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Effect of Dietary Nitrogen Content on the Urine Metabolite Profile of Dairy Cows Assessed by Nuclear Magnetic Resonance (NMR)-Based Metabolomics

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Supporting Information

ABSTRACT: NMR-based metabolomics was applied on urine samples from 32 cows that were fed four levels of crude protein (124, 135, 151, and 166 g/kg DM, respectively) in a crossover design with the aim of identifying urinary metabolites related to nitrogen intake and nitrogen efficiency. Principal component analysis (PCA) on selected regions of the obtained ^{1}H NMR spectra revealed an effect of crude protein intake on NMR signals in the 0.5−3.0 and 5.0−10.0 ppm regions. Partial least-squares (PLS) regressions confirmed a correlation between the NMR metabolite profile and both nitrogen intake and efficiency. The NMR signals that correlated with nitrogen intake and efficiency included urea, hippurate, phenylacetylglutamine, and *p*-cresol sulfate, which all contributed to the prediction of nitrogen intake and efficiency. Thus, it was not possible to identify a single metabolite that could be used as a marker to predict nitrogen efficiency, and it can be concluded that a wide-ranging urinary metabolite profile is needed to evaluate nitrogen efficiency in ruminants.

KEYWORDS: nitrogen efficiency, *p*-cresol sulfate, nuclear magnetic resonance, bovine urine, crude protein, protein marker

INTRODUCTION

Efficiency of nitrogen utilization in ruminants is low (around 25%) and highly variable (10−40%) compared with the higher efficiency of other production animals.[1] The low efficiency has implications for production performance and the environment. Consequently, there is a great interest in improving nitrogen utilization in dairy cows. This may be achieved through a better understanding of nitrogen requirements and metabolism. However, a prerequisite for optimizing nitrogen utilization in diary cows is an efficient tool for the assessment of nitrogen utilization. Therefore, establishment of biomarkers related to nitrogen efficiency would be valuable, as it would facilitate a fast and reliable method to determine how different factors such as feeding strategy influence nitrogen utilization.

Nuclear magnetic resonance (NMR)-based metabolomics has been proven to be a very useful tool for elucidating how diet influences the urine metabolite profile in dietary intervention studies. Thus, by using NMR-based metabolomics it was shown that protein source (meat protein versus milk protein) had an impact on creatinine and hippurate excretion in young boys,[2] and it has also been demonstrated that the technique could discriminate between vegetarians and lactovegetarians on the basis of the urine metabolite profile.[3] In pigs NMR-based metabolomics has been successfully applied to study the effects of arginine supplementation[4] and a whole grain diet on the urine metabolite profile,[5] and it has been suggested that betaine could be a potential marker for intake of whole grain rye.[6] Proton NMR spectroscopy enables a simultaneous detection of multiple metabolites in a high-throughput analysis of biofluids without any labor-demanding sample preparation. This fact together with an excellent repeatability[7−9] makes the technique an attractive tool useful for screening and identifying biomarkers in biofluids.

It is well-known that the level of dietary nitrogen affects the urinary excretion of urea and purines in dairy cows[10,11] and thereby the urinary metabolite profile. However, most studies investigating the effect of dietary nitrogen in diary cows have employed targeted analyses, which means that the results are limited to a discrete number of metabolites that are expected to be influenced by dietary nitrogen level. The aim of the present study is to investigate if an untargeted NMR-based metabolomic approach could identify urinary markers related to dietary nitrogen in dairy cows fed four different levels of crude protein in a crossover design.

MATERIALS AND METHODS

Animals, Feeding, and Sampling. The study included 32 Danish Holstein cows. The cows were randomly allocated to four different experimental diets composed of maize silage, grass/clover silage, barley (rolled), and soybean meal (decortic) in a full crossover design with 21 day periods. Thus, all 32 cows were fed the four different experimental diets in four different periods. In the 21 day periods, the cows were fed the experimental diets for the first 14 days, and during the last 7 days, a wash-out diet was provided. The dietary treatments consisted of feeding the cows with four different levels of nitrogen corresponding to 124, 135, 151, and 166 g crude protein/kg DM, respectively. Diets were offered ad libitum and fed in three equally sized

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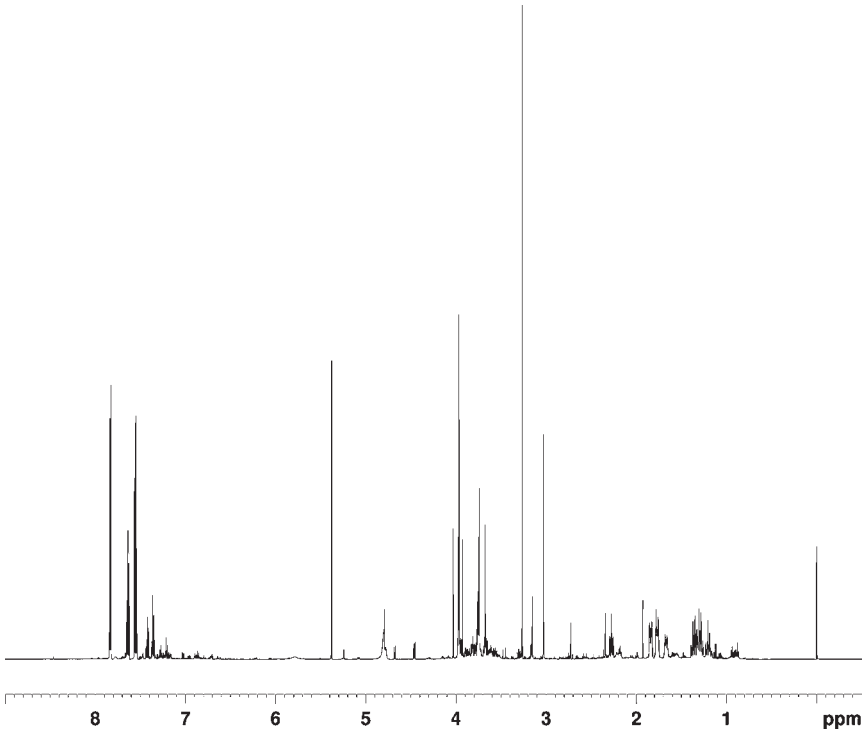

