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Application of Gas Chromatography−Mass Spectrometry Metabolite Profiling Techniques to the Analysis of Heathland Plant Diets of Sheep

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Little is known about how plant biochemistry influences the grazing behavior of animals consuming heterogeneous plant communities. The biochemical profiles of grassland species are mostly restricted to major nutritional characteristics, although recent developments in analytical techniques and data analysis have made possible the detailed analysis of minor components that may influence animal feeding preferences, performance, and health. In the present study, gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF/MS) was used to profile the abundances of metabolites in nine specific heathland plant groups and in three mixed forage diets containing 10, 20, or 30% heather (*Calluna vulgaris*) and also in plasma and feces from sheep offered one of the three diets. Statistical and chemometric approaches, that is, principal component analysis (PCA) and hierarchical cluster analysis (HCA), were used to discriminate between these diets and between individual animals maintained on these diets. It is shown that GC-TOF/MS analysis of sheep plasma allowed distinction between the very similar diets by PCA and HCA, and, moreover, the plant metabolites responsible for the differences observed have been identified. Furthermore, metabolite markers of herbage mixtures and individual plant groups have been identified, and markers have been detected in sheep plasma and feces.

KEYWORDS: Diet composition; GC-TOF/MS; ruminant nutrition; metabolite markers

INTRODUCTION

Free-ranging ruminants are significant components of the agri-ecosystem, and their health and production performance depend upon the nutritive value of the complex plant communities available for consumption. Choices made by large herbivores regarding the type and quantity of plant material grazed can have a profound effect on species richness and diversity and, consequently, on the structure and function of the agri-ecosystem. Furthermore, the dynamic distribution of nutrients and minerals through trampling and excretion can also affect the ecosystem (*1*). Therefore, a key element to understanding factors affecting long-term sustainability of ecosystems is an understanding of foraging preferences on heterogeneous swards. This would provide objective guidance for efficient range management through habitat restoration and maintenance and the development of management guidelines for grazing sensitive ecosystems.

Many plants consumed by herbivores contain the nutrients needed to meet basic requirements, but they can also contain a diverse and complex array of secondary compounds that provide some degree of defense against predation, disease, competition, and adverse climatic conditions. These compounds are frequently antinutritional or toxic, yet little is known about how they influence the choices and aversions of animals in their grazing behavior and the associated impact on health and performance. In addition, the composition of primary metabolites varies greatly among sward components and has significant effects on animal performance and dietary selection. Few detailed studies have been conducted with free-ranging animals grazing complex plant communities. This is primarily due to difficulties in accurately determining animal intake and diet composition in such environments. Earlier investigations involved direct observation of the grazing animal (*2*, *3*). However, although the method proved to be simple, problems in species identification and quantification of plants consumed were major disadvantages. More recent approaches include microhistological procedures (*1*, *3*−*5*), stable C-isotope discrimination (*1*, *6*−*8*), use of plant wax marker compounds (*1*, *9*−*11*), and near-infrared spectroscopy (NIRS) (*12*−*15*).

The advent of the postgenomic era has brought powerful high-throughput analytical methods coupled to advanced chemometric techniques. One area that has benefited directly from these

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advances is metabolite profiling, and various strategies are being used to explore large populations of metabolites in biological fluids (*16*). Although many of the reported techniques allow identification of both ruminant diet and botanical composition, the use of gas chromatography with time-of-flight mass spectrometry (GC-TOF/MS) as a profiling tool permits identification of metabolites within samples. Whereas alternative GC-MS technologies, for example, quadrupole MS, would also allow the profiling of metabolites in complex samples, TOF/MS, a nonscanning technology, is highly reproducible and allows much higher rates of data acquisition, which increases analytical resolution and permits more rigorous mass spectral deconvolution and metabolite identification. In the present study, we hypothesized that it is possible to correlate the presence or absence of specific compounds in blood plasma or feces with the consumption of specific plants in the diet of sheep. The objective of this study was to use GC-TOF/MS to profile metabolites present in a range of plants at different concentrations in diets fed to sheep and to determine the presence or absence of these metabolites and their derivatives in blood and feces. Furthermore, we report on how information on plant chemical composition can be used to discriminate between animals on each of the different dietary regimens on offer.

MATERIALS AND METHODS

The experimental design and sampling procedures have been reported in detail elsewhere (*17*). Briefly, during a zero-grazing experiment, material from two indigenous vegetation communities, a *Molinia caerulea* dominated grassland (hill grass mix) and a *Calluna vulgaris* dominated dwarf-shrub community (heather mix), was offered to sheep in three different ratios: 10% heather mix/90% hill grass mix; 20% heather mix/80% hill grass mix; and 30% heather mix/70% hill grass mix. All ratios were prepared on a fresh matter basis. Each diet was offered to four animals each of a group of 12 mature Welsh Mountain ewes, for a period of 29 days, with 22 days for adaptation and 7 days for measurements. Samples of the sward mixtures were collected daily, bulked over the course of the measurement week, and stored at −20 °C. These bulked samples were subsequently thoroughly mixed and subsampled and separated into categories based on botanical classification. These plant samples were freeze-dried and ground to pass through a 1 mm sieve in preparation for analysis.

The amount of feed refused by each animal were recorded daily, and a subsample was taken to determine dry matter content by drying overnight at 100 °C. The remaining refusals were then bulked on an individual animal basis, before being separated to determine botanical composition. This information, together with the botanical composition of the diet as offered, was used to calculate the composition of the diet consumed by each animal. Total fecal output was recorded daily, and a subsample from each animal was retained and stored at −20 °C. At the end of the measurement week the daily fecal samples were bulked on an individual animal basis, thoroughly mixed, and subsampled. This sample was then freeze-dried and milled to pass through a 1 mm sieve in preparation for chemical analysis.

A blood sample was collected from each animal at the end of the measurement period. Blood was collected from the jugular vein into Vacuette evacuated collection tubes containing lithium heparin (Greiner Labortechnik, Kremsmünster, Austria) and immediately placed on ice. Within an hour of collection, blood cells were separated from plasma by centrifugation (approximately 1700*g* for 25 min at 4 °C), and the plasma was transferred in approximately 1 mL aliquots into microcentrifuge tubes for storage at −80 °C.

