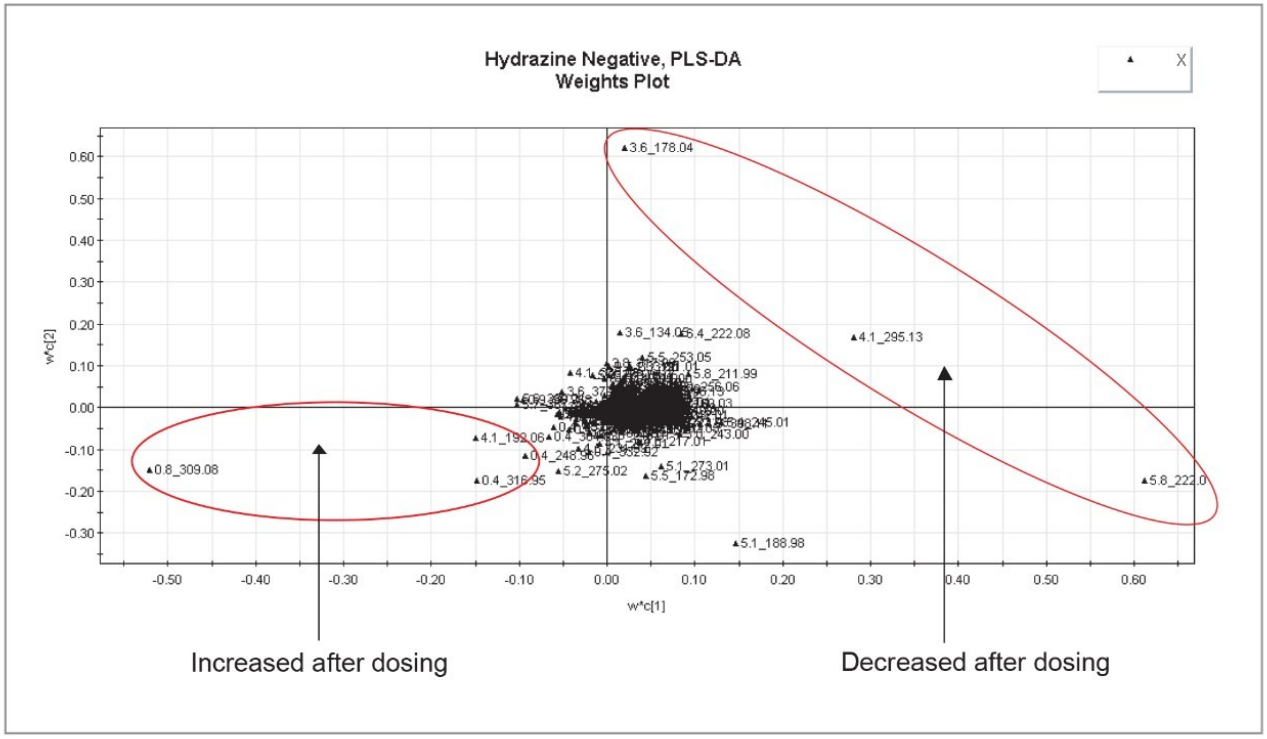

Figure 9. Weights plot for negative ion data.

A similar separation was observed between the high dose and the controls and low dose samples as had been observed with the positive ion data.

Exact mass MS/MS product ion analysis on the *m/z* 178 observed in negative ion mode confirmed it as being hippuric acid (see Figure 10) which was also observed as decreasing after dosing in the NMR study on the same sample set.