Figure 1. Representative 600 MHz ^{1}H NMR spectrum obtained from a bovine urine sample from a cow fed 135 g crude protein/kg DM for a 14-day period prior to sampling.

portions daily. Urine samples were collected on day 14 of each period by stimulating the cow to urinate by hand sweeping the supramammary region, resulting in a total of 128 urine samples, which were subjected to NMR analysis and subsequently multivariate data analysis.

Efficiency of nitrogen utilization was determined as (g N in milk/g N intake) × 100%.

NMR Spectroscopy. The NMR measurements were performed at 298 K on a Bruker Avance III 600 spectrometer, operating at a ^{1}H frequency of 600.13 MHz and equipped with a 5 mm ^{1}H TXI probe (Bruker BioSpin, Rheinstetten, Germany). Prior to the measurements, urine samples were thawed and 400 μL aliquots were mixed with 200 μL of D_2O containing 0.05% w/w TSP. ^{1}H NMR spectra were obtained using a single 90° pulse experiment, and solvent suppression was achieved by irradiating the solvent peak during the relaxation delay of 5 s. A total of 64 transients of 16K data points spanning a width 12.15 ppm were collected. All spectra were referenced to the TSP signal at 0 ppm.

In addition, to aid assignment two-dimensional (2D) ^{1}H−^{1}H correlation (COSY), 2D ^{1}H−^{1}H total correlation (TOCSY), 2D ^{1}H−^{1}H NOESY, and 2D ^{1}H−^{13}C HSCQ spectra were recorded on selected samples (see the Supporting Information). The TOCSY spectra were acquired with a width of 6250 Hz in both dimensions, 4K data points, 512 increments with 32 transients per increment, and a 60 ms spinlock period. The NOESY spectra were acquired with a size and number of data points similar to that of the TOCSY and a mixing time of 800 ms. The HSCQ spectra were acquired with widths of 6250 Hz in the F2 dimension and 21128 Hz in the F1 dimension, a data matrix with a size of 2048 × 512 data points, and 64 transients per increment.

Postprocessing and Multivariate Data Analysis. NMR spectra were aligned using the icoshift procedure[12] on 15 manually selected intervals, and subsequently the ^{1}H NMR spectra were divided into 0.0074 ppm integral regions and integrated in the region 0.5−10.0 ppm. The reduced spectra consisting of 1466 independent variables were normalized to the TSP standard. Multivariate data analysis was performed using Unscrambler software, version 9.2 (Camo, Oslo, Norway). Principal component analysis (PCA) was applied to the centered data to explore any clustering behavior of the samples. In addition, partial least-squares (PLS) regressions were performed with the NMR variables as X and content of crude protein in the diet as well as calculated nitrogen efficiency as y-variables. During PLS regressions, Martens' uncertainty test[13] was used to eliminate noisy variables. All models were validated by full cross-validation.

LC-MS Analysis. Urine samples for LC-MS analysis were thawed, and 100 μL was transferred to a new eppendorf tube and immediately acidified by the addition of 20 μL of 10% formic acid. The samples were centrifuged for 20 min at 20000g, and the supernatant was collected and diluted by 25 times with 10 mM sodium formate solution to facilitate the later recalibration. The LC-MS analyses were performed on a Micro-OTOF (Bruker, Bremen, Germany) coupled with an Agilent 1200 series capillary HPLC (Agilent, Santa Clara, CA). The HPLC was operated at a flow rate of 15 μL/min, and the mobile phases consisted of A (water/formic acid, 99.9:0.1 (v/v)) and B (acetonitrile/formic acid, 99.9:0.1 (v/v)). For each analysis, 2.5 μL of a diluted cow urine sample was loaded to a Luna C8 column, 3 μm, 100 Å, 150 mm × 0.5 mm i.d. (Phenomenex, Torrance, CA) and sequentially eluted at 30 °C using the following gradient: linear gradient of 20−80% B in 16 min, 80−100% B in 4 min, 100% B for 4 min, 100−20% B in 2 min, and 20% B for 9 min. The MS was operated in the negative mode, and the data were collected in the profile format at a frequency of 1 Hz with Active Focus mode off. The m/z range was set as 80−800, and other optimized mass parameters were as follows: end plate offset, −500 V; capillary voltage, +3400 V; nebulizer pressure, 0.4 bar; dry gas flow, 4.0 L/min; dry temperature, 180 °C; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; ISCID energy, 0 eV; hexapole RF, 100 Vpp; quadrupole ion energy, 5.0 eV; collision energy, 6.0 eV; collision RF, 60 Vpp; transfer time, 98.4 s; prepulse storage, 1.0 s. All reagents used were of LC-MS grade purchased from Sigma-Aldrich. Pure water was obtained from an SG ultrapure water system (SG Water, Barsbuttel Germany).