Sample Preparation for Analysis by GC-TOF/MS. Samples were prepared for derivatization to the methoxime/trimethylsilyl (TMS) derivative. Portions of plasma (0.2 mL) were mixed with 0.4 mL of ice-cold acetone and incubated at 4 °C for 1−2 h to precipitate protein. The plasma samples were then centrifuged at 13000*g* for 5 min in a benchtop centrifuge, and a portion of the supernatant (350 μL) was dried at 40 °C under a stream of nitrogen. Samples of animal feces and plant material were prepared for derivatization using an alternative method. Portions of each sample (60 mg) were extracted into 300 μL of ice-cold methanol using a Retsch mill (3 × 2 min). The samples were then mixed with 200 μL of chloroform and incubated with shaking for 5 min at 37 °C, after which a further 400 μL of water was added. The samples were vortexed and then centrifuged for 5 min at 13000*g* in a benchtop centrifuge to separate the phases. A portion (80 μL) of the upper phase was removed and transferred to a clean microcentrifuge tube and dried in a centrifugal vacuum desiccator until dry at 30 °C. Samples were derivatized for analysis by the addition of 40 μL of freshly prepared methoxyaminehydrochloride in pyridine (20 mg/mL) and incubated in a shaking incubator at 30 °C for 1.5 h. A 10 μL aliquot of a mixture of alkanes (C10−C37) was added to each vial to allow measurement of mass spectral metabolite tag (MST) retention index, and the samples were derivatized to the TMS form by the addition of 70 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and incubation at 37 °C for 45 min. For sample analysis, a GC 6890 (Agilent Technologies, Palo Alto, CA) was coupled to a time-of-flight (TOF) mass spectrometer (Pegasus II MS system; LECO, St. Joseph, MI). The GC was operated under electronic pressure control and equipped with a split/splitless capillary inlet. Injection was 1 mL in the splitless mode with a 2 min pulse at 110 psi and the injection temperature set to 230 °C. The GC capillary column used was a 30 m × 0.25 mm i.d. Rtx-5Sil MS with an integrated guard column and a 0.25 mm film (Restek GmbH, Bad Homburg, Germany). Helium was used as carrier gas with constant flow at 1 mL/min. The temperature program was 2 min at 80 °C followed by a 15 min ramp to 350 °C and final heating for 2 min at 350 °C. The transfer line to the mass spectrometer was set to 250 °C. The mass spectrometer source was set to 200 °C. Mass spectra were monitored with an acquisition rate of 6 spectra/s in the mass range *m*/*z* 70−600. Tuning and all other settings of the mass spectrometer were according to the manufacturer's recommendations. Mass spectral metabolite tags were obtained by automated deconvolution of GC-MS chromatograms using Chromatof software (LECO) and identified tentatively by automated comparison of mass spectra match and retention time index with deconvoluted MSTs in a public access retention indexed mass spectral library containing 6205 mass spectra (http://csbdb.mpimp-golm.mpg.de/). Peak identification was made by best retention index match hit, and identities greater than (±) 20 retention index units were eliminated. Compounds that appeared in three or fewer samples were discarded as erroneous. Peak abundances of compounds occurring as multiple peaks in the chromatogram were combined and exported to Matlab version 6.5.1 (The Mathworks, Cambridge, U.K.), and Genstat version 8.1 (VSN International Ltd., Hemel Hempstead, U.K.) for statistical analysis.

Chemometric Data Analysis. Blood plasma GC-MS data were analyzed by principal component analysis (PCA) (*18*) using the statistics toolbox of Matlab. Compound total ion abundances were standardized to proportion of total ion current to account for differences in sample concentration and injection. Data were standardized for some analyses using the "prestd" function of the neural network toolbox of Matlab. This function mean centered the data from each sample to a standard deviation of one. Principal components (PCs) were calculated for the complete data set on both raw and normalized data. Pairs of PCs were then plotted in a series of ordination diagrams for visual inspection. Where separation between animals in different dietary groups was found, the PC loadings were interrogated to identify compounds that contributed to this separation. High positive or negative loadings indicated the variables that were most influential on the spatial distribution of the samples on the respective ordination diagrams. Furthermore, hierarchical cluster analysis (HCA) was used to estimate linkages between diet classes within the data set. Euclidean distance on the PCs with Ward's linkage methods was used to derive a similarity matrix, which was processed by agglomerative or divisive clustering algorithms to construct a dendrogram. PCA was also carried out on the fecal GC-MS data as described above. Where separation between the three groups was evident by visual inspection of score plots, HCA was carried out as above. Due to the limited number of plant samples (i.e., one sample per dietary treatment), PCA and HCA were not carried out on the plant metabolite data.

Table 1. Percentage Composition of Diet Components in Each of the Three Dietary Treatments, As Consumed

plant species	treatment (heather in diet) 10%	20%	30%
Calluna vulgaris (heather)	12.3	23.8	34.6
Erica tetralix (bell heather)	0.07	0.14	0.20
Molinia caerulea (purple moor grass)	40.8	35.1	29.8
Vaccinium myrtillus (bilberry)	0.16	0.31	0.44
Juncus effuses (soft rush)	11.5	10.0	8.7
Festuca spp. (grass, fescue)	1.56	1.39	1.24
Carex spp. (sedge)	3.71	3.20	2.72
dead grass (all species)	22.7	19.5	16.6
moss (unclassified)	7.28	6.52	5.81

Data and Statistical Analysis. Plant metabolite data were examined in Microsoft Excel to determine those compounds that were predominantly associated with a particular plant group. Compounds that had ≥70% of their total relative abundance (across all plant groups) associated with one plant group were deemed to be predominantly attributable to that plant group.

Pearson's product-moment correlation coefficients (R) were calculated between metabolites present in the plant, plasma, and feces samples with the amount of heather present in the diet using the "corrcoef" function of Matlab. PCA and HCA were then used to verify that the relative abundances of these metabolites allowed data separation according to dietary regimen. Furthermore, correlations were calculated for the first 10 PC scores of the plasma and feces samples against the total amount of heather eaten and the total amount eaten of each plant subgroup in the mixed diet.

Compounds found to be significantly ($P < 0.05$) correlated with diet were subjected to further investigation by analysis of variance (ANOVA) using Genstat to establish if there were significant differences in the mean abundances of plant components present in the plasma and fecal samples. Treatment effects were partitioned into linear and other effects using polynomial contrasts.

RESULTS AND DISCUSSION

The mean compositions of the three experimental diets are presented in **Table 1**. The mean quantities of *C. vulgaris* in the diets were close to the amounts originally specified but differed due to selection of dietary components by individual animals.

Relationship of Metabolites in Blood Plasma, Feces, and Plants to the Proportion of Heather in the Diet. *Analysis of Plasma Samples.* PCA of standardized/normalized plasma data sets showed >99.5% of the total variance was retained by the first 10 components. The first five PCs accounted for >87% of the variance. It was possible to separate samples from animals maintained on the 10% heather diet from samples from animals on the 20 and 30% diets through the combined effect of both PC1 (35.85% of total variance) and PC4 (10.12% of total variance) axes (**Figure 1A**). Generally, animals maintained on the 20 and 30% diets were separated from animals on the 10% diet along the PC1 axis, whereas there were negative correlations (both $P < 0.05$) between the percentages of *Calluna* and *Erica* in the diet and PC1. Samples taken from animals on the 10% diet had mean positive PC1 scores and negative PC4 scores, whereas samples from animals offered the 20 and 30% diets had mean negative PC1 scores and positive PC4 scores. Examination of the loadings plot (**Figure 1B**) for these two PCs showed that the 10% diet differed from the other diets in a manner dependent on the concentration of pyroglutamic acid, L-proline, L-isoleucine, 3-aminopiperidin-2-one, and hydrogen cyanamide. Samples from the 20 and 30% diets had lower concentrations of these compounds but greater amounts of malonic acid and L-valine. These two compounds both had high negative PC1 loadings and relatively high positive loadings on PC4. It was not possible to discriminate between animals on each of the three diets by HCA.

