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Urinary biomarkers of forage feeding from ESI-MS/MS fingerprinting: exploratory studies

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ABSTRACT

The combination of metabolic profiling by a combination of broad-spectrum analytical methods and multivariate statistics offers new opportunities for obtaining a global over-view of metabolism. It also facilitates the discovery of metabolites which can serve as useful markers of biological processes or states (“biomarkers”). Fast liquid chromatography and electrospray ionisation mass spectrometry with collision-induced dissociation in an ion-trap (fast LC-ESI-MS/MS) has been applied to determine metabolic profiles of bovine urine samples. The samples were collected twice daily from groups of five lactating dairy cows grazing in four different forage management systems comprising different spatial and temporal allocations of ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). The data have been analysed by different multivariate statistical methods, and a number of candidate biomarkers of forage feed and diurnal processes have been identified.

Keywords: Biomarker, urine, dairy cows, ryegrass, white clover, LC-MS/MS

INTRODUCTION

Recently analytical approaches have been developed that aim at comprehensive global metabolic profiles of biofluids, including urine under the guise of “metabonomics” (or “metabolomics”). These involve the application of multivariate statistics to analyse metabolite profiles detected by a broad-spectrum analytical technique, such as nuclear magnetic resonance (NMR) or mass spectrometry (MS) (e.g. Shockor & Holmes, 2002; Wilson et al., 2005). These approaches offer the prospect of the discovery and measurement of significant metabolites unconstrained by the investigators preconceptions of likely targets. The investigation of broad patterns of urinary metabolites has been shown to be a valuable approach in human health, for instance in the investigation of in-born errors of metabolism in infants from urinary organic acid profiles (Kumps et al., 2002). Urinary metabolites which are markers of particular dietary components (“biomarkers”) are an established tool in relating food intake to physiological outcomes in human nutrition (e.g. Lampe, 2003). Investigations of specific urinary metabolites of farmed ruminants are well-established as a valuable scientific approach for the investigation of diet-related disorders, such as the estrogenic effects of *Trifolium* species (e.g. Kramer et al., 1996). A recent report has suggested the potential of urinary metabolites as biomarkers of forage selection by ruminants grazing mixed swards (Keir et al., 2001). We suggest broad spectrum urinary metabolic profiling is of potential value in agricultural research, providing a global overview of metabolic

differences due to different experimental treatments and aiding the discovery of metabolites which can serve as useful biomarkers of metabolic processes or diets.

We have been investigating the effects of forage feeding regimes on the formation of products of amino acid degradation in the rumen, particularly the flavour compounds skatole and indole from tryptophan, and *p*-cresol from tyrosine, and their appearance in milk, meat and body fluids including urine (Lane et al., 2002; Young et al., 2003; Schreurs et al., 2003; Fraser et al., 2004). In a recent trial in which dairy cows were offered forage diets, including spatially and temporally separated offerings of ryegrass and white clover, highly elevated levels of skatole and indole were found in the milk of cows on diets with high white clover content (Cosgrove et al., 2006). Metabolite profiles of urine samples from this trial have been determined by a methodology based on fast liquid chromatography and electrospray ionisation mass spectrometry with collision-induced dissociation in an ion-trap (fast LC-ESI-MS/MS).

MATERIALS AND METHODS

Dairy Cow Trial

The trial design and methodology is described in detail elsewhere (Cosgrove et al., 2006). In brief, the trial was carried out on the Massey University No 1 Dairy Unit in April (late lactation) and October (early lactation) 2005. In each experimental period, 4 groups of 5 spring-calving cows were allocated to four forage feed treatments with different spatial and temporal

allocations of ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) as follows:

Grass-only, 24 hrs a day (GRASS); mixed ryegrass-white clover pasture, 24 hrs a day (MIX); continuous, free access to ryegrass and white clover, growing separately side-by-side as monocultures (G/C); grass-only at night and clover-only during the day (GnCd).