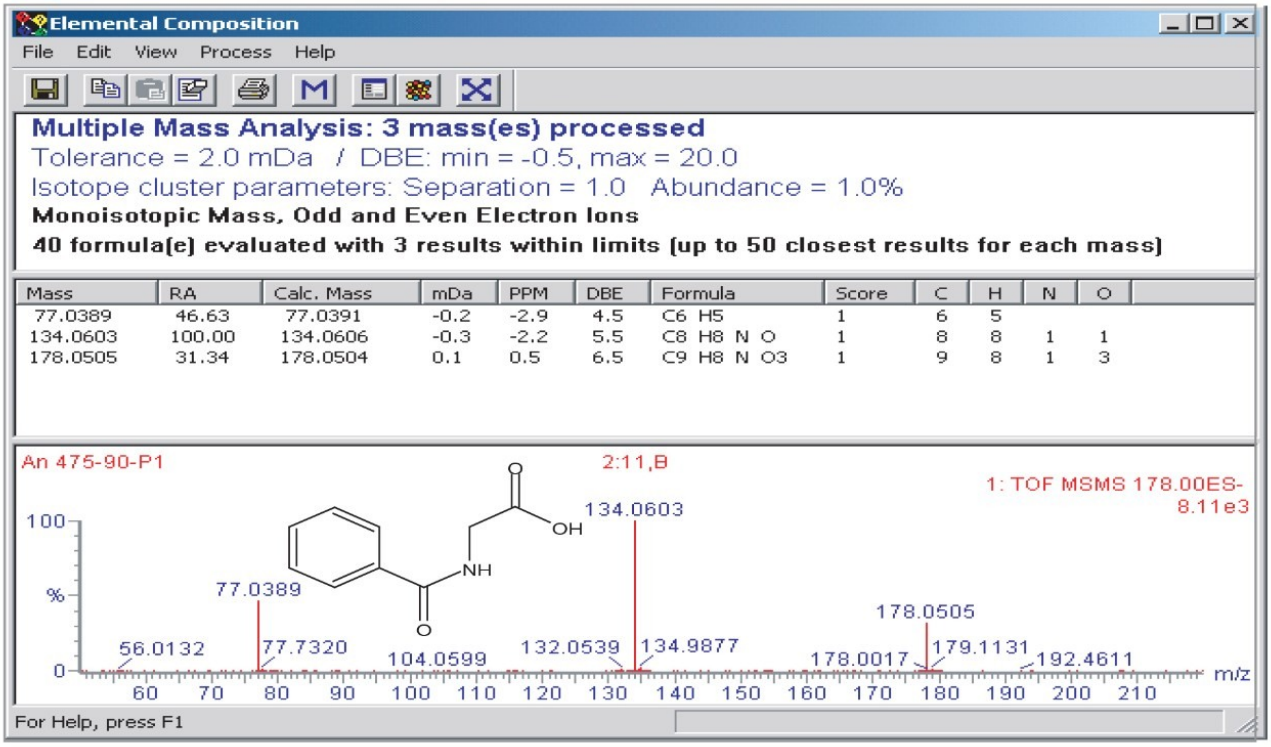

Figure 10. Exact mass MS/MS measurements of m/z 178 from hippuric acid.

Conclusion

The separation achieved by MS was very similar to that seen by NMR, with a bacterial contamination of the samples showing greater variability than the effect of the low dose of hydrazine (see Figure 11). However the PCA and PLS-DA do show that i) MS can differentiate high dose samples from the controls, and ii) the ions responsible for the separation can be identified. Further elucidation of these potential biomarkers is required by exact mass MS/MS before their biological significance can be determined.