Analysis of the plasma data by Pearson's product-moment correlation indicated other metabolites were significantly correlated ($P < 0.05$) with diet. Metabolites that were positively correlated with increasing proportion of heather in the diet included L-alanine ($R = 0.61$), L-isoleucine ($R = 0.64$), L-valine ($R = 0.677$), 4-aminobutyric acid ($R = 0.63$), 2-propenoic acid ($R = 0.66$), 3-methylundecane ($R = 0.69$), methoxymethylcyclopropane ($R = 0.65$), *N*,*N*-diethyl-*N*′-methyl-1,2-ethanediamine ($R = 0.59$), malonic acid ($R = 0.80$), succinic acid ($R = 0.73$), and galactaric acid ($R = 0.73$). In contrast, nicotinamide ($R = -0.58$) was negatively correlated with increasing *Calluna* proportion in the diet ($P < 0.05$).

PCA of the peak areas of the metabolites identified by Pearson correlation showed distinct clustering of the three treatment groups through the combined effect of PC1 (54.0% total variance) against PC4 (1.42% total variance) (**Figure 2A**). Animals offered the 10% heather diet were distinguished by their PC1 score from animals on the 20 and 30% diets, whereas the latter two diet groups were distinguished by their score for PC4. The loadings plot (**Figure 2B**) shows this grouping was dependent on several metabolites. In particular, plasma from animals maintained on the 10% diet contained higher levels of 3-methylundecane, nicotinamide, L-isoleucine, and 4-aminobutyric acid, all of which had positive PC1 loadings. The grouping of the 20 and 30% samples corresponded to the concentrations of methoxymethylcyclopropane and *N*,*N*-diethyl-*N*′-methyl-1,2-ethanediamine, relative concentrations of which were higher in samples from the 20% heather diet. Samples from animals on the 30% heather diet also had relatively higher amounts of L-valine and malonic acid (as indicated by PCA eigenvectors). Similarly, HCA of PC1 and PC4 scores by Euclidean distance separated the samples into two clusters (**Figure 2C**), with one cluster comprising three of the 10% heather samples. The second cluster was subdivided further into two clusters with all of the 20 and 30% heather samples correctly grouped.

Analysis of Feces Samples. Analysis of the GC-MS data from analysis of the sheep feces samples by PCA and HCA failed to distinguish between animals on each of the three diets. Pearson's correlation was carried out to identify fecal metabolite(s) that were strongly correlated with diet. Malic acid ($R = 0.63$) was found to be positively correlated ($P < 0.05$), whereas 2-butyl-2-ethyloxazolidine ($R = -0.69$) was inversely related to diet ($P < 0.05$). Due to the small number of variables identified, it was not possible to conduct PCA or HCA on the data.

Analysis of Plant Samples. Pearson correlation was used to identify those metabolites present in samples of the mixed diets that correlated most with the amount of heather present in the diet. Results indicated that the concentrations of 5-*tert*-butyl-2-thiophenecarboxylic acid ($R = 0.99$) and 2-imino-4(5*H*)-thiazolone ($R = 0.99$) in the diet mix were most dependent on the amount of heather in the diet ($P < 0.05$). In contrast, nicotinic acid ($R = -0.99$), malic acid ($R = -0.99$), xylose ($R = -0.99$), and 2-aminoadipic acid ($R = -0.99$) were all negatively correlated ($P < 0.05$).

Relationship of Metabolites in Blood Plasma, Feces, and Plant Samples to Botanical Dietary Composition. *Analysis of Plasma Samples.* Correlation analysis of the first 10 PC scores of the metabolite abundances in the plasma samples against the amount of each plant subgroup eaten showed a negative correlation between the amounts of *Calluna* ($R = -0.61$, $P < 0.05$) and *Erica* ($R = -0.62$, $P < 0.05$) consumed and PC1.

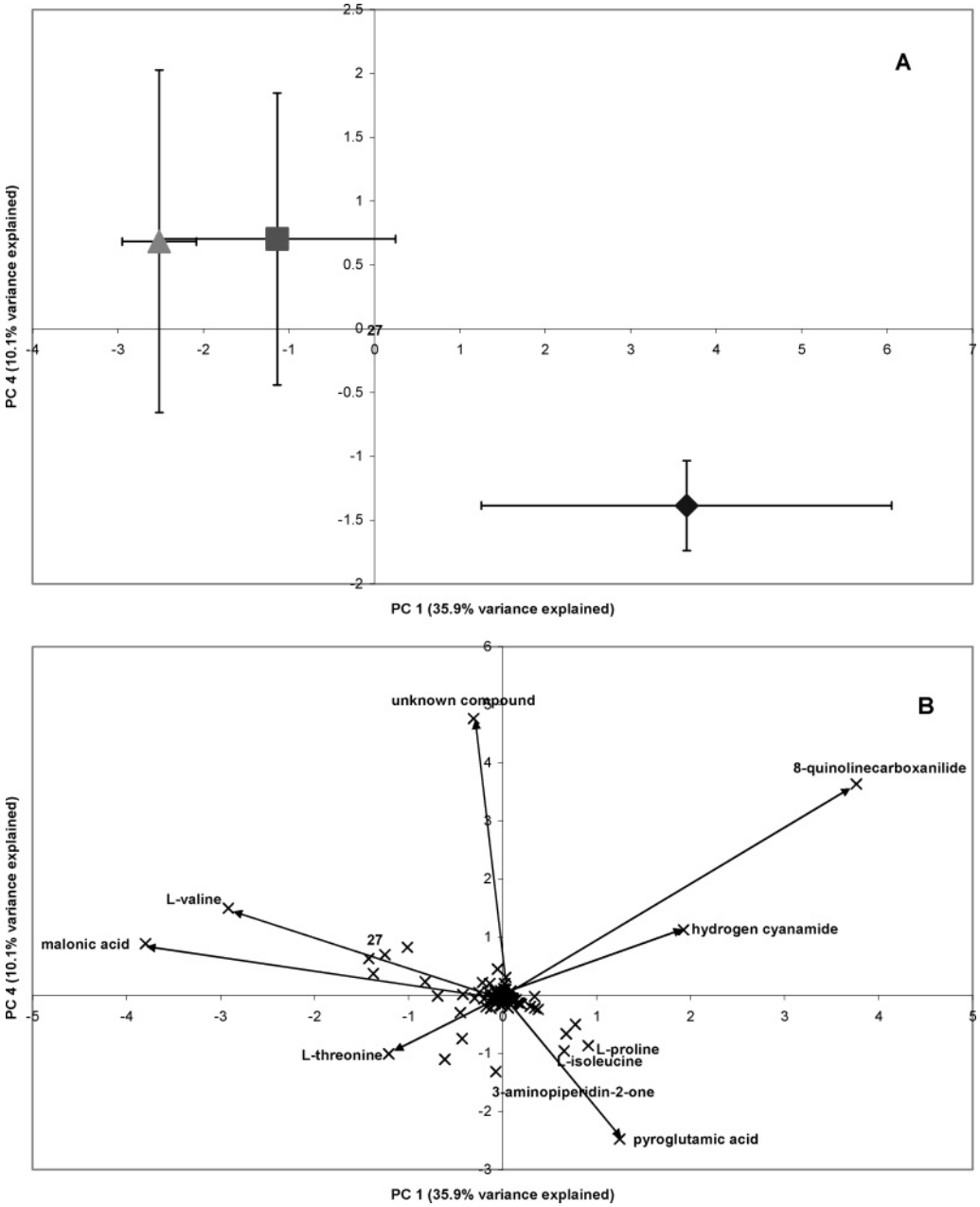

Figure 1. Plots of PC1 and PC4 scores (**A**) and PC1 and PC4 loadings (**B**) (standard error bars represent standard error of means for each of the three diet groups) of plasma samples from sheep maintained on diets of 10% heather (●), 20% heather (■), and 30% heather (▲).