Cows were allocated to treatments for 8 days. Urine was sampled prior to milk collection on the afternoon of day 7 (PM) and the morning of day 8 (AM) and stored at -20 °C for subsequent analysis. After morning milking on day 8, the cows were moved directly to the next assigned treatment in a balanced, cross-over design. Urine samples collected in April were frozen, and subsequently thawed and samples from cows within groups were pooled prior to analysis. Samples collected in October were pooled prior to freezing and thawed for analysis.

Fast LC-MS/MS

A sub-sample (30 µl) of each pooled urine sample was diluted with 1.5 ml 0.1% formic acid and centrifuged at 12000 rpm for 2 minutes to precipitate particulates. A portion (1 ml) of this solution was transferred to a 1.5 ml auto-sampler vial for analysis. Samples were analysed using a Thermo Surveyor pump and auto-sampler connected to a Thermo LTQ linear ion-trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) using negative electrospray ionisation. A 20 µl aliquot was loaded onto a strata-X on-line extraction cartridge (20 x 2.0 mm, Phenomenex, Torrance, CA, USA) with water (300 µl min⁻¹). For the first 0.5 minutes the flow from the cartridge was diverted away from the mass spectrometer to waste, after which all flow entered the electrospray source. Samples were eluted with a gradient comprising water (A), and acetonitrile containing 0.1% formic acid (B). The column was eluted with solvent A for 1.5 minutes, then with a linear gradient to 100% B over 3 minutes and held at 100% B for 3 minutes. The initial solvent (100% A) was restored over a 0.5 min linear gradient and eluted for 1.5 min at 600 µl min⁻¹, and for 0.5 min at 300 µl min⁻¹. The ion trap was programmed to collect a MS1 spectrum from 150-1000 *m/z* followed by MS2 and MS3 spectra from collision induced dissociation of the most intense ions in the MS1 spectrum, using a 12 second exclusion list to cycle through co-eluting ions.

Data processing

For each sample the averaged mass spectrum profile for the whole chromatogram from 1.5 min to 8 min was exported from the instrument

software (Xcalibur 1.4, Thermo Electron Corporation, San Jose, CA, USA) into a Microsoft Excel spreadsheet. The data were aligned into unit *m/z* bins taking the maximum intensity of ions assigned to a bin as the value for that bin. The binned data were normalised to the mean ion intensity per sample, and filtered to exclude low intensity *m/z* bins where the maximum intensity did not exceed 5% of the mean in any samples, and low intensity bins identified as ¹³C isotope peaks.

Multivariate analysis

Principal components analysis (PCA) and discriminant function analysis (DFA) was carried out with Minitab 14 software (Minitab Ltd). The data for the April and October experiments were analysed separately (by correlation). Random Forest analysis (Breiman, 2001) was carried out in R (R development core team, 2005) using the randomForest package (Liaw and Wiener 2002). The complete data set (both April and October experiments) was analysed together to examine consistent discrimination between the forage treatments and times of daily sampling across both experimental periods. The data were also analysed by analysis of variance using a linear model in R and *m/z* bins ranked for probable significance of effects.

Extracted ion chromatograms for discriminating *m/z* bins were examined to determine whether they corresponded to a single ion species, and collision-induced fragmentation data (if available) were examined to characterise candidate molecules by comparison with literature information.

RESULTS

An example of an averaged mass spectrum recorded from a fast LC-MS/MS run is shown in Fig 1. After initial processing, on average 605 (+/- 66) of the unit bins between *m/z* 150 -1000 contained data for detected ions. The data set was reduced to 426 bins after filtering to remove low intensity ions. Mass fragmentation data was collected for approximately 160 distinct MS1 ions in each scan. An example of the MS2 fragmentation data for the intense *m/z* 187 ion (identified as *p*-cresol sulphate) is shown in Fig. 1.