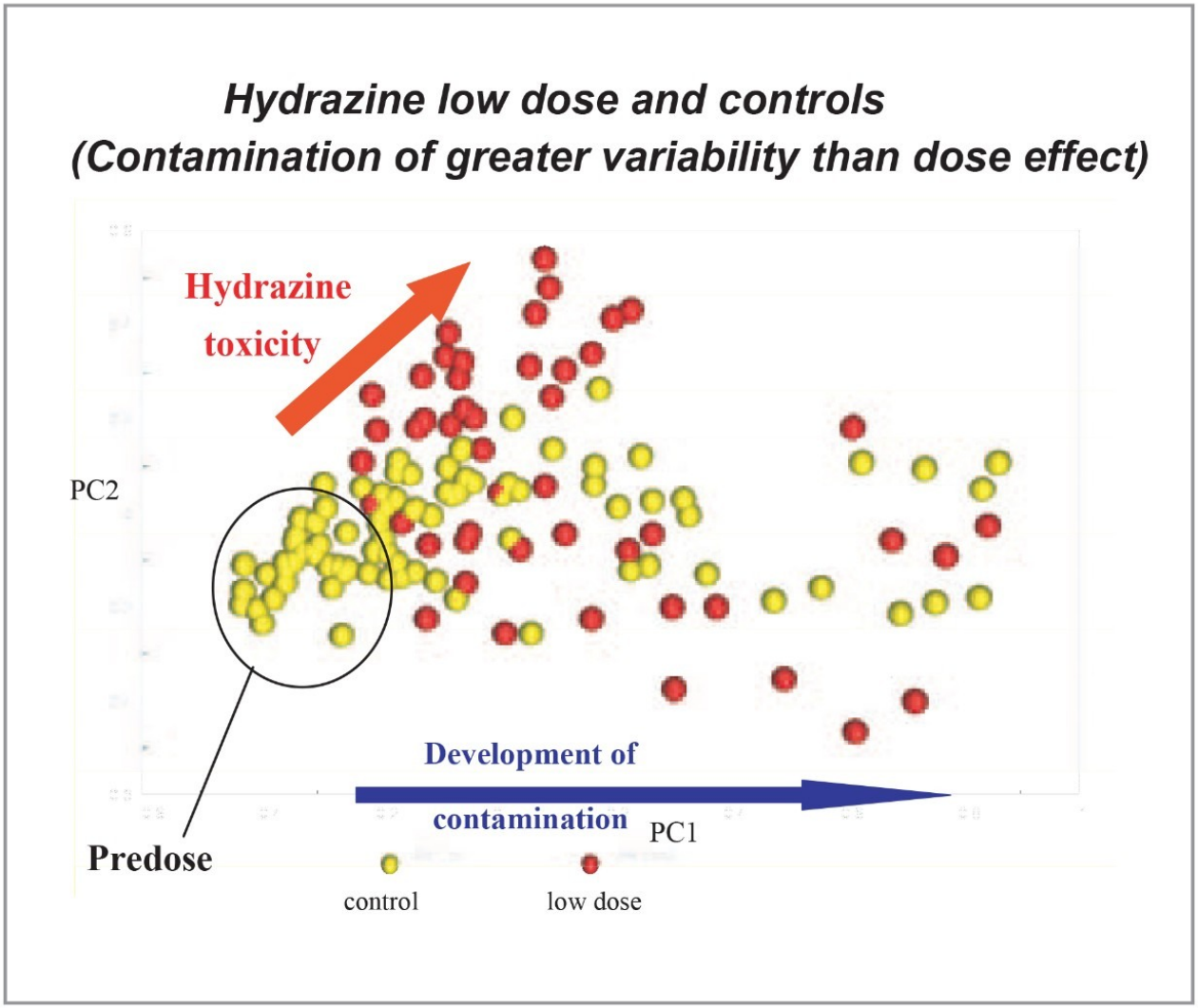

Figure 11. NMR PCA data showing controls and low dose samples.

Creatine was identified as increasing after dosing by MS. Increased urinary levels of creatine and taurine observed in the NMR study were associated with reduced liver function. Many of the highly polar species such as amino acids, organic acids and sugars identified as being biomarkers by ^{1}H NMR are not retained under the reversed-phase chromatography conditions employed here. The identification of these species will be the subject of future studies.

However, MS has advantages in that there are compound classes e.g. sulphates, which can not be detected or

where their concentration is too low to be seen by NMR. The results presented here clearly show that LC-MS can provide complementary information to ^{1}H NMR in metabonomic applications and may be a viable alternative in the field of drug discovery and development.

Potential Applications

- Biomarkers of disease state (for diagnosis and measuring therapeutic effect)
- Safety and efficacy - biomarker monitoring
- Mechanisms that lead to a disease state
- Better understanding of the pharmacology and toxicology of a drug (particularly in early drug discovery and development to prevent late stage attrition)
- Explain and identify gender or strain differences
- Phenotypic characterization of genetic mutants

References

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