Consistent with this trend, the total amount of heather in the diet, that is, *Calluna* and *Erica* combined ($R = -0.65$, $P < 0.05$), was also negatively correlated with PC1. In contrast, the amount of *Molinia* in the diet was positively related to PC1 ($R = 0.58$, $P < 0.05$). Similarly, the 12 metabolites identified previously by Pearson correlation analysis as being most significantly correlated to the amount of heather in the diet correlated strongly with the first principal component from the plasma PCA.

Statistical analysis by ANOVA showed significant differences in the mean concentrations of L-valine ($P < 0.01$), 4-aminobutyric acid ($P < 0.05$), galactaric acid ($P < 0.05$), succinic acid ($P < 0.05$), 2-propenoic acid ($P < 0.05$), and methoxymethylcyclopropane ($P < 0.05$) in plasma samples from sheep on each of the dietary treatments. Samples from animals on the 20 and 30% heather diets contained significantly higher concentrations of these compounds than samples from animals on the 10% diet (**Table 2**). Similarly, there were significant between-treatment differences in the concentrations of L-alanine ($P < 0.01$) and malonic acid ($P < 0.001$) as the proportion of heather in the diet increased. Differences in the mean concentrations of several other compounds were observed that fell below 95% level of confidence. For example, concentrations of L-isoleucine and *N*,*N*-diethyl-*N*′-methyl-1,2-ethanediamine were higher in samples from animals on the 20 and 30% diets compared to the 10% diet, whereas the concentration of nicotinamide decreased with increased proportion of heather in the diet.

Analysis of Feces Samples. Examination of the correlation between PCs 1−10 for the GC-MS fecal data against diet composition showed that the amount of *Calluna* ($R = -0.54$, $P < 0.1$) and the total content of heather ($R = -0.55$, $P < 0.1$) in the diet were negatively correlated with PC4. In contrast, the amount of *Molinia* ($R = 0.68$, $P < 0.05$) consumed was positively related to PC4. Furthermore, *Festuca* ($P < 0.001$) and *Carex* ($P < 0.05$) showed strong positive correlations,

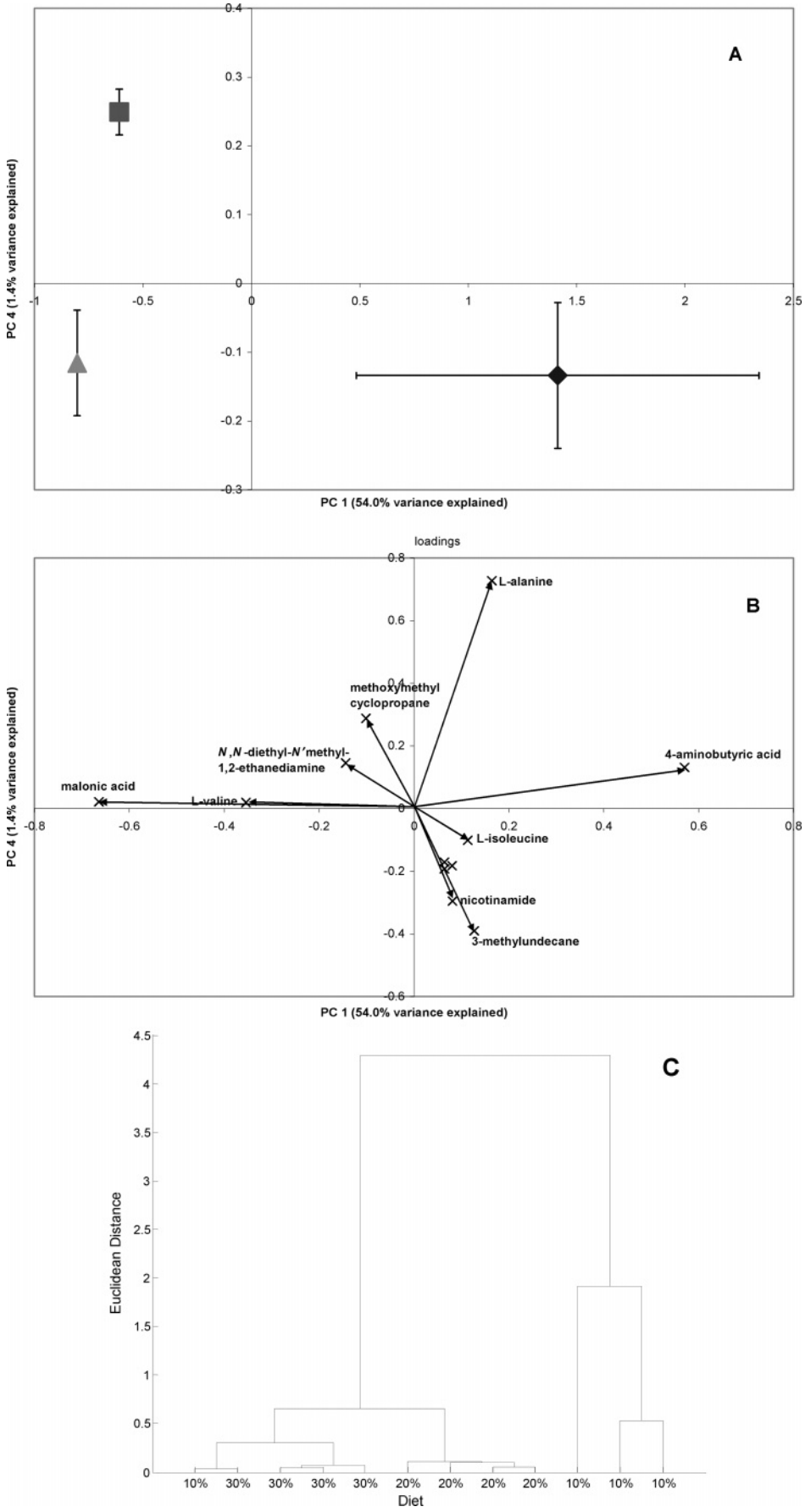

Figure 2. Plots of PC1 and PC4 scores (**A**) and PC1 and PC4 loadings (**B**) (standard error bars represent standard error of means for each of the three diet groups) of plasma samples (normalized peak areas that correlated strongly with diet) from sheep maintained on diets of 10% heather (●), 20% heather (■), and 30% heather (▲). (**C**) HCA of PC1 and PC4 scores for sheep plasma maintained on one of the three diets.