Table 1. Resonance Assignments for Bovine Urine

^{1}H (ppm)	multiplicity[a]	^{13}C (ppm)	assignments
8.46	s		formate, CH
7.83	d	127.2	hippurate, CH
7.64	t	132.2	hippurate, CH
7.54	t	128.8	hippurate, CH
7.43	m	128.9	phenylacetylglutamine/ phenylacetylglycine, CH
7.37	m	129.4, 127.3	phenylacetylglutamine/ phenylacetylglycine, CH
7.29	m	130.0	*p*-cresol sulfate, CH
7.22	m	128.8	*p*-cresol sulfate, CH
7.20	d	128.7	tyrosine, CH
6.88	d	116.0	tyrosine, CH
5.80	s		urea, NH_2
5.38	s	63.9	allantoin, CH
4.46	m	103.0	β-galactose, CH
4.03	s	56.7	creatinine, CH_2
3.97	d	44.8	hippurate, CH_2
3.93	s	68.6	creatine, CH_2
3.75	m	44.5	phenylacetylglutamine, CH_2
3.67	s	42.9	phenylacetylglutamine, CH_2
3.45	s	44.2	Glycine, CH_2
3.26	s	60.1	trimethylamine-*N*-oxide (TMAO), $N(CH_3)_3$
3.15	s	41.6	unidentified
3.03	s	37.1	creatinine, CH_3
3.03	s	30.4	creatine, CH_3
2.72	s		unidentified
2.66	q		propionate, CH_2
2.34	s	19.9	*p*-cresol sulfate, CH_3
2.28	m	45.4	unidentified
1.93	s		acetate, CH_3
1.84	m	29.1	lysine, CH_2
1.76	m	26.0	lysine, CH_2
1.48	d	17.0	alanine, CH_3
1.21	t	15.8	propionate, CH_3
1.12	d	30.3	unidentified

[a] Symbols for multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

The raw data were calibrated by DataAnalysis software (Bruker) using the cluster signals from sodium formate. The metabolite formula was determined by SmartFormula software on the basis of high mass accuracy (<2 mDa) and well-matched isotopic pattern (mSigma Score < 10).

RESULTS AND DISCUSSION

Optimization of nitrogen utilization in ruminants imposes an efficient tool for assessment of nitrogen utilization. Therefore, establishment of biomarkers related to nitrogen efficiency could be valuable, as it would be useful in the elucidation of how different factors such as feeding strategy influence nitrogen utilization. In the present study ^{1}H NMR-based metabolomics was applied on urine samples from dairy cows fed four different levels of crude protein with the aim of elucidating the relationship between the urine metabolite profile and nitrogen intake and efficiency. A typical ^{1}H NMR spectrum obtained from a urine sample from a cow fed 135 g crude proten/kg DM is seen in Figure 1. Several signals are observed, which have been assigned to various metabolites on the basis of the existing literature,[14−16] the human metabolome database,[17] and 2D NMR experiments (Table 1).

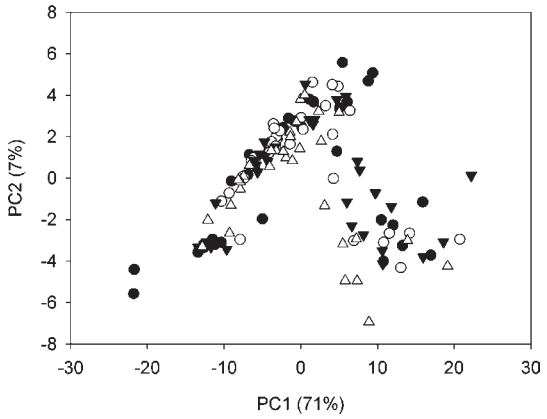

Figure 2. PCA score scatter plot showing the two first principal components obtained for NMR spectra (10.0−5.0 and 4.6−0.5 ppm) acquired on urine samples collected from cows fed four different levels of crude protein: (●) 124 g/kg DM; (○) 135 g/kg DM; (▼) 151 g/kg DM; (△) 166 g/kg DM.

PCA. To elucidate possible similarities and dissimilarities among the different feeding regimens, PCA was carried out on NMR data including all variables in the regions 0.5−4.6 and 5.0−10.0 ppm. No clear grouping of samples according to feeding was observed in the score plots (Figure 2). Therefore, PCA was also carried out on parts of the NMR metabolite profile including only variables in the aromatic region (5.0−10.0 ppm), including only variables in the sugar region (3.0−4.0 ppm) and including only variables in the low ppm region (0.5−2.9 ppm) (Figure 3B,D,F). Score scatter plots of the first two PCs showed grouping according to feeding regimen along PC2 for PCA based on the aromatic region (Figure 3A) and the low ppm region (Figure 3E), whereas no grouping could be identified for PCA based on variables in the 3−4 ppm region (Figure 3C). Analysis of the corresponding PC2 loadings reveals that grouping of feeding regimen in the aromatic region can be ascribed to a higher level of urea (broad peak at 5.8 ppm), *p*-cresol sulfate (peaks at 7.22 and 7.29 ppm), and phenylacetylglutamine (peaks at 7.37 and 7.43 ppm) together with a lower level of hippurate (peaks at 7.55, 7.64, and 7.83 ppm) in cows fed a high level of crude protein (Figure 3B). For the 0.5−2.9 ppm region, PC2 loadings reveal that grouping of feeding regimen in the aromatic region mainly can be ascribed to higher intensity of a singlet at 2.34 ppm that has been assigned to *p*-cresol sulfate (Figure 3F).

