DOI:10.1021/jf904321p

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Seasonal Changes in the Metabolic Fingerprint of 21 Grass and Legume Cultivars Studied by Nuclear Magnetic Resonance-Based Metabolomics

Hanne Christine Bertram,*,† Martin Riis Weisbjerg,‡ Christian S. Jensen,§ Morten Greve Pedersen,§ Thomas Didion,§ Bent O. Petersen,# Jens Ø. Duus,# Mette K. Larsen,† and Jacob Holm Nielsen†

†Department of Food Science, Faculty of Agricultural Sciences, University of Aarhus, P.O. Box 102, DK-5792 Årslev, Denmark, ‡Department of Animal Health and Bioscience, Faculty of Agricultural Sciences, University of Aarhus, P.O. Box 50, DK-8830 Tjele, Denmark, §Research Division, DLF-Trifolium A/S, Højerupvej 31, 4660 Store Heddinge, Denmark, and #Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark

A nuclear magnetic resonance (NMR)-based approach was introduced for metabolic fingerprinting of 21 grass and legume cultivars in the present study. Applying principal component analysis (PCA) on the fingerprints obtained on water extracts, it was possible to elucidate the variation between cultivars and the magnitude of changes in the metabolic fingerprint between the spring growth and the second regrowth. Consequently, the potential of the method for tracking differences and changes related to cultivar and season was demonstrated. In addition, partial least-squares (PLS) regressions revealed correlations between the NMR fingerprints and the value of the grasses as animal feed evaluated as concentration of sugars, neutral detergent fibres (NDF) (R = 0.82), indigestible neutral detergent fibres (iNDF) (R = 0.90), and in vitro organic matter digestibility (IVOMD) (R = 0.75). The correlations between these parameters and the NMR fingerprint could mainly be ascribed to differences in spectral intensities from signals assigned