Table 2. Mean Concentrations of Metabolites (Relative Abundance) in Plasma and Feces Samples from Sheep Maintained on One of Three Diets

	treatment (heather in diet)				
	10%	20%	30%	SED	significance[a]
plasma metabolites					
L-alanine	3.46	13.87	11.19	2.24	<0.001
L-isoleucine	0.209	0.385	0.445	0.092	<0.05
L-valine	5.6	23.6	23.0	5.20	<0.001
4-aminobutyric acid	3.75	6.71	7.30	1.19	<0.05
galactaric acid	0.0042	0.0131	0.0361	0.0097	<0.001
malonic acid	9.0	24.7	32.6	5.8	<0.001
2-propenoic acid	0.124	0.295	0.327	0.072	<0.05
succinic acid	0.121	0.259	0.329	0.064	<0.001
methoxymethylcyclopropane	3.5	9.6	10.6	2.6	<0.05
3-methylundecane	0.398	0.759	0.835	0.145	<0.05
N,*N*-diethyl-*N*′-methyl-1,2-ethanediamine	5.4	12.1	12.5	2.9	<0.05
nicotinamide	0.0456	0.0053	0.0058	0.0170	<0.05
fecal metabolites					
2-butyl-2-ethyloxazolidine	0.0072	0.0027	0.0004	0.0024	<0.05
malic acid	0.0027	0.0058	0.0134	0.0044	<0.05

[a] Significance of linear effects.

whereas *Vaccinium* was negatively correlated ($P < 0.05$) with this component.

Analysis of these data by ANOVA showed that concentrations of 2-butyl-2-ethyloxazolidine ($P < 0.05$) increased significantly with decreasing amounts of heather in the diet. In contrast, malic acid ($P < 0.1$) increased with the proportion of heather in the diet (**Table 2**).

Identification of Potential Markers of Dietary Components. Comparison was made of the amount of each metabolite in the samples of each specific plant group present in the mixed diet. Compounds were identified that showed a high abundance in any one of the plant groups (>70%) relative to the combined amount present in all of the plant subgroup samples. These compounds (**Table 3**) were taken to be potential marker compounds of intake of a particular plant subgroup within the mixed diet. Some of the plant subgroups, *Calluna*, *Molinia*, and moss plant groups, contained metabolites that were largely species specific. These marker metabolites were detected in the sheep plasma and feces (**Table 4**).

Potential Use of Metabolite Profiling Technique To Help Determine Dietary Composition. The relationship between health and diet are of increasing concern to the public and agricultural sector. Studies relating human health (as opposed to animal health) to diet are at a more advanced stage and have used a variety of postgenomic approaches including proteomic and metabolomic technologies (*16*, *19*−*23*). Metabolomic-based methods combined with bioinformatics are currently being explored to identify and validate targets to improve nutritional health, steer the next generation of food and crops (*19*−*22*), and improve our understanding of the effects of diet on health, with a view to gaining a better understanding of the mechanisms of action and health benefits of particular food components. Furthermore, metabolomics has the potential to explore homeostatic control and how metabolic balance is altered by excesses or deficiencies of dietary components (*20*). Recent studies have demonstrated the usefulness of GC-TOF/MS analysis to identify a large number of metabolites from plant and animal sources (blood plasma and feces) (*24*−*29*). Although this method shows promise for rapidly and efficiently identifying a large number of metabolites, to date, no work has been reported using GC-TOF/MS to identify plant metabolites within ruminant forage mixtures and ruminant diets.

We have previously shown that FT-IR analysis coupled with chemometric methods allowed discrimination of sheep offered the 10, 20, and 30% heather diets using metabolic fingerprints in plasma and feces (unpublished data). We have shown chemical differences between the three diets by infrared spectroscopy but were unable to characterize these differences due to the inherent nature of the technique. In the current study, we utilized high chromatographic resolution of gas chromatography coupled with a high rate of data acquisition to identify metabolites present in a variety of plant groups present at different concentrations in three diets fed to sheep and determined the presence or absence of these and other metabolites in blood and feces. With this approach we were able to identify those chemicals responsible for the structure observed in PCA and HCA plots.

Analysis of Plant Subgroups and Species. We identified potential markers of the three herbage diets and individual plant groups that are predominantly associated with a diet or specific to a plant species. Plant markers tentatively identified as 2-hydroxy-4-pyrimidinamine, 2,5-dihydroxypyrazine, *erythro*-pentitol, erythritol, and *N*-(1-phenylethyl)-1-naphthalene carboxamide were specific to the *Molinia* species. These compounds were also detected in the 10% heather diet but not in the 20 or 30% herbage mixtures. We propose that this may be due to levels being below the detectable threshold level necessary to supply a detectable quantity of the metabolites. 2-Ethyl-*N*-(2-ethylhexyl)-*N*-methyl-1-hexanamine and *N*,*N*-diethyl-*N*′-methyl-1,2-ethanediamine were specific to the *Calluna* species. The former compound was detected in the 30% heather diet but not in the 10 and 20% herbage mixtures, and, again, this may be due to metabolite levels being below the threshold in the 10 and 20% dietss, whereas the latter compound was abundant in all three dietary mixtures. However, further work is required to confirm the identification of the metabolites.

Analysis of Sheep Plasma. The chemical composition of blood plasma, in contrast to that of animal feces, is tightly regulated, and the concentrations of many key metabolites, for example, glucose, cholesterol, triglycerides, and amino acids, are maintained within limits by homeostasis. However, plasma from animals also contains, often at low levels, secondary metabolites derived from dietary components [e.g., flavonoids (*30*), lupine alkaloids (*31*)] or from contaminating microorganisms [e.g., ergot alkaloids (*32*)]. Metabolites that have been produced by microbial metabolism in the gut and absorbed across the intestinal lining are also present in blood plasma. In the case of ruminant animals the influence of microbial metabolism on

Table 3. Metabolites Detected in the Diet Mixtures That Are Predominantly Attributable to Individual Plant Subgroups or Species (>70% Relative Abundance)

compound >70% relative abundance in one plant group	predominant source in the mixed diet	detectable in 10% heather?	detectable in 20% heather?	detectable in 30% heather?
β-alanine	*Molinia*	yes	yes	yes
DL-homocysteine	*Molinia*	yes	yes	yes
glycine	*Molinia*	yes	yes	no
L-alanine	*Molinia*	yes	yes	yes
L-glutamic acid	*Molinia*	yes	yes	yes
L-glutamine	*Molinia*	yes	yes	yes
L-homoserine	*Molinia*	yes	yes	yes
L-leucine	*Molinia*	yes	yes	yes
L-phenylalanine	*Molinia*	yes	yes	yes
L-proline	*Molinia*	yes	yes	no
L-serine	*Molinia*	yes	yes	yes
L-threonine	*Molinia*	yes	yes	yes
L-valine	*Molinia*	yes	yes	yes
N-acetylglutamine	*Molinia*	yes	yes	yes
2,4-bis(hydroxybutanoic acid)	*Calluna*	yes	yes	yes
2-aminoadipic acid	*Molinia*	yes	yes	yes
citric acid	*Molinia*	yes	yes	yes
D-(-)-citramalic acid	*Molinia*	yes	yes	no
nonanoic acid	*Molinia*	yes	yes	no
picolinic acid	*Molinia*	yes	no	no
succinic acid	*Molinia*	yes	no	no
1,6-anhydroglucose	*Molinia*	yes	yes	yes
xylose	*Molinia*	yes	yes	yes
3-deoxyglucitol	*Molinia*	yes	yes	yes
erythritol	*Molinia*	yes	no	no
erythro-pentitol	*Molinia*	yes	no	no
mthoxymethylcyclopropane	*Molinia*	yes	yes	yes
2,5-dihydroxypyrazine	*Molinia*	yes	no	no
2-ethyl-*N*-(2-ethylhexyl)-*N*-methyl-1-hexanamine	*Calluna*	no	no	yes
2-hydroxy-4-pyrimidinamine	*Molinia*	yes	no	no
5-aminocarboxy-4,6-dihydroxypyrimidine	*Molinia*	yes	yes	yes
8-quinolinecarboxanilide	*Molinia*	yes	yes	yes
ethanolamine	*Molinia*	yes	yes	yes
N-(1-phenylethyl)-1-naphthalenecarboxamide	*Molinia*	yes	no	no
N,*N*-di(2-hydroxyethyl)methanamine	*Molinia*	yes	yes	yes
N,*N*-diethyl-*N*′-methyl-1,2-ethanediamine	*Calluna*	yes	yes	yes
N-benzenesulfonyloxy-2,2-bis(trifluoromethyl)aziridine	*Molinia*	yes	yes	no