Correlation between NMR Urine Metabolite Profiles and Nitrogen Intake and Efficiency. To investigate the ability of the NMR metabolite profile to predict feeding regimen, a PLS regression was carried out with NMR variables as *X* and crude protein as *y*, which revealed a relative high correlation ($R = 0.80$) (Figure 4A), implying that the NMR urine metabolite profile could explain approximately 64% of the variation in crude protein intake. The regression coefficients, which show the NMR signals contributing to the prediction of crude protein, include a substantial contribution from *p*-cresol sulfate signals at 2.34, ∼7.22, and ∼7.29 ppm, glycine at 3.68 ppm, urea at 5.80 ppm,

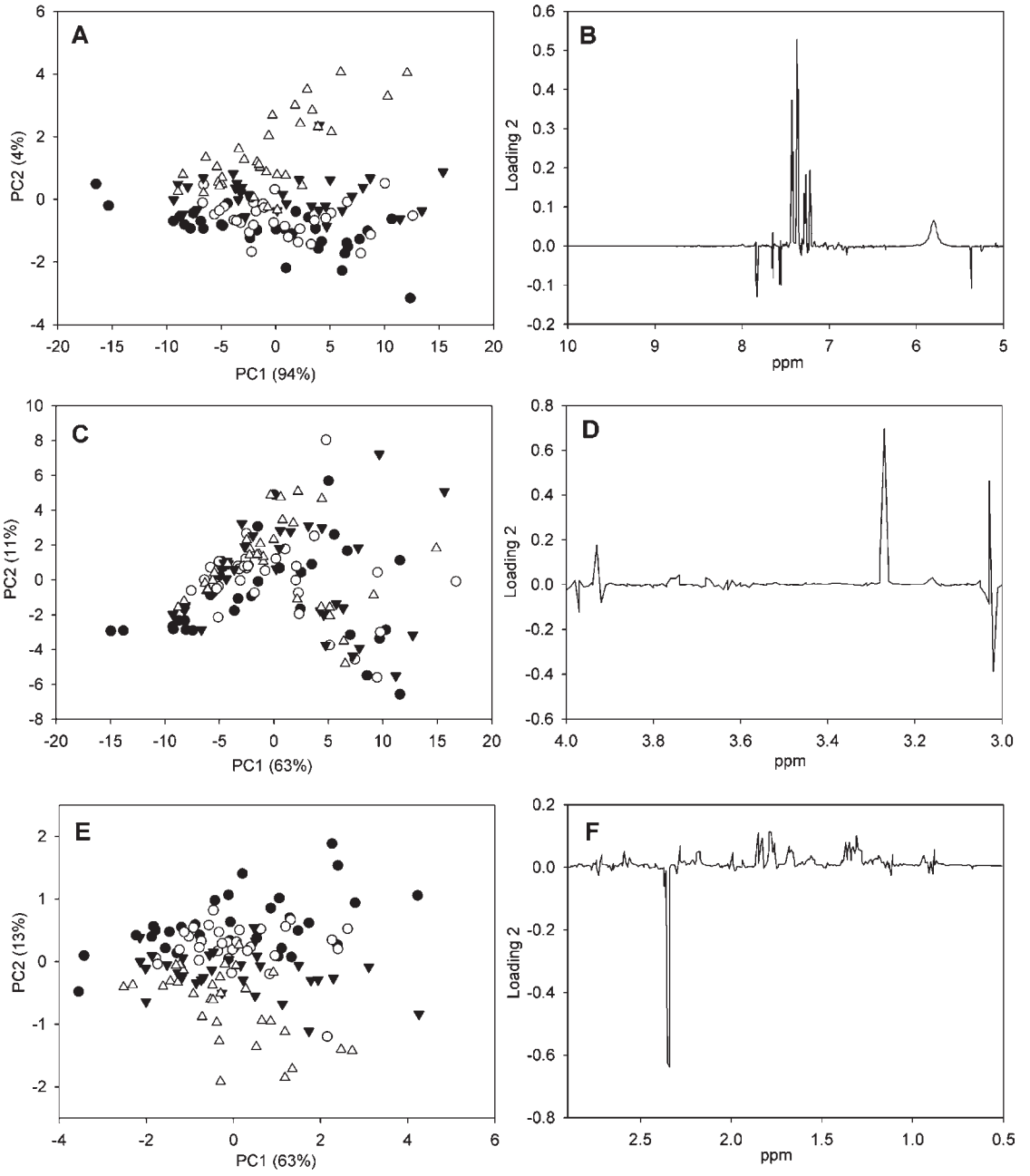

Figure 3. PCA score scatter plot showing the two first principal components obtained for NMR variables in (A) the 10.0−5.0 ppm region, (C) the 4.0−3.0 ppm region, and (E) the 2.9−0.5 ppm region. Symbols indicate crude protein intake: (●) 124 g/kg DM; (○) 135 g/kg DM; (▼) 151 g/kg DM; (△) 166 g/kg DM. Panels B, D, and F show loading plots of the corresponding second principal component.

and hippurate signals at ∼7.55 and ∼7.83 ppm, which are all positively correlated with crude protein intake (Figure 4B). In addition, the regression coefficients contain considerable contribution from creatinine at 3.04 ppm and allantoin at 5.37 ppm, which are negatively correlated with crude protein intake (Figure 4B). Nitrogen efficiency was calculated, and PLS regression was also carried out with NMR variables as *X* and nitrogen efficiency as *y*, which resulted in relatively high correlation (R = 0.74) (Figure 5A). To the authors' knowledge, the present study is the first to report the use of NMR metabolite profiling for the prediction of nitrogen efficiency in ruminants, and a correlation of R = 0.74 must be considered promising. However, the present data are based on a controlled experimental design, and further studies are needed to elucidate if a correlation can also be established when animals are fed a broader and more heterogeneous range of feed than the experimental diets investigated here.

