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# A combined $^1$H NMR and HPLC–MS-based metabonomic study of urine from obese (fa/fa) Zucker and normal Wistar-derived rats

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**Abstract**

$^1$H NMR and HPLC–MS were used to generate metabolite fingerprints for the metabonomic analysis of urine obtained from both male and female Zucker obese (fa/fa) rats, used as a model of type II diabetes, and normal male Wistar-derived animals. The resulting data were subjected to chemometric analysis (principal components analysis and partial least squares discriminant analysis) to investigate the effects of strain, diurnal variation is strain, diurnal variation and gender and gender on metabolite profiles. In the case of strain, $^1$H NMR spectroscopic analysis revealed increased taurine, hippurate and formate and decreased betaine, α-ketoglutarate, succinate and acetate in samples from Zucker-obese compared to Wistar-derived rats. HPLC–MS analysis detected increased hippurate and ions at *m*/*z* 255.0640 and 285.0770 in positive, and 245.0122 and 261.0065 in negative electrospray ionisation (ESI), respectively, for the Zucker obese samples. Both techniques enable the detection of diurnal variation in the urine of male and female Zucker rats, marked by increases in taurine, creatinine, allantoin and α-ketoglutarate by $^1$H NMR, and ions at *m*/*z* 285.0753, 291.0536 and 297.1492 (positive ESI) and 461.1939 (negative ESI) using HPLC–MS, in the evening samples. Differences between male and female Zucker rats were also observed. Compared to samples from male rats hippurate, succinate, α-ketoglutarate and dimethylglycine ($^1$H NMR) were elevated in the urine of female animals together with ions at, e.g., *m*/*z* 431.1047, 325.0655, 271.0635 and 447.0946 (positive ESI) and *m*/*z* 815.5495 and 459.0985 (negative ESI) by HPLC–MS. Both analytical techniques used in this study were able to detect differences between normal and Zucker obese rats, which may provide markers of metabolic disease.


*Keywords:* $^1$H NMR; HPLC–MS; Zucker rat urine; Diurnal variation; Gender; Strain variation

## 1. Introduction

The application of information rich analytical strategies for producing comprehensive metabolic fingerprints of biological fluids and tissues, combined with the use of chemometric techniques based on multivariate statistical analysis (e.g., principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA)) has resulted in the rapid development of the field of metabonomics [1]. Inherently a "systems" approach, metabonomics also provides a mechanism for understanding variations in low molecular weight metabolites in complex, mutli-cellular systems by providing a "global" metabolite profile. Such data, especially when combined with that from proteomic and genomic studies, should result in a more complete view of the system under study, which facilitates examination of biological responses to external stimuli.

The majority of the metabonomic research to date has been conducted using NMR spectroscopy (e.g., see [2–4]) and numerous examples exist of the successful application of metabonomics for toxicology and clinical studies [2,3,5]. More recently, HPLC–MS approaches have begun to be used for metabonomic analysis, either alone or in combination with NMR analysis [6–11]. This approach has been successfully applied in toxicity studies in rats [6] and in studies of

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genetic, diurnal and gender differences in mice [7]. In studies, where both HPLC–MS and $^1$H NMR have been used [10,11] it is clear that the combination offers advantages over the use of either alone especially for structural moieties which are invisible by NMR, e.g. sulfates, or which are not easily detected by MS (e.g., glucose).

As part of a series of studies aimed at obtaining a better understanding of normal variation in biofluid composition and determining the differences between "normal" animals and models of disease, we have examined a variety of rodent strains using metabonomic techniques. The obese (fa/fa) Zucker rat is important as it represents a widely used rodent model of obesity and insulin-resistance [12]. As such the Zucker rat is of interest due to the increasing prevalence of type II diabetes in Western societies [13].

Here we describe the application of both $^1$H NMR and HPLC–MS, combined with pattern recognition techniques, to investigate the urinary metabolic fingerprints of male and female obese (fa/fa) Zucker rats and male Alderely Park (AP) (Wistar-derived) rats. Further, as previous studies in rodents have shown differences in metabolite composition as a result of normal physiological variation in addition to the effects of toxicity or disease, effects on metabolite profiles due to diurnal variation and gender differences were also examined for Zucker rats.