plasma composition is considerable, an extreme example being the presence of large concentrations of volatile fatty acids produced as a consequence of microbial carbohydrate metabolism. We investigated the potential to discriminate between animals on each of the different dietary regimens on offer, and we tentatively identified the plant metabolites thought to be responsible for this segregation.

Using PCA of data obtained by GC-MS analysis of blood plasma it was possible to distinguish between animals fed three very similar diets, and samples from animals maintained on the 10% heather diet could be distinguished from samples from animals offered the 20 and 30% diets by PCA and HCA. The PC loadings showed that plasma samples taken from animals on the 10% diet contained higher concentrations of pyroglutamic acid, L-proline, L-isoleucine, 3-aminopiperidin-2-one, and hydrogen cyanamide and lower concentrations of L-valine and malonic acid relative to the 20 and 30% diets. Conversely, the abundances of the latter two compounds increased, whereas the others decreased, with the proportion of heather in the diet.

Hydrogen cyanamide is used in agricultural chemicals (fertilizers and as a plant growth regulator in herbicides) (*33*, *34*); furthermore, it is a dehydration product of urea ($H_2NCONH_2 \leftrightarrow H_2NCN + H_2O$). We detected cyanamide in low levels in samples of live *Carex* and in plasma samples taken from animals on the three dietary treatments. As plants in this study were not exposed to or treated with fertilizers or herbicides, and it is unlikely that cyanamide would be produced under biological conditions, we propose that the cyanamide originated from the *Carex* in the diet of the animals. This view is supported by the recent identification of cyanamide in another upland plant, hairy vetch (*Vicia villosa*) (*35*).

Furthermore, PCA and HCA based upon analysis of the peak areas of the 12 compounds that most strongly correlated with diet as determined by Pearson's correlation coefficient succeeded in discriminating between animals on each of the three diets. Plasma samples taken from animals on the 10% diet were distinguished from those taken from animals fed the 20 and 30% diets by virtue of the PC1 score. The loadings showed that the 10% diet contained higher concentrations of 3-methylundecane, nicotinamide, and 4-aminobutyric acid relative to the 20 and 30% samples. Conversely, the abundances of these compounds decreased as the proportion of heather increased in the diet. Similarly, samples from animals maintained on the 20% heather diet could be distinguished from samples from animals on the 30% diet by PC4 score. The 20% samples contained higher concentrations of methoxymethylcyclopropane and *N*,*N*-diethyl-*N*′-methyl-1,2-ethanediamine relative to the 30% diet. Results obtained from ANOVA of plasma metabolites best correlated with the percentage of heather in the diet were consistent with these findings. Furthermore, these compounds were present in all three experimental diets, with *N*,*N*-diethyl-*N*′-methyl-1,2-ethanediamine being largely specific to the *Calluna* species. However, contrary to expectations plasma samples from animals on the 10% diet contained lower levels of 3-methylundecane and 4-aminobutyric acid as compared to animals on the 20 and 30% diets. We presume that this may be

Table 4. Metabolites Detected in Blood Plasma and Feces Samples from Domestic Sheep on One of Three Diets

	Amino Acids	
β-alanine	L-glutamine	L-phenylalanine
DL-homocysteine	L-glycerol-3-phosphate	L-proline
glycine	L-homoserine	L-serine
L-alanine	L-isoleucine	L-threonine
L-asparagine	L-leucine	L-valine
L-aspartic acid	L-lysine	*N*-acetylglutamic acid
L-cysteine	L-methionine	*N*-acetylglutamine
L-glutamic acid	L-norvaline	pyroglutamic acid
	Organic Acids	
1,4-benzenedicarboxylic acid	benzoic acid	malic acid
2,4-bishdroxybutanoic acid	carbonic acid	malonic acid
2-aminoadipic acid	cis-aconitic acid	nicotinic acid
2-aminobutanoic acid	citric acid	nonanoic acid
2-hydroxyglutaric acid	D-(-)-citramalic acid	pentanoic acid
2-ketoglutaric acid	dodecanoic acid	pentonic acid
2-methyl-2-butenedioic acid	erythronic acid	picolinic acid
2-piperidinecarboxylic acid	*erythro*-pentonic acid	shikimic acid
2-propenoic acid	ethanedioic acid	suberylglycine
3-hydroxypropanoic acid	fumaric acid	succinic acid
4-aminobutyric acid	galactaric acid	succinic anhydride
4-aminomethylcyclohexane carboxylic acid	gluconic acid	tartaric acid
4-hydroxybutanoic acid	glutaric acid	threonic acid
5-aminovaleric acid	gyceric acid	xylonic acid
acetic acid	hydroxybenzoic acid	
benzeneacetic acid	maleic acid	
	Sugars	
1,6-anhydroglucose	β-D-methylfructopyranoside	fucose
4-ketoglucose	D-fructose	rhamnose
arabinofuranose	D-*gluco*-hexodialdose	ribose
arabino-hexos-2-ulose	D-ribofuranose	xylose
arabinose	D-xylofuranose	
β-D-methylfructofuranoside	erythrose	
	Alcohols	
1-phenylethanol	glycerol	threitol
3-deoxyglucitol	*meso*-erythritol	xylitol
erythritol	pentitol	
erythro-pentitol	ribitol	
	Amides	
2,2,2-trifluoro-*N*-(1-methylpropyl)acetamide	hydrogen cyanamide	nicotinamide
2,2,2-trifluoro-*N*-methylacetamide	*N*-(1-phenylethyl)-1-naphthalenecarboxamide	urea
4-(dimethylamino)-*N*,*N*-dimethylbenzene-sulfonamide	*N*,*N*-diethyldodecanamide	
	Alkanes	
1-(2,3-dichlorophenyl)-2-(1,2,4-triazol-1-yl)-2-oxime-ethanedione	3-amino-2,3-di(hydroxyamino)propanenitrile	*n*-decane
1,2,3-trihydroxybutane	3-methylundecane	*n*-dodecane
2-(2-trifluoromethylphenyl)ethane-1,1,2-tricarbonitrile	4-methyldodecane	*n*-pentadecane
3,8-dioxa-2,9-disiladecane	methoxymethyl cyclopropane	
	Alkenes	
acetylene		
	Lactones	
erythronic acid-1,4-lactone	pentonic acid-1,4-lactone	threonic acid-1,4-lactone
	Nitrogen Compounds	
1,2,3-trihydroxybenzene	3-aminopiperidin-2-one	*N*,1,1,1-tetramethyl-*N*-1-methyl-2-phenyl-2-(hydroxyethyl)silanamine
1,3-bis(ethynylbenzene)	4,6-diamino-3-[*p*-methoxyphenyl]-1-methyl-pyrazolo[3,4-*d*]pyrimidine	*N*,*N*-di(2-hydroxyethyl)-methanamine
2,3-dimethylquinoline-1-oxide	4-*tert*-butyl-2,6-dimethyl-3,5-dinitroacetophenone	*N*,*N*-diethyl-*N*′-methyl-1,2-ethanediamine
2,4(1*H*,3*H*)-pyrimidinedione	5-aminocarboxy-4,6-dihydroxypyrimidine	*N*,*N*′-ditrifluoroacetyl-1,2-ethandimine
2,5-dihydroxypyrazine	5-isopropyl-1*H*-indole-2,3-dione	ornithine
2-butyl-2-ethyloxazolidine	5-isopropylidene-3,3-dimethyl-dihydrofuran-2-one	phenylethanolamine
2-ethyl-*N*-(2-ethylhexyl)-*N*-methyl-1-hexanamine	cytosine	putrescine
2-hydroxy-4-pyrimidinamine	ethanolamine	uracil