Even though proton NMR spectroscopy can be considered a high-throughput analysis when compared to many other analytical techniques, identification of specific biomarkers for nitrogen efficiency that may be determined by faster assays would assist in the use of biomarkers for nitrogen efficiency evaluation. It is therefore of interest to elucidate the signals in the NMR metabolite fingerprints that seem to be related to nitrogen efficiency. The regression coefficients reveal a positive contribution from multiple signals at ∼1.0−2.5 ppm (Figure 5B). All of

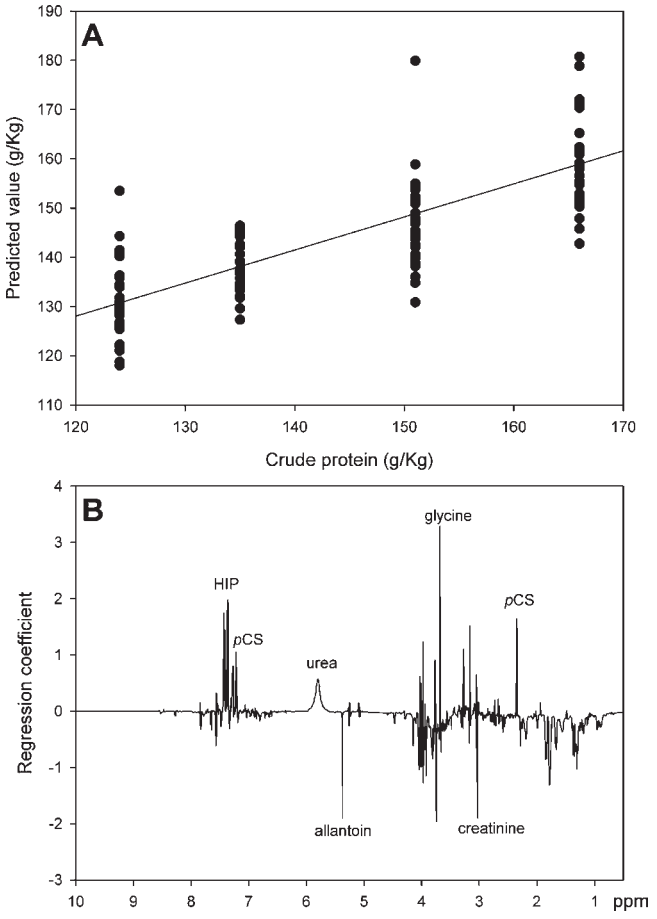

Figure 4. (A) Predicted crude protein versus actual crude protein (g/kg DM) obtained from PLS regression with NMR urine spectra as *X* and crude protein (g/kg DM) as *y* (n = 128). (B) Corresponding regression coefficients, which reveal the NMR signals used in the prediction of crude protein. The most influential signals have been assigned (HIP, hippurate; *p*CS, *p*-cresol sulfate).

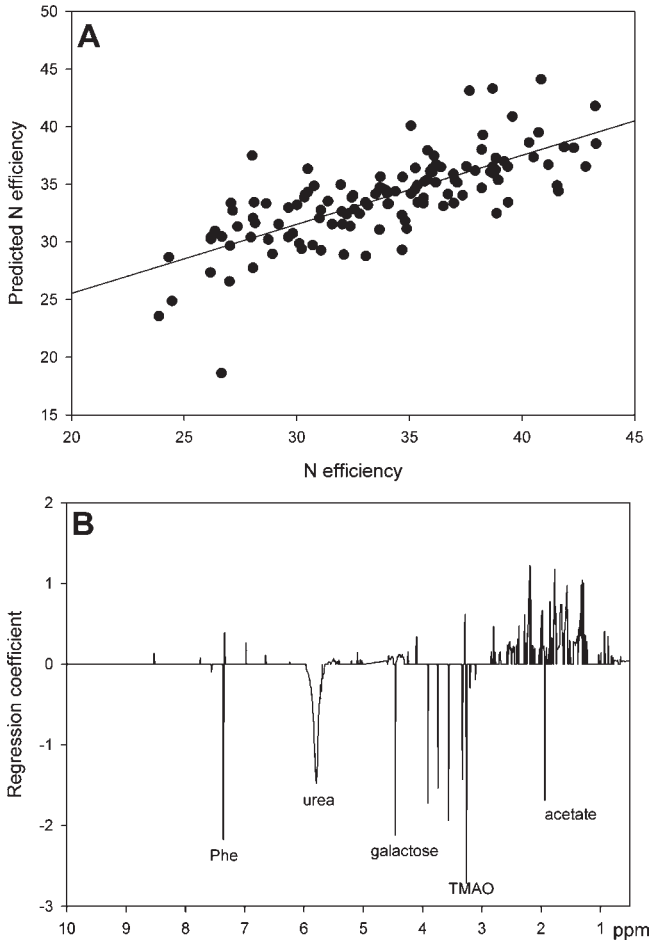

Figure 5. (A) Predicted nitrogen efficiency versus nitrogen efficiency (%) obtained from PLS regression with NMR urine spectra as *X* and nitrogen efficiency (%) as *y* (n = 128). (B) Corresponding regression coefficients, which reveal the NMR signals used in the prediction of crude protein. The most influential signals have been assigned (Phe, phenylalanine/phenylacetylglutamine; TMAO, trimethylamine-*N*-oxide).

these signals have not been identified, but probably include different amino acids including glutamate and various branched-chain amino acids. In addition, the regression coefficients reveal a negative contribution from acetate at 1.95 ppm, from urea at 5.80 ppm, and signals at 3.26, 4.46, and 7.37 ppm, which probably can be assigned to trimethylamine-*N*-oxide (TMAO), β-galactose, and phenylacetylglutamine, respectively (Figure 5B). The strong signal from urea can be anticipated because urea is an end product of protein catabolism, and it is well-known that the level of dietary nitrogen affects the urinary excretion of urea and purines in dairy cows.[10,11] The present study also showed that allantoin and nitrogen efficiency were negatively correlated. Allantoin is a product of purine metabolism, and a strong correlation between urinary excretion of purine metabolites and allantoin has been reported.[18] Urinary purine metabolite excretion seems to be an indicator of microbial protein supply in ruminants.[19,20] Some evidence suggests that a proportion of purines are degraded to xanthines and other purine derivatives that will not be reused and will therefore represent an irreversible loss of N,[21] which is in agreement with the negative correlation between allantoin and nitrogen efficiency found in the present study.