## 2. Materials and methods

### 2.1. Chemicals

All aqueous solutions were prepared using purified distilled water (18.2 MΩ) from a Millipore MillQ system (MA, USA). HPLC grade acetonitrile was purchased from JT Baker (NJ, USA). Formic acid, extra pure grade (98–100%), was purchased from Fluka (WI, USA). The "metabonomics performance test mix" (containing theophylline, caffeine, nortriptyline, hippuric acid, and 4-nitrobenzoic acid) was obtained from Waters Corporation (MA, USA). All other materials were purchased from Sigma–Aldrich (MO, USA).

### 2.2. Samples

Urine samples were collected from male and female obese (fa/fa) Zucker rats (BABU, Alderley Park) by minimal bladder manipulation at two time periods, morning and evening. The males were aged 8 ($n = 5$) and 12 ($n = 10$) weeks and the females 8 ($n = 10$) weeks. Samples were also collected, in the morning only, from male Alderley Park rats (Wistar-derived) aged 12 ($n = 5$) weeks. Samples were stored at −20 °C prior to preparation and analysis.

### 2.3. Sample preparation

Samples for HPLC–MS analysis were centrifuged at 13,000 rpm for 5 min 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. For $^1$H NMR analysis, urine samples were buffered 2:1 with sodium phosphate buffer (0.2 M; pH 7.4) in deuterium oxide containing 3-trimethylsilyl-propionic acid (TSP; 0.5 mg/ml).

### 2.4. Sample analysis

#### 2.4.1. $^1$H NMR

NMR analysis was carried out using a Bruker DRX500 NMR spectrometer (Bruker Spectrospin Ltd., Coventry, UK) operating at 500.13 MHz for proton. $^1$H NMR spectra were acquired at 30 °C, with 90° pulse widths over a spectral width of 9980.04 Hz into 64 K data points. Typically 128 scans were acquired with an acquisition time of 3.28 s, a pulse width of 4.3 μs and a relaxation delay of 1 s. Water suppression was achieved by employing the standard 'Noesypresat' pulse sequence (Bruker Spectrospin Ltd.) with secondary irradiation of the dominant water signal during the mixing time of 150 ms and the relaxation delay of 2 s. All spectra were referenced to the internal reference standard TSP ($\delta 1_H = 0.0$) and corrected for phase and baseline distortions. No line broadening/apodization functions were applied.

#### 2.4.2. HPLC–MS

Chromatography was performed on a Waters Metabonomics System comprised of an Alliance® 2795XC, equipped with a column oven and 2996 PDA detector, coupled to a Micromass® Q-Tof micro™ equipped with an electrospray source operating in either positive or negative ion mode and a LockSpray™ interface for accurate mass measurements. The source temperature was set at 120 °C with a cone gas flow of 50 L h$^{-1}$, a desolvation gas temperature of 250 °C and a nebulization gas flow of 450 L h$^{-1}$. The capillary voltage was set at 3.2 or 2.6 kV for positive or negative ion mode, respectively, with a cone voltage of 40 V, a scan time of 1.0 s and an interscan delay of 0.10 s. A collision energy of 10 V was employed with a collision gas pressure of $5.3 \times 10^{-5}$ Torr. Leucine enkephalin was employed as the lockmass at a concentration of 50 fmol/μL (in 50:50 ACN:$H_2O$, 0.1% formic acid) at a flow rate of 30 μL/min via a lock spray interface. All mass spectral data was collected in centroid mode.

A 10 μL aliquot of diluted rat urine was injected onto a 2.1 mm × 10 cm Symmetry® C18 3.5 μm column. The column was eluted with a linear gradient of 0–20% B over 2–16 min, 20–95% B over 16–32 min; the composition was held at 95% B for 1 min, then returned to 100% A at 34 min at an eluent flow rate of 250 μL/min; where A = 0.1% formic acid (aq) and B = 0.1% formic acid in acetonitrile. A "purge-wash-purge" cycle was employed on the autosampler, with 75% aqueous methanol used for the wash solvent and 0.1% aqueous formic acid used as the purge solvent; this ensured that the carry-over between injections was minimized. The mass spectrometric data was collected in full scan mode from

100 to 850 *m/z* from 0 to 20 min. In order to ensure accurate intraday and interday HPLC–MS performance, an injection of a "metabonomics performance test mix" (containing theophylline, caffeine, nortriptyline, hippuric acid and 4-nitrobenzoic acid) was made after every 10 urine injections. This standard mixture provides both a benchmark of mass accuracy and also allowed chromatographic performance to be monitored via retention time reproducibility and peak shape. For the individual urine samples, the same function was performed by monitoring the endogenous hippuric acid peak.