Principal component analysis (PCA) of the data showed evidence of discrimination within the data between the forage treatments and milking times for each experimental period. A plot of the sample scores for the first two principal components from the October data set (accounting for 28.5% and 19.1% of the variance respectively)

FIGURE 1: Mass spectrum of a pooled urine sample collected in the morning from lactating dairy cows grazing grass overnight after grazing clover during the previous day (GnCd AM treatment). The data is the averaged MS1 profile between 1.5 min and 8 min of a fast LC-MS/MS chromatogram. The scale relative to the most intense ion (100%) has been increased by a factor of 5 for data above 400 m/z . The inset shows an example MS2 spectrum from collision induced fragmentation of the m/z 187 ion (*p*-cresol sulphate) to *p*-cresol anion (m/z 107) and sulphate (m/z 80).

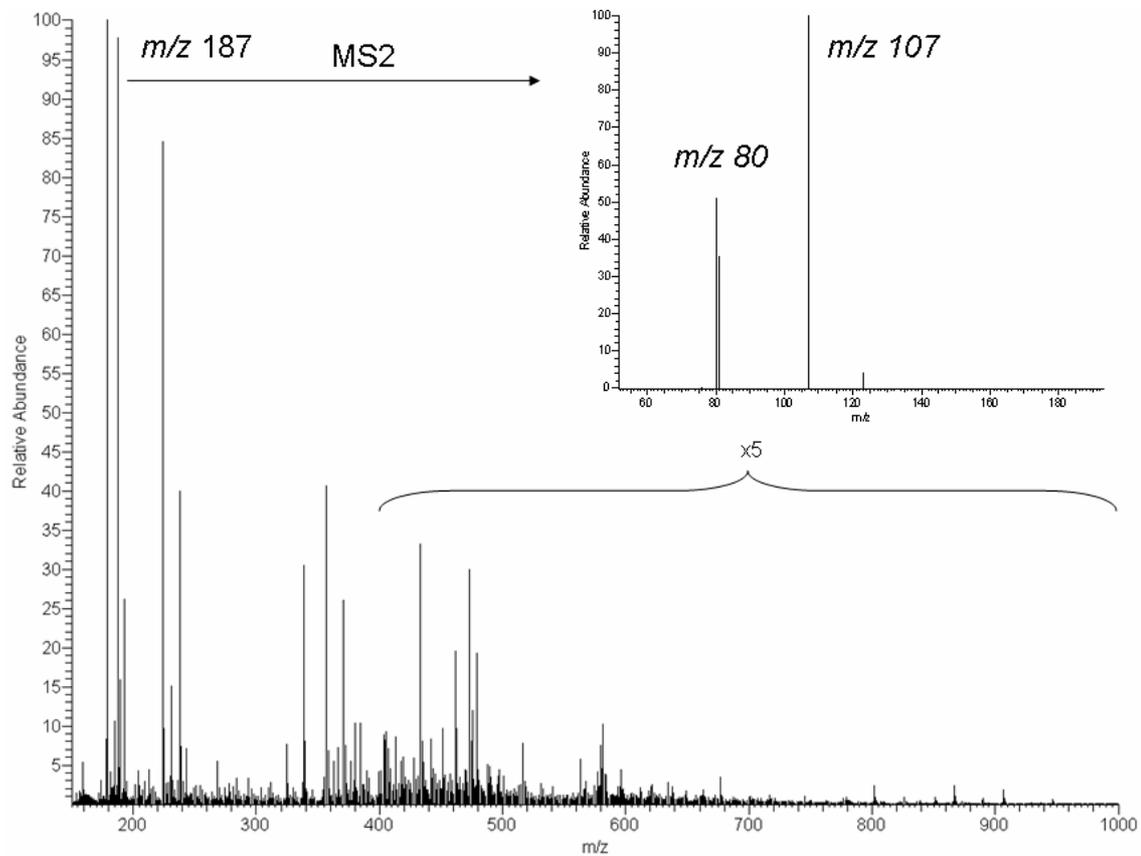


FIGURE 2: a) Plot of sample scores for the first two principal components, PC1 and PC2 from principal component analysis of urine metabolite MS profiles from samples collected from lactating dairy cows in October 2005; b) Plot of sample scores for discriminant functions DF1 and DF2 from linear discriminant analysis of urine metabolite MS profiles from samples collected from lactating dairy cows in October 2005 (● = G/C AM, ○ = G/C PM, ▼ = GnCd AM, ▽ = GnCd PM, ■ = GRASS AM, □ = GRASS PM; ◆ = MIX AM, ◇ = MIX PM).