Urinary TMAO was likewise found to be negatively correlated with nitrogen efficiency, which probably also can be explained by the fact that TMAO is involved in protein catabolism. A previous metabolomics study on humans reported that a meat-rich diet was associated with increased urinary TMAO compared with a vegetarian diet,[3] which also indicated that TMAO was a marker for high protein intake and protein catabolism. TMAO also has often been associated with fish intake,[2,22] and it remains unknown if TMAO is a general protein marker or if it is related to the catabolism of specific proteins.

Identification of Specific NMR Signals. Noticeably, a singlet at 2.34 ppm appeared to be important for the discrimination of samples in both PCA and PLS regressions. Two-dimensional NMR experiments revealed that this singlet originates from a molecule that also has two signals present as an AABB system at 7.22 and 7.29 ppm in the aromatic region and that the three signals have ^{13}C chemical shift values of 19.9, 128.8, and 130.0 ppm, respectively. Accordingly, a possible assignment is *p*-cresol or *p*-cresol sulfate, for which similar chemical shift values have been reported according to HMDB.[17] The 7.22 and 7.29 ppm signals have previously also been reported in human urine[23] as well as sheep urine where they were tentatively assigned to *p*-cresol sulfate.[16] Unfortunately, the assignment cannot be verified by spiking as *p*-cresol sulfate is not stable and therefore

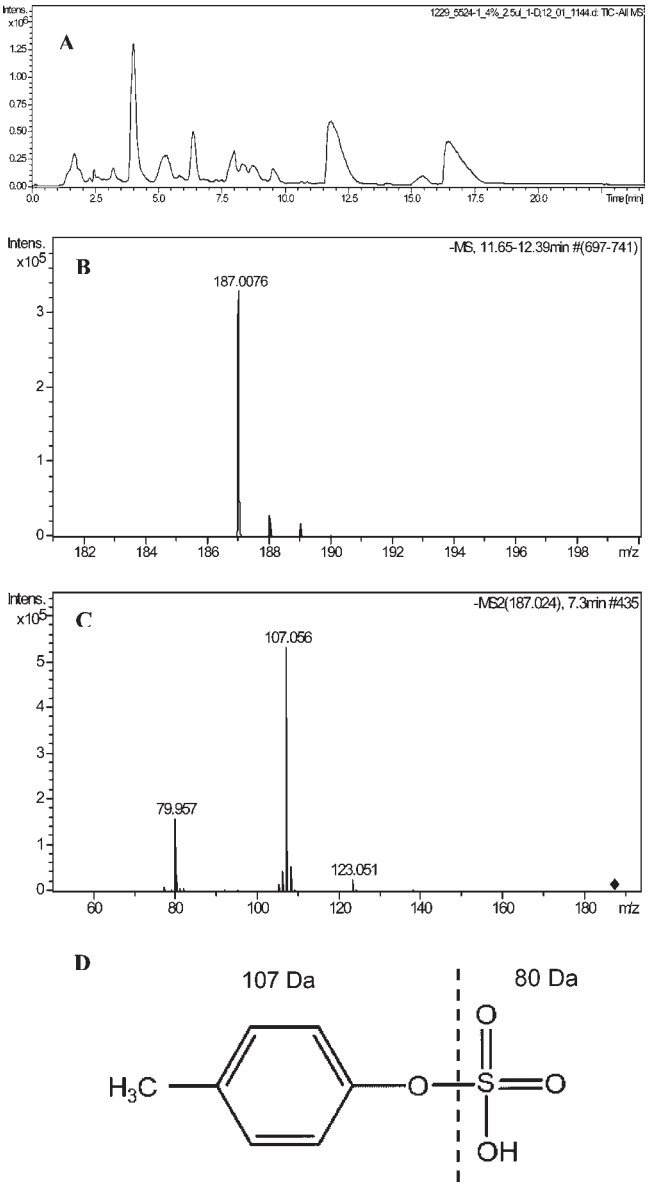

Figure 6. Identification of *p*-cresol sulfate by LC-MS and MS/MS in negative mode: (A) BPC chromatogram obtained on a representative urine sample; (B) MS spectrum acquired at 12 min (the molecular formula of this compound (m/z 187.0076) was determined as $C_7H_7O_4S$ with an error of 0.5 mDa and mSigma value of 1.5 according to SmartFormula software (Bruker, Bremen, Germany)); (C) MS/MS spectrum of the ion m/z 187.0076; (D) molecular structure of the detected compound.

not available as a pure reagent. Therefore, to obtain evidence for this assignment, LC-MS was carried out on selected urine samples from two cows fed the four different levels of nitrogen (n = 8), which revealed the presence of a compound with m/z 187 and a fragmentation pattern (Figure 6) that is identical to that of *p*-cresol sulfate.[24] Quantification of this compound detected by LC-MS was strongly correlated with the integrals of the NMR signals at 2.34 ppm (R^2 = 0.96) and 7.20−7.30 ppm (R^2 = 0.80), and increases in the intensity of the LC-MS peak representing *p*-cresol sulfate also increased with increasing crude protein intake (Table 2). Consequently, it seems reasonable to suggest that the NMR signals at 2.34, 7.22, and 7.29 ppm should

Table 2. Relative Amount of *p*-Cresol Sulfate Determined by LC-MS Analysis

crude protein intake (g/kg DM)	intensity of *p*-cresol sulfate peak[a]
124	130.3 ± 0.6
135	163.0 ± 10.1
151	189.4 ± 26.6
166	232.5 ± 11.2

[a] Reported values are mean values ± standard deviations obtained from urine samples of two cows.