Table 4. (Continued)

	Nitrogen Compounds	
2-methyl-1(2*H*)-isoquinolinone	ethyl pipecolinate	vinylbital
3-4-diethylamino-1-methylbutylamino]-1-methoxynaphthalene	hydroxylamine	
	Sulfur Compounds	
2,3-dimethyl-5-methylamino-1,3,4-thiadiazole	5-*tert*-butyl-2-thiophenecarboxylic acid	*N*-benzenesulfonyloxy-2,2-bis(trifluoromethyl)aziridine
2-imino-4(5*H*)-thiazolone	8-quinolinecarboxanilide	
	Phosphorus Compounds	
phosphoric acid		

a consequence of microbial metabolism in the rumen or intestinal tract.

Examination of the correlation between the PC scores and feed consumption showed that the amounts of *Calluna*, *Erica*, and *Molinia* in the diet were strongly correlated with PC1 axis. Principal components 1 and 4 generally separated all three classes on the basis of the combined effect of the dietary components, *Calluna*, *Erica*, *Molinia*, *Festuca*, and *Carex*, with a high score on the PC1 axis, indicating a lower consumption of *Calluna* and *Erica* but conversely a higher relative consumption of *Molinia*, *Festuca* and *Carex*. Moreover, the percent of variance accounted for in PC1 increased to 54%, clearly demonstrating structural similarities between the two PCA models.

Analysis of Sheep Feces. It was not possible to distinguish between animals on each of the three diets by PCA or HCA of data from the fecal samples. There was, however, a significant correlation ($P < 0.05$) between the amounts of each of *Molinia*, *Vaccinium*, *Carex*, and *Festuca* consumed and PC4 scores. The variance accounted for by this principal component was 1.4%, and this component may indicate the relative amounts of hill grass plants as compared to heather in feces. The metabolome of blood plasma reflects the uptake of metabolites from the diet, both before and after microbial modification, and of the interchange of nutrients about the body. The metabolome of feces comprises parts of the diet that are not digested or that have been modified by microbial fermentation and intestinal digestion but which have not been absorbed by the animal. Metabolites can also be transferred from the animal's body into gut digesta and be excreted in feces. Therefore, it is interesting, but perhaps not surprising, that this work has shown that correlations can be made between the chemical composition of plasma and dietary composition but not with feces and diet. One reason for this may be the processes of digestion and absorption of nutrients from the diet, leading to very different metabolomes; the correlation of plasma with diet is better than that of feces with diet. In a previous study (unpublished data) we have shown that it was possible to distinguish between animals fed the different diets using FT-IR analysis of feces.

In conclusion, this is the first report of GC-TOF/MS being used to discriminate between agricultural livestock animals maintained on mixed, complex but distinct diets. The tandem technique of gas chromatography with mass spectrometry is gaining increasing popularity in the field of metabolomics as it is highly sensitive and quantitative over a wide range of metabolite concentrations. It is also particularly suited for the resolution and quantification of low molecular mass compounds in plant tissues, animal feces, and blood plasma and provides a unique combination of compound-specific mass spectra and retention information from which metabolites may be identified using mass spectrometric libraries. In this study, GC-TOF/MS metabolite profiles of blood plasma and feces were correlated with the consumption of specific plants in the diet, and we have demonstrated in a posteriori manner the validity of this technique to discriminate between these very similar diets. However, after further work to validate and develop the approach, it is anticipated that the primary application of this technique will be a priori, that is, to help with the determination of dietary intake from information of plasma metabolite composition. This study demonstrates clearly the suitability of metabolomic strategies for the identification of biomarkers related to dietary intervention and as such could provide an invaluable tool for developing effective and sustainable livestock management regimens for animals grazing diverse mixed swards in agroecosystems that are potentially fragile.

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LITERATURE CITED

(1) Mayes, R. W.; Dove, H. Measurement of dietary nutrient intake in free-ranging mammalian herbivores. *Nutr. Res. Rev.* **2000**, *13* (1), 107−138.

(2) Holechek, J. L.; Vavra, M.; Pieper, R. D. Botanical composition determination of range herbivore diets—a review. *J. Range Manage.* **1982**, *35* (3), 309−315.

(3) Henley, S. R.; Smith, D. G.; Raats, J. G. Evaluation of 3 techniques for determining diet composition. *J. Range Manage.* **2001**, *54* (5), 582−588.

(4) Bartolome, J.; Franch, J.; Gutman, M.; Seligman, N. G. Physical factors that influence fecal analysis estimates of herbivore diets. *J. Range Manage.* **1995**, *48* (3), 267−270.

(5) Alipayo, D.; Valdez, R.; Holechek, J. L.; Cardenas, M. Evaluation of microhistological analysis for determining ruminant diet botanical composition. *J. Range Manage.* **1992**, *45* (2), 148−152.

(6) Tieszen, L. L.; Boutton, T. W.; Tesdahl, K. G.; Slade, N. A. Fractionation and turnover of stable carbon isotopes in animal-tissues—implications for delta-C-13 analysis of diet. *Oecologia* **1983**, *57* (1−2), 32−37.

(7) Coates, D. B. Reliability of extrusa samples collected from steers fistulated at the oesophagus to estimate the diet of resident animals in grazing experiments. *Aust. J. Exp. Agric.* **1987**, *27*, 739−745.

(8) Bennett, L. L. Diet selection by steers using microhistological and stable carbon isotope ratio analyses. *J. Anim. Sci.* **1999**, *77*, 2252−2258.