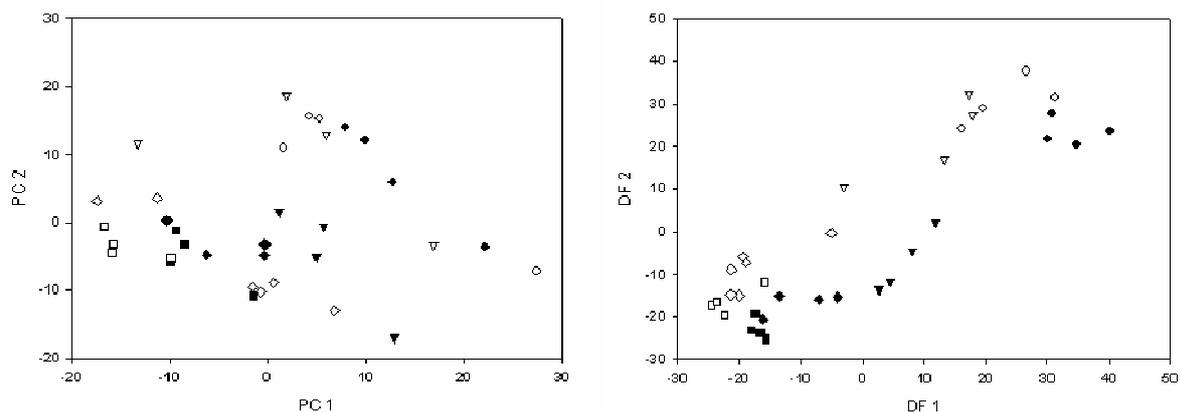
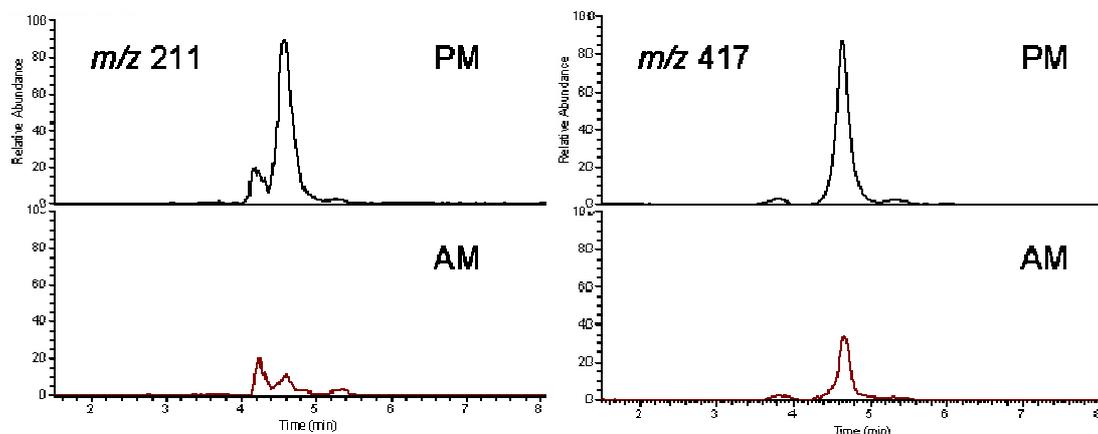


FIGURE 3: Extracted ion chromatograms for m/z 211 (candidate metabolite: trimethoxybenzoate) and m/z 417 (candidate metabolite: equol glucuronide) from fast LC-MS/MS of urine samples collected from lactating dairy cows on the GnCd forage treatment after grazing clover during the day (PM) and grazing grass overnight (AM).