Table 3. Squared Pearson Correlation Coefficients (R^2) for the Correlation between Relative Amount of Selected Metabolites in Cow Urine Determined by Integration of ^{1}H NMR Signals and Calculated Nitrogen Efficiency

chemical shift (ppm)	assignment	R^2 N efficiency
1.95	acetate	0.13
5.40	allantoin	0.11
5.80	urea	0.27
5.85	uridine	0.17
7.36	phenylalanine/phenylacetylglutamine	0.05
7.43	phenylalanine/phenylacetylglutamine	0.05

be assigned to *p*-cresol sulfate. Noticeably, in a human study in which a standard diet was compared with a protein-rich diet, a significant increase in urinary excretion of *p*-cresol was observed,[23] which in agreement with the present findings suggests that urinary *p*-cresol and/or *p*-cresol sulfate levels could be a marker for protein intake and potentially also for nitrogen utilization. *p*-Cresol sulfate is a microbial metabolite and probably derives from secondary metabolism of *p*-cresol, and it has been suggested that the most likely origin of *p*-cresol are tyrosine residues in proteins, which through the action of colonic bacteria generate volatile phenolic compounds excreted in the urine.[23] *p*-Cresol sulfate is probably derived from tyrosine sulfate; in humans at least four cytosolic sulfotransferases have been identified.[25]

NMR peaks at 7.37 and 7.43 ppm were also found to be correlated with nitrogen intake and nitrogen efficiency. On the basis of the existing literature[14,15] and the human metabolome database,[17] these peaks may potentially be assigned to either phenylalanine, phenylacetylglycine, or phenylacetylglutamine. On the basis of 2D NMR experiments, it was not possible to determine if these signals should be ascribed to phenylalanine, phenylacetylglycine, or phenylacetylglutamine, as only weak cross-peaks were observed. LC-MS analysis detected phenylacetylglycine and phenylacetylglutamine in the urine, whereas phenylalanine was not detected by the applied LC-MS analysis. Phenylacetylglutamine is produced from phenylalanine, and phenylalanine is a precursor for tyrosine. Thus, if tyrosine is the origin of *p*-cresol sulfate, the relationship between nitrogen efficiency and urinary phenylacetylglutamine/phenylalanine and *p*-cresol sulfate levels is probably linked.

Contribution from Individual Metabolites. To investigate the correlation between individual metabolites and nitrogen efficiency, the relative amounts of selected metabolites were determined by integration of the NMR peaks, and results are listed in Table 3. The highest correlation was obtained for urea, which could explain 27% of the variation in nitrogen efficiency

(R^2 = 0.27), and rather low correlations were obtained for the remaining metabolites.

In conclusion, the present study demonstrates a correlation between the NMR urinary metabolite profile of dairy cows and nitrogen intake as well as nitrogen efficiency. Several NMR signals contributed to the prediction of nitrogen intake and efficiency, and it was not possible to identify a single metabolite that could be used as a marker to predict nitrogen efficiency. It can therefore be concluded that a wide-ranging urinary metabolite profile is needed to evaluate nitrogen efficiency in ruminants.

■ ASSOCIATED CONTENT

Supporting Information. Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Calsamiglia, S.; Ferret, A.; Reynolds, C. K.; Kristensen, N. B.; van Vuuren, A. M. Strategies for optimizing nitrogen use by ruminants. *Animal* **2010**, *4*, 1184–1196.

(2) Bertram, H. C.; Hoppe, C.; Petersen, B. O.; Duus, J. Ø.; Mølgaard, C.; Michaelsen, K. F. An NMR-based metabonomic investigation on effects of milk and meat protein diets given to 8-year-old boys. *Br. J. Nutr.* **2007**, *97*, 758–763.

(3) Stella, C.; Beckwith-Hall, B.; Cloarec, O.; et al. Susceptibility of human metabolic phenotypes to dietary modulation. *J. Proteome Res.* **2006**, *5*, 2780–2788.

(4) He, Q. H.; Kong, X. F.; Wu, G. Y.; Ren, P. P.; Tang, H. R.; Hao, F. H.; Huang, R. L.; Li, T. J.; Tan, B.; Li, P.; Tang, Z. R.; Yin, Y. L.; Wu, Y. N. Metabolomic analysis of the response of growing pigs to dietary L-arginine supplementation. *Amino Acids* **2009**, *37*, 199–208.

(5) Bertram, H. C.; Bach Knudsen, K. E.; Serena, A.; Malmendal, A.; Nielsen, N. C.; Fretté, X. C.; Andersen, H. J. NMR-based metabonomic studies reveal changes in the biochemical profile of plasma and urine from pigs fed high-fibre rye bread. *Br. J. Nutr.* **2006**, *95*, 955–962.

(6) Bertram, H. C.; Malmendal, A.; Nielsen, N. C.; Straadt, I. K.; Larsen, T.; Bach Knudsen, K. E.; Lærke, H. N. NMR-based metabonomics reveals that plasma betaine increases upon intake of high-fiber rye buns in hypercholesterolemic pigs. *Mol. Nutr. Food Res.* **2009**, *53*, 1055–1062.