(9) Sibbald, A. M.; Davidson, G. C.; Mayes, R. W. Effect of dosing regime on intake estimation using the *n*-alkane technique in sheep fed pelleted grass meal. *J. Sci. Food Agric.* **2000**, *80* (8), 1206−1210.

(10) Ali, H. A. M.; Mayes, R. W.; Lamb, C. S.; Hector, B. L.; Verma, A. K.; Orskov, E. R. The potential of long-chain fatty alcohols and long-chain fatty acids as diet composition markers: development of methods for quantitative analysis and faecal recoveries of these compounds in sheep fed mixed diets. *J. Agric. Sci.* **2004**, *142*, 71−78.

(11) Ali, H. A. M.; Mayes, R. W.; Hector, B. L.; Orskov, E. R. Assessment of *n*-alkanes, long-chain fatty alcohols and long-chain fatty acids as diet composition markers: the concentrations of these compounds in rangeland species from Sudan. *Anim. Feed Sci. Technol.* **2005**, *121* (3−4), 257−271.

(12) Lyons, R. K.; Stuth, J. W. Fecal NIRS equations for predicting diet quality of free-ranging cattle. *J. Range Manage.* **1992**, *45* (3), 238−244.

(13) Leite, E. R.; Stuth, J. W. Fecal NIRS equations to assess diet quality of free-ranging goats. *Small Ruminant Res.* **1995**, *15* (3), 223−230.

(14) Volesky, J. D.; Coleman, S. W. Estimation of botanical composition of esophageal extrusa samples using near infrared reflectance spectroscopy. *J. Range Manage.* **1996**, *49* (2), 163−166.

(15) Walker, J. W.; Clark, D. H.; McCoy, S. D. Fecal NIRS for predicting percent leafy spurge in diets. *J. Range Manage.* **1998**, *51* (4), 450−455.

(16) Whitfield, P. D.; German, A. J.; Noble, P. J. M. Metabolomics: an emerging post-genomic tool for nutrition. *Br. J. Nutr.* **2004**, *92* (4), 549−555.

(17) Fraser, M. D.; Theobald, V.; Moorby, J. M. Determining the diet composition of animals grazing complex swards using n-alkanes and long-chain fatty alcohols. *Ecol. Appl.* **2006**, *16* (5), 1901−1910.

(18) Jolliffe, I. T. *Principle Component Analysis*; Springer-Verlag: New York, 1986.

(19) Watkins, S. M.; Hammock, B. D.; Newman, J. W.; German, J. B. Individual metabolism should guide agriculture toward foods for improved health and nutrition. *Am. J. Clin. Nutr.* **2001**, *74* (3), 283−286.

(20) German, J. B.; Roberts, M. A.; Watkins, S. M. Personal metabolomics as a next generation nutritional assessment. *J. Nutr.* **2003**, *133* (12), 4260−4266.

(21) Solanky, K. S.; Bailey, N. J.; Beckwith-Hall, B. M.; Davis, A.; Bingham, S.; Holmes, E.; Nicholson, J.; Cassidy, A. Application of biofluid ^{1}H nuclear magnetic resonance-based metabonomic techniques for the analysis of the biochemical effects of dietary isoflavones on human plasma profile. *Anal. Biochem.* **2003**, *323*, 197−204.

(22) German, J. B.; Watkins, S. M. Metabolic assessment—a key to nutritional strategies for health. *Trends Food Sci. Technol.* **2004**, *15*, 541−549.

(23) Solanky, K. S.; Bailey, N. J.; Beckwith-Hall, B. M.; Bingham, S.; Davis, A.; Holmes, E.; Nicholson, J. K.; Cassidy, A. Biofluid H-1 NMR-based metabonomic techniques in nutrition research metabolic effects of dietary isoflavones in humans. *J. Nutr. Biochem.* **2005**, *16* (4), 236−244.

(24) Schauer, N.; Zamir, D.; Fernie, A. Metabolic profiling of leaves and fruit of wild species tomato: a survey of the *Solanum lycopersicum* complex. *J. Exp. Bot.* **2004**, *56* (410), 297−307.

(25) Schauer, N.; Steinhauser, D.; Strelkov, S.; Schomburg, D.; Allison, G.; Moritz, T.; Lundgren, K.; Roessner-Tunali, U.; Forbes, M. G.; Willmitzer, L.; Fernie, A. R.; Kopka, J. GC-MS libraries for the rapid identification of metabolites in complex biological samples. *FEBS Lett.* **2005**, *579* (6), 1332−1337.

(26) Roessner, U.; Wagner, C.; Kopka, J.; Trethewey, R. N.; Willmitzer, L. Simultaneous analysis of metabolites in potato tuber by gas chromatography−mass spectrometry. *Plant J.* **2000**, *23* (1), 131−142.

(27) Wagner, C.; Sefkow, M.; Kopka, J. Construction and application of a mass spectral and retention time index database generated from plant GC/EI-TOF-MS metabolite profiles. *Phytochemistry* **2003**, *62* (6), 887−900.

(28) Birkemeyer, C.; Kolasa, A.; Kopka, J. Comprehensive chemical derivatization for gas chromatography−mass spectrometry-based multi-targeted profiling of the major phytohormones. *J. Chromatogr.* **2003**, *993* (1−2), 89−102.

(29) Fiehn, O.; Kopka, J.; Trethewey, R. N.; Willmitzer, L. Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. *Anal. Chem.* **2000**, *72* (15), 3573−3580.

(30) Radtke, J.; Linseisen, J.; Wolfram, G. Fasting plasma concentrations of selected flavonoids as markers of their ordinary dietary intake. *Eur. J. Nutr.* **2002**, *41* (5), 203−209.

(31) Gay, C. C.; Panter, K. E.; Mealey, K. L.; Gay, J. M.; Hjartarson, S. W.; Tibary, A.; Motteram, E. S.; Wierenga, T.; James, L. F. Comparison of plasma disposition of alkaloids after lupine challenge in cattle that had given birth to calves with lupine-induced arthrogryposis or clinically normal calves. *Am. J. Vet. Res.* **2004**, *65* (11), 1580−1583.

(32) Stuedemann, J. A.; Hill, N. S.; Thompson, F. N.; Fayrer-Hosken, R. A.; Hay, W. P.; Dawe, D. L.; Seman, D. H.; Martin, S. A. Urinary and biliary excretion of ergot alkaloids from steers that grazed endophyte-infected tall fescue. *J. Anim. Sci.* **1998**, *76* (8), 2146−2154.

(33) Son, L.; Kuden, A. B. Dormex and Promalin affects fruit set and earliness of apricot (*Prunus armeniaca*) and plum (*Prunus domestica*) cultivars. *N. Z. J. Crop Hortic. Sci.* **2005**, *33* (1), 59−64.

(34) George, A. P.; Lloyd, J.; Nissen, R. J. Effects of hydrogen cyanamide, paclobutrazol and pruning date on dormancy release of the low chill peach cultivar, Flordaprince, in subtropical Australia. *Aust. J. Exp. Agric.* **1992**, *32* (1), 89−95.

(35) Kamo, T.; Hiradate, S.; Fujii, Y. First isolation of natural cyanamide as a possible allelochemical from hairy vetch *Vicia villosa*. *J. Chem. Ecol.* **2003**, *29* (2), 275−283.

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