is shown in Fig 2a. Discriminant function analysis (DFA) of the data across the eight combinations of forage feed treatments and milking times within each experimental period showed partial resolution with an estimated error rate of 8.8% (Fig 2b.). The analysis suggested the forage feed treatments prior to urine collection could be classified into two classes “clover” (G/C and GnCd pm) and “grass” (GnCd am, GRASS, MIX), with misclassifications only within these classes. DFA analysis by milking time alone (am or pm) or by “clover” or “grass” feed classes reduced the error rate with no incorrectly categorised samples (am vs. pm) or a single sample misclassified (“clover” vs. “grass”) for both experimental periods.

By Random Forest analysis (Breiman, 2001) the complete data set could be classified by milking time (am or pm) with an estimated error rate of 11.6%, and the “clover” or “grass” forage classes with an estimated error rate of 2.9%. Classification by a combination of the classes (“clover” am; “clover” pm; “grass” am; “grass” pm) was achieved with an estimated error rate of 8.7%

Lists of the top fifty discriminating ions selected by DFA, Random Forest analysis and ANOVA did not coincide but showed considerable overlap, particularly between the latter two methods. Examination of extracted ion chromatograms for the corresponding m/z values in most cases showed a single major component, and confirmed the treatment differentials extracted by statistical analysis. Examples are shown in Fig 3.

From these discriminating ions, a number of candidate molecular species were tentatively identified by investigation of the LC-MS/MS data

(details not shown). These are listed in Table 1 together with their possible dietary precursors.

DISCUSSION

Sampling for biomarker discovery

The discovery of biomarkers from complex metabolite profiles by statistical analysis poses challenges for experimental design and sampling (e.g. Kristal *et al.*, 2005). In this study we aimed to find consistent biomarkers for differences in forage class and milking time. We have thus examined pooled urine samples from treatment groups of cows at each of four sampling occasions at two seasonal periods (32 samples for each of the binary comparisons). The analysis of individual animal samples could provide a more robust basis for discriminating forage feed effects, and perhaps enable more subtle differences to be detected, but with significantly increased analysis time and costs.

Instrumental methodology and data analysis

Due to the high content of urea and other highly ionic material in urine, rapid direct infusion MS methods as pioneered by Smedsgaard (Smedsgaard & Frisvad, 1996) are not directly applicable. We have adopted a fast LC-MS/MS procedure to achieve high throughput analysis compared to high resolution LC-MS (Wilson *et al.*, 2005), eliminating sample clean-up by discarding highly ionic material and urea to waste before collecting the LC-MS/MS data over 8 min. Some highly polar metabolites (e.g. creatinine) were lost by this method, but the process provided sufficient chromatography to provide distinct metabolite peaks in ion chromatograms (Fig 3).

Without the power of multivariate statistics, the analysis of these data sets would be limited to major metabolites and a few presumptive candidates. The handling and analysis of LC-MS data is a very active field of research, and sophisticated methods are being developed (e.g. Idborg *et al.*, 2004). For this preliminary study we have applied a very simplified approach based on the mass spectrum profile for the whole chromatogram (Experimental), and this has provided useful data.

We have applied the Random Forest technique (Breiman, 2001) to analysing the data, as well as the more commonly used principal component analysis (PCA) and discriminant function analysis (DFA). Random Forest is a machine learning approach with good characteristics for avoiding over-fitting where (as here) the number of measured variables far exceeds the number of samples measured. The more conservative classification accuracies estimated by the Random Forest analysis compared to DFA (above) are probably more robust.

This procedure can identify candidate biomarkers, providing some chromatographic and mass spectrometric evidence from fast LC-MS/MS. It is a discovery methodology rather than a robust analytical procedure. Candidate biomarkers require confirmation of their chemical structures and validation using appropriate targeted analytical methods.