(7) Potts, B. C. M.; Deese, A. J.; Stevens, G. J.; Reily, M. D.; Robertson, D. G.; Theiss, J. J. NMR of biofluids and pattern recognition: assessing the impact of NMR parameters on the principal component analysis of urine from rat and mouse. *Pharm. Biomed. Anal* **2001**, *26*, 463–476.

(8) Keun, H. C.; Ebbels, T. M. D.; Antti, H.; Bollard, M. E.; Beckonert, O.; Schlotterbeck, G.; Senn, H.; Niederhauser, U.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. *Chem. Res. Toxicol.* **2002**, *15*, 1380–1386.

(9) Bertram, H. C.; Malmendal, A.; Petersen, B. O.; Madsen, J. C.; Pedersen, H.; Nielsen, N. C.; Hoppe, C.; Mølgaard, C.; Michaelsen, K. F.; Duus, J. Ø. Effect of magnetic field strength on NMR-based metabonomic human urine data — a comparative study of 250, 400, 500 and 800 MHz. *Anal. Chem.* **2007**, *79*, 7110–7115.

(10) Kristensen, N. B.; Storm, A. C.; Larsen, M. Effect of dietary nitrogen content and intravenous urea infusion on ruminal and portal-drained visceral extraction of arterial urea in lactating Holstein cows. *J. Dairy Sci.* **2010**, *93*, 2670–2683.

(11) Dewhurst, R. J.; Davies, L. J.; Kim, E. J. Effects of mixtures of red clover and maize silages on the partitioning of dietary nitrogen between milk and urine by dairy cows. *Animal* **2010**, *4*, 732–738.

(12) Savorini, F.; Tomasi, G.; Engelsen, S. B. Icoshift: a versatile tool for the rapid alignment of 1D NMR spectra. *J. Magn. Reson.* **2010**, *202*, 190–202.

(13) Martens, H.; Martens, M. Modified jack-knife estimation of parameter uncertainty in bilinear modelling by partial least squares regression (PLSR). *Food Qual. Preference* **2000**, *11*, 5–16.

(14) Lindon, J. C.; Nicholson, J. K.; Everett, J. R. NMR spectroscopy of biofluids. *Annu. Rep. NMR Spectrosc.* **1999**, *38*, 1–88.

(15) Clayton, T. A.; Baker, D.; Lindon, J. C.; Everett, J. R.; Nicholson, J. K. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 14728–14733.

(16) Nyberg, N. T.; Nielsen, M. O.; Jaroszewski, J. W. Metabolic trajectories based on ^{1}H NMR spectra of urines from sheep exposed to nutritional challenges during prenatal and early postnatal life. *Metabolomics* **2010**, *6*, 489–496.

(17) Wishart, D. S.; Knox, C.; Guo, A. C.; Eisner, R.; Young, N.; Gautam, B.; Hau, D. D.; Psychogios, N.; Dong, E.; Boutra, S.; Mandal, R.; Sinelnikov, I.; Xia, J.; Jia, L.; Cruz, J. A.; Lim, E.; Sobsey, C. A.; Shrivastava, S.; Huang, P.; Liu, P.; Fang, L.; Peng, J.; Fradette, R.; Cheng, D.; Tzur, D.; Clements, M.; Lewis, A.; De Souza, A.; Zuniga, A.; Dawe, M.; Xiong, Y.; Clive, D.; Greiner, R.; Nazyrova, A.; Shaykhutdinov, R.; Li, L.; Vogel, H. J.; Forsythe, I. HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res.* **2009**, *37* (Suppl. 1), D603–D610.

(18) Shingfield, K. J.; Offer, N. W. Evaluation of the spot urine sampling technique to assess urinary purine derivative excretion in lactating dairy cows. *Anim.Sci.* **1998**, *66*, 557–568.

(19) Chen, X. B.; Mejia, A. T.; Kyle, D. J.; Orskov, E. R. Evaluation of the use of purine derivative-creatinine ratio in spot urine and plasma samples as an index of microbial protein supply in ruminants — studies in sheep. *J. Agric. Sci.* **1995**, *125*, 137–143.

(20) Shingfield, K. J. Estimation of microbial protein supply in ruminant animals based on renal and mammary purine excretion. A review. *J. Anim. Feed Sci.* **2000**, *9*, 169–212.

(21) McAllan, A. B. The fate of nucleic acids in ruminants. *Proc. Nutr. Soc.* **1982**, *41*, 309–316.

(22) Lenz, E. M.; Bright, J.; Wilson, I. D.; et al. Metabonomics, dietary influences and cultural differences: a ^{1}H NMR-based study of urine samples obtained from healthy British and Swedish subjects. *J. Pharm. Biomed. Anal.* **2004**, *36*, 841–849.

(23) Geypens, B.; Claus, D.; Evenepoel, P.; Hiele, M.; Maes, B.; Peeters, M.; Rutgeerts, P.; Ghoos, Y. Influence of dietary protein supplements on the formation of bacterial metabolites in the colon. *Gut* **1997**, *41*, 70–76.

(24) Cao, L.; Kirk, M. C.; Coward, L. U.; Jackson, P.; Whitaker, J. N. *p*-cresol sulphate is the dominant component of urinary myelin basic protein like material. *Arch. Biochem. Biophys.* **2000**, *377*, 9–21.

(25) Falany, C. N. Sulfation and sulfotransferases. 3. Enzymology of human cytosolic sulfotransferases. *FASEB J.* **1997**, *11*, 206–216.