### 2.5. Data analysis

Following data reduction to segmented integral values (as described previously [14]), the resulting $^1$H NMR data was analysed by PCA and PLS-DA using Umetrics AB Simca-P software Version 10.0.2 (Umeå, Sweden). The data were mean-centered for this analysis. All HPLC–MS data was processed using the Micromass MarkerLynx Applications Manager Version 1.0 (Waters Ltd.) to yield a data matrix containing information as to retention time, mass and normalized peak intensity. For each sample, 20 ions are detected within 0.2 min retention time windows across each chromatogram, then *m/z* and retention time values are aligned across all samples in a data set. The intensity of each ion is normalised to the samples' total signal intensity. The processed data list was subsequently analysed either by PCA within the MarkerLynx program or by PLS-DA using Pirouette Lite Version 3.10 (2) (Infometrix Inc., Woodinville, WA, USA). All elemental compositions were calculated from exact mass data within MassLynx using an error window of 5 ppm, which corresponds to the instrumental mass accuracy of the mass spectrometer.

## 3. Results and discussion

### 3.1. Comparison of AP and Zucker Rats

Given that the Zucker obese rat represents an abnormal animal an obvious biological comparison to be made is between the $^1$H NMR and HPLC–MS data from them and a "normal" strain as represented by the AP (Wistar-derived) rat. A simple visual inspection of the NMR and HPLC–MS results from male AP and Zucker rat urine shows several obvious differences. Thus, Fig. 1A and B present representative $^1$H NMR spectra for typical 12-week-old male AP and Zucker rat urine samples and Fig. 1C–F the corresponding HPLC total ion current chromatogram (TIC) mass spectral data in both positive (C and D) and negative (E and F) ion mode for the same samples. Examination of the two NMR spectra shows that elevated amounts of taurine, hippurate and formate were present in the urine of Zucker compared to AP rats. Conversely, urine from AP rats contained relatively higher concentrations of betaine, α-ketoglutarate, succinate and acetate than samples from the Zucker strain (Fig. 1A and B). A direct comparison of the HPLC–MS TIC traces shown in Fig. 1C–F also reveals obvious differences between the metabolite profiles of the AP and Zucker rats although clearly, the identity of the compounds responsible for these differences cannot be inferred from visual inspection of the TIC itself. Examination of the mass spectral data showed that, consistent with

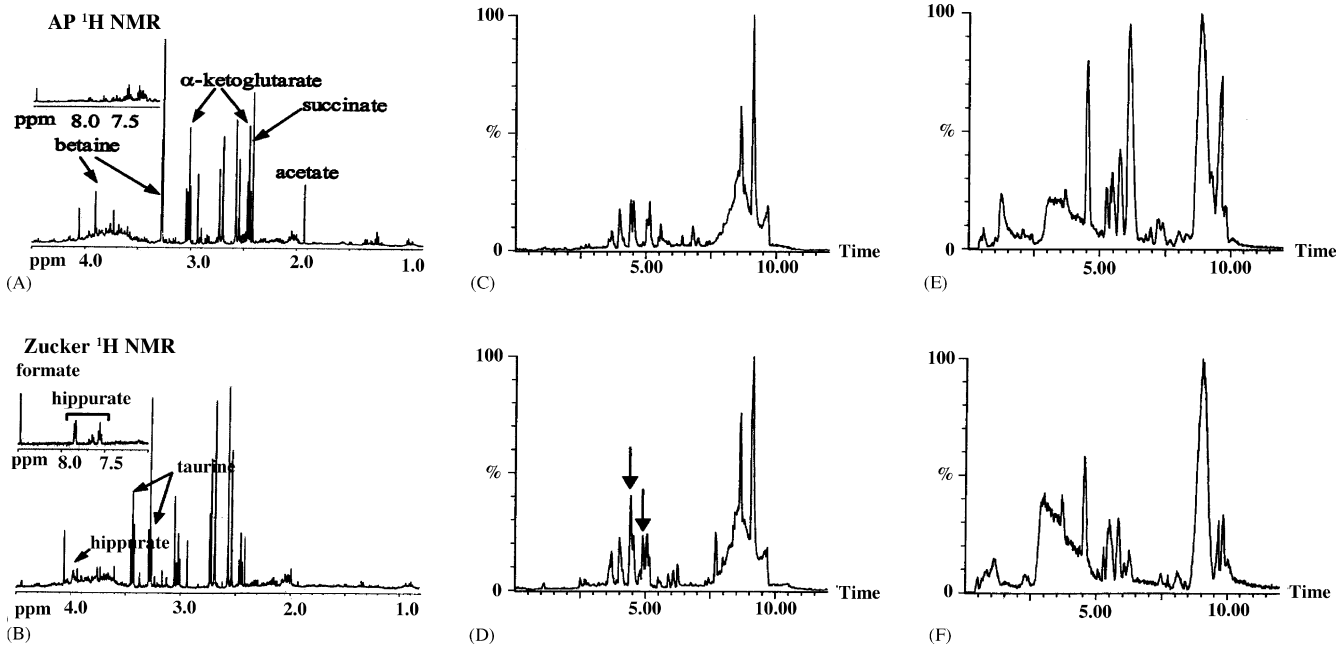


Fig. 1. $^1$H NMR spectra (0.8–4.5 and 7.0–8.5 ppm) and positive and negative ion TICs (HPLC–MS analysis) obtained from urine samples collected from a 12-week-old male AP rat (A, C, E) and a male Zucker rat (B, D, F), respectively. The arrows on the TICs highlight the peaks discussed in the text as differing between the two strains.

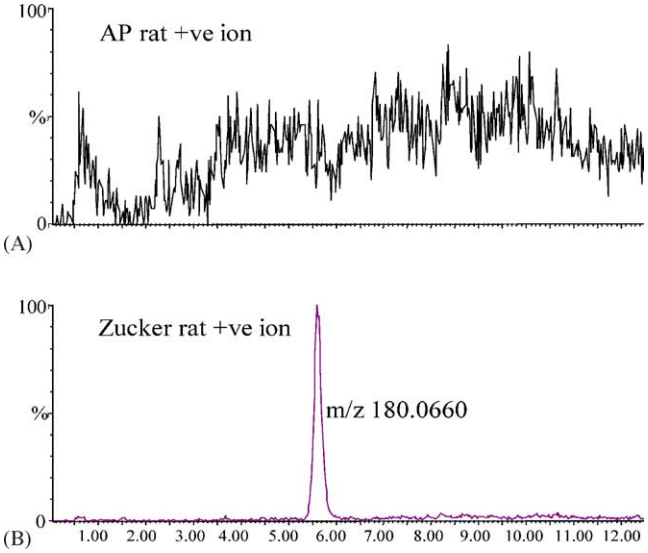


Fig. 2. Extracted ion chromatograms for hippurate (5.60 min; *m/z* 180.0660; positive ion mode) obtained from HPLC–MS analysis of urine from 12-week-old (A) male AP and (B) male Zucker rats, respectively.

the NMR data, the extracted ion chromatograms (EICs) for hippurate in positive ion mode (5.60 min, $m/z = 180.0660$) show an elevated amount of this component in Zucker rat urine as illustrated in Fig. 2. Negative ion analysis showed a corresponding elevation in the $[M–H]^-$ ion, *m/z* 178.0504 (data not shown). Hippurate is largely derived via gut microfloral metabolism of dietary compounds [15–19] and the differences observed between normal and Zucker rats may, therefore, reflect differences in gut microfloral populations rather than differences in biochemistry between the two strains. Interrogation of the positive ion MS data for two peaks which were more prominent in the urine of Zucker rats, at retention times of 4.14 and 5.05 min, showed them to have masses of 255.0640 and 285.0770 Da, respectively. These masses corresponded to atomic compositions of $C_{15}H_{11}0_4$ or $C_7H_{15}N_2O_6S$ for *m/z* 255.0640 (possibly 3,4-dihydroxyflavone or 5-glutamyl-taurine) and $C_8H_{17}N_2O_7S$ or $C_{16}H_{13}O_5$ (possibly 5,6-dihydroxy-7-methoxyflavone) for *m/z* 285.0770, respectively. In contrast, the negative ion MS data pointed to two prominent ions present at higher concentrations in the AP rat urine. These were found at retention times of 4.18 and 5.12, with masses of 261.0065 ($C_9H_9O_7S$) and 245.0122 ($C_9H_9O_6S$), respectively

Whilst visual comparison of this type of data, from both NMR spectroscopy and HPLC–MS data sets, could be performed such an analysis would be tedious and inefficient and prone to subjective error. Examination of the data was, therefore, undertaken using PCA. The scores and loadings plots shown in Fig. 3A–F show the results of PCA of the $^1$H NMR and HPLC–MS data obtained from the 12-week-old male AP and Zucker rats. In all scores plots (Fig. 3A, C and E) there is a clear separation of the urine from the two strains with the AP samples clustering away from the Zucker ones. The reason for this clustering is manifest from the corresponding loadings plots, where the effects of taurine, hippurate, formate, betaine and α-ketoglutarate, etc., dominate the spearation between the Zucker and AP rat urine samples in the case of $^1$H NMR (Fig. 3B). In the loadings plot for the HPLC–MS data

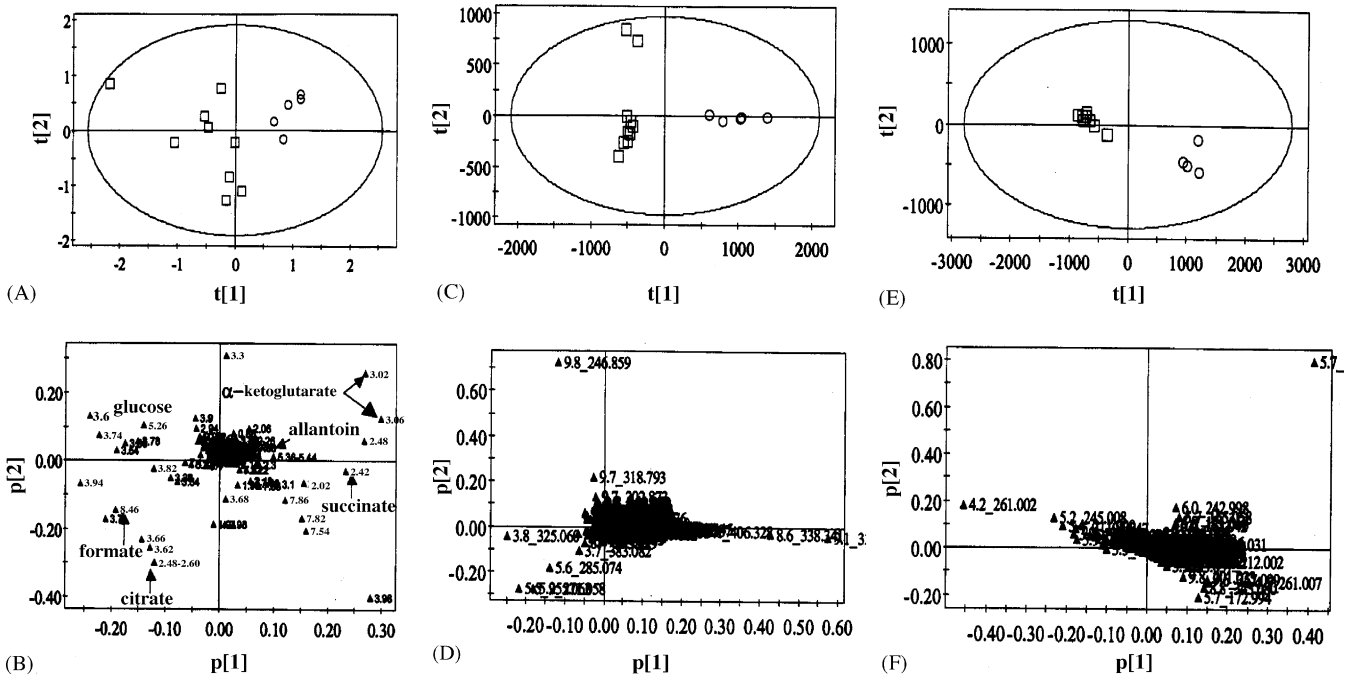


Fig. 3. PCA scores (A, C, E) and loadings (B, D, F) plots (component 1 vs. component 2) obtained from $^1$H NMR spectra (A, B) and positive (C, D) and negative (E, F) ion HPLC–MS data from urine samples collected from 12-week-old male AP (◯) and male Zucker (□) rats (each point represents a single sample). Loading shown are the mid-segment value (ppm) for $^1$H NMR data and the retention time and *m/z* for HPLC–MS data.

(Fig. 3D and F), *m/z* = 255 and 285 are the most significant contributors for positive ion data and *m/z* = 250 and 261 for negative ion data to the cluster in the scores plot.

This example clearly illustrates the complimentary nature of $^1$H NMR and HPLC–MS, as although clustering was observed for both analytical approaches, the markers underlying these separations were different. In part, this reflects the fact that the detection of species such as taurine, formate, succinate and acetate by MS without prior derivatization, etc. can be difficult. This is the result of a number of factors including the polar nature of the analytes, which makes them poorly retained under the chosen chromatographic conditions and/or poor ionisation properties. However, if required it would be possible to employ alternative chromatographic or mass spectroscopic conditions to detect these compounds.

### 3.2. Diurnal variation—Zucker rats

The ability to see clustering due to diurnal differences amongst the Zucker rats is another point of comparison for the $^1$H NMR and HPLC–MS data. Fig. 4A–C presents three scores plots from partial least squares discriminant analysis of the data from 8-week-old female Zucker rats. In both instances, clustering is observed in the scores plot between samples collected in the morning and evening. In the case of $^1$H NMR the compounds responsible for the clustering of the morning samples include formate and citrate, whilst the evening samples are characterized by increased taurine, creatinine, allantoin and α-ketoglutarate. From the positive ESI data, the ions responsible for the HPLC–MS clustering included *m/z* 285.0753, 255.0661, 377.1466, 291.0536, 170.0580 and 297.1492. Of these, the ions at *m/z* 377.1466 and 170.0580 were present in elevated quantities in urine samples collected in the morning whilst those at *m/z* 285.0753, 291.0536 and 297.1492 were more prevalent in the evening samples. In the case of the negative ESI data, ions at *m/z* = 245.0096, 275.0172, 459.0985, and 461.1939 gave the largest contribution to the observed clustering in the scores plot. Of these ions, those at *m/z* 275.0172 and 459.0985 were present at higher concentrations in urine samples collected during the morning, whereas *m/z* 461.1939 was most abundant in the evening samples. A postulated elemental composition for each of these ions is given in Table 1, with some proposed identities, however, as with the ions responsible for distinguishing between strain, these compounds are as yet unidentified with the exception of the ion at *m/z* 377.1466, which was riboflavin.

Similar clustering was also observed within the data set obtained from the urine of male Zucker rats (data not shown) with higher amounts of urinary formate and citrate being observed in the morning samples and increased taurine, creatinine, allantoin and α-ketoglutarate in the evening ($^1$H NMR data). Analysis of the HPLC–MS data for male Zucker rats showed that the same ions were responsible for the observed clustering as was seen for the female urine samples.

### 3.3. Gender variation—Zucker rats

The scores plots for gender variation in 8-week-old male and female Zucker rats are presented in Fig. 5A–C. In this instance, good clustering is seen for both NMR and the HPLC–MS data. For $^1$H NMR data discrimination was based

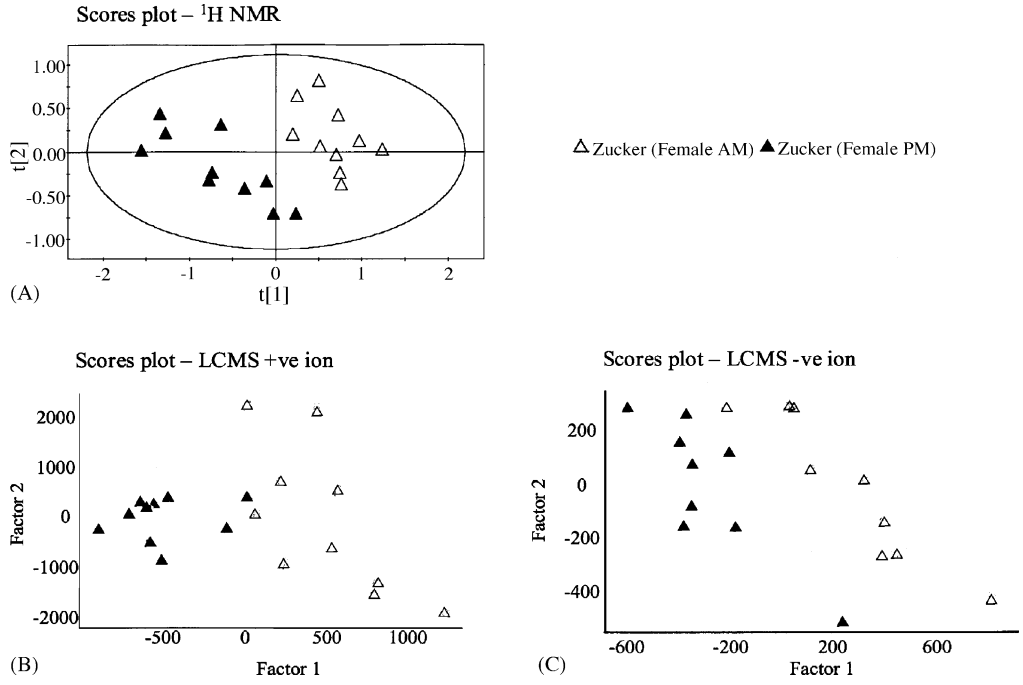


Fig. 4. PLS-DA scores plots (component 1 vs. component 2) obtained from (A) $^1$H NMR spectra and (B) and (C) positive and negative ion HPLC–MS data, respectively, acquired from urine samples collected in the morning and evening from 8-week-old female Zucker rats (each point represents a single sample).

Table 1
Key metabolites with corresponding chemical shifts (ppm; $^1$H NMR data) and positive and negative mass ions (HPLC–MS data) responsible for defining the diurnal and gender variation observed using PLS-DA ( proposed)

| | $^1$H NMR | | LCMS (positive ion) | | LCMS (negative ion) | |
|---|---|---|---|---|---|---|
| | Chemical shift (ppm) | Assignment | *m/z* | Proposed elemental composition | *m/z* | Proposed elemental composition |
| Diurnal variation | ∼2.48–2.74 | Citrate (AM) | 170.0580 | $C_8H_{10}O_4$ | 275.0172 (AM) | $C_5H_{11}N_2O_9S$ |
| | 8.46 | Formate (AM) | 377.1466 | $C_{17}H_{21}N_4O_6$[a] | 459.0985 (AM) | $C_{13}H_{27}N_6O_4S_4$ |
| | 2.46, 3.02 | α-Ketoglutarate (PM) | 255.0661 (PM) | $C_{11}H_{14}NO_4P$ | 461.1939 (PM) | $C_{15}H_{33}N_4O_{10}S$ |
| | 3.06, 4.06 | Creatinine (PM) | 285.0753 (PM) | $C_{16}H_{13}O_5$[b] | 245.0096 (slight increase in AM) | $C_5H_{13}N_2O_3S_3$ |
| | 3.26, 3.42 | Taurine (PM) | 291.0536 (PM) | $C_{11}H_{15}O_7S$ | | |
| | 5.40 | Allantoin (PM) | 297.1492 (PM) | $C_{11}H_{25}N_2O_5S$ | | |
| Gender variation | 3.98, 7.56, 7.64, 7.84 | Hippurate (F) | 431.1047 (F) | $C_{15}H_{27}O_{10}S_2$ | 815.5495 (F) | $C_{48}H_{79}O_8S$ |
| | 2.42 | Succinate (F) | 325.0655 (F) | $C_{11}H_{19}NO_6S_2$ | 459.0985 (F) | $C_{17}H_{31}O_6S_4$ |
| | 2.46, 3.02 | α-Ketoglutarate (F) | 271.0635 (F) | $C_4H_{11}N_6O_8$ | 201.0226 (M) | $C_8H_9O_4S$ |
| | 2.93 | Dimethylglycine (F) | 255.0634 (F) | $C_8H_{16}O_7P$ | 343.0830 (M) | $C_{18}H_{15}O_7$[c] |
| | 2.06/2.10/2.22/ 2.26 | Small unassigned resonances (F) | 447.0946 (F) | $C_{16}H_{21}N_3O_{10}S$ | | |

[a] Riboflavin.
[b] Proposed identities: 5,6,-dihydroxy-7-methoxyflavone.
[c] Proposed identities: 3′,5-dihydroxy-4′,6,7, trimethoxyflavone.

on increased levels of urinary formate and succinate in the urine of the females and increased glucose, creatinine, allantoin and citrate in that of the males. In the case of the positive ion HPLC–MS data, gender discrimination centered on ions at *m/z* 431.1047, 325.0655, 271.0635, 255.0634 and 447.0946, which were all elevated in the urine samples obtained from female animals. For the negative ion data, *m/z* = 815.5495 and 459.0985 were dominant in the female urine samples whilst ions at *m/z* 201.0226 and 343.0830 were observed at increased concentrations in urine samples from the male animals. Metabolites and proposed elemental compositions of ions responsible for the observed clustering for gender variation are given in Table 1.

For strain, diurnal and gender differences the identification of the components detected by $^1$H NMR spectroscopy was aided by the large body of information already avail-

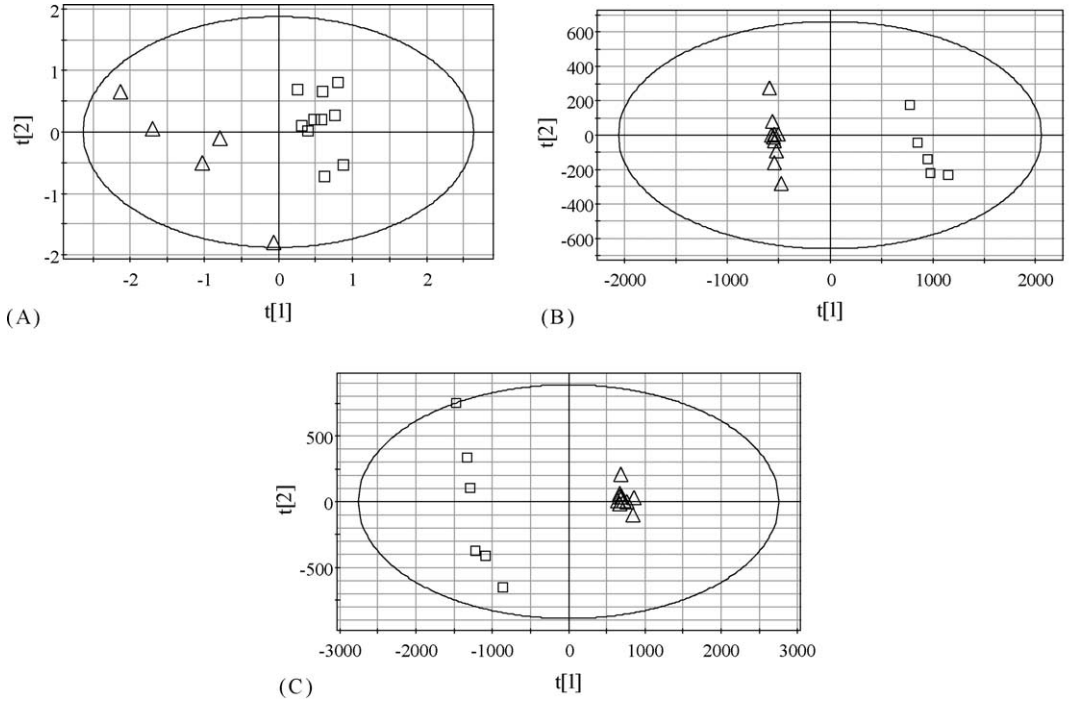


Fig. 5. PLS-DA scores plots (component 1 vs. component 2) obtained from (A) $^1$H NMR spectra and (B) and (C) positive and negative ion HPLC–MS data acquired from urine samples collected from 8-week-old male and female Zucker rats (each point represents a single sample). Open triangles: females, open squares: males.

able on the normal components of urine (e.g., in chemical shift lists for endogenous metabolites [20]). In comparison, the available data for metabolite identification by HPLC–MS is less extensive and the identity of most of the molecules contributing to the discrimination is currently not known, although elemental compositions can be postulated based on the accurate masses. Further investigations are under way to attempt to characterise and identify these compounds, including metabolite isolation followed by NMR and MS analysis. Once identified, specific assays will be required for absolute quantification as ion suppression, inherent to electrospray ionisation in HPLC–MS, may well affect the response observed for certain ions.

## 4. Conclusions

As found in previous studies on mouse urine [7,21,22], both HPLC–MS and $^1$H NMR were able to detect differences in rat urinary metabolic profiles based on strain and gender and on diurnal variation. The ready detection of such differences reinforces the care that must be taken in designing and performing metabonomic studies to ensure that, e.g., the samples are collected at the same time of day to ensure that any differences seen relate to treatment rather than diurnal variation, etc. Apart from hippurate, which was detected using both techniques, discrimination by HPLC–MS and $^1$H NMR was based on different sets of markers confirming the complementary nature of the two methods of metabolic fingerprinting. Exploitation of the different analytical selectivities that each technique provides should lead to complimentary datasets that allow a greater insight into the systems biology under investigation. Currently, HPLC–MS-based metabonomics is at an early stage of development, and databases enabling rapid identification of the urinary components responsible for the clustering are not yet available. Studies to identify the biomarkers, and to establish suitable databases are ongoing. It is clear, however, from the data presented to date that HPLC–MS analysis has considerable potential for metabonomic studies both alone and, more especially, in combination with $^1$H NMR.

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