Metabolic origin of candidate biomarkers

Accepting these limitations, the candidate molecular species characterised from the list of

discriminating *m/z* bins (Table 1) show interesting evidence of diurnal and forage-derived effects linked to protein degradation in the rumen, and the metabolism of plant phenolics. These analyses have been carried out in parallel with studies of products of ruminal protein degradation (skatole, indole) accumulating in the milkfat of dairy cows from this trial (Cosgrove *et al.*, 2006). Two of the candidate biomarkers identified here, hydroxymethyloxindole sulphate and dihydroxymethyloxindole sulphate, represent putative products of skatole metabolism. They were detected as diurnal markers, showing a pattern of elevation in the morning urine collection, but not as markers of forage class, in marked contrast to the pattern of skatole accumulation in milkfat observed in the parallel study.

Most of the remaining metabolites identified are phenolic products of the metabolism in the rumen and animal of plant phenolics. Equol, dihydrogenistein and O-demethylangolensin are associated with the “clover” forage treatment and are known as estrogenic rumen products of isoflavones of *Trifolium* species (Kramer *et al.*, 1996 and references cited). Of the other phenolics only the putative quinol sulphate is linked to the “grass” forage treatments. More targeted studies would be required to further resolve relationships between forage diet components and metabolic markers including questions of diurnal phasing of intake and excretion suggested by these data. As the characterisation of candidates (Table 1) was based mainly on known urine metabolism and chemistry, the true novelty of this work may lie in the as yet uncharacterised species.

TABLE 1: Selected discriminating *m/z* bins identified by multivariate statistical analysis of urine metabolite MS profiles from samples collected from lactating dairy cows in October 2005, and associated treatment differentials (G, C refer to “grass” and “clover” forage feed treatments prior to urine collection; GAM, CPM etc refer to diurnal effects within “grass” or “clover” feed treatments only; brackets show a secondary effect). Candidate molecules corresponding to each *m/z* bin were characterised by mass spectrometric data.

<i>m/z</i>	Treatment Differential	Candidate Molecule	Likely Dietary Precursor
159	GAM>GPM	4-aminobutyryl glycine	-
184	G>C	2-oxo-hexenoylglycine	-
189	G>C (PM>AM)	Quinol sulphate	Phenolics
194	C>G	Salicylic acid	Lignin or precursors
197	C>G (PM>AM)	Syringic acid	Lignin or precursors
204	GAM>GPM	Indolelactate	Protein - Tryptophan
208	AM>PM	4-Hydroxyphenylacetyl glycine	Protein - Tyrosine
211	CPM>CAM	Trimethoxybenzoic acid	Lignin or precursors
242	AM>PM	Hydroxymethyloxindole sulphate	Protein - Tryptophan (Skatole)
258	AM>PM	Dihydroxymethyloxindole sulphate	Protein - Tryptophan (Skatole)
321	AM>PM (C>G)	Equol sulphate	Phytoestrogen - formononetin
351	C>G (PM>AM)	Dihydrogenistein sulphate	Phytoestrogen - genistein
387	C>G	Pinosylvin glucuronide	Lignin or intermediates
417	C>G (AM>PM)	Equol glucuronide	Phytoestrogen - formononetin
433	C>G (AM>PM)	O-desmethylangolensin glucuronide	Phytoestrogen - genistein
447	C>G (PM>AM)	Dihydrogenistein glucuronide	Phytoestrogen - genistein

CONCLUSIONS

This exploration of fast LC-MS/MS fingerprinting of bovine urine has identified a number of candidate molecular species of different classes which systematically differ between forage treatments and milking times across experimental periods and sampling occasions (Table 1). Successful discrimination of the mass spectrum profiles of the urine samples by sampling time and forage feed (Fig 2) has made possible the “unbiased” discovery of discriminating molecules between the treatments and provided a global overview of the molecular species and metabolic processes involved.

The results are encouraging in terms of the identification of biomarkers of forage selection in mixed swards (cf. Keir *et al.*, 2001) which may be used to relate animal performance to actual feed intake. The discovery power of fast LC-MS/MS fingerprinting demonstrated here also offers the prospect of identifying presently unknown pasture factors adversely affecting animal performance through the detection of their urinary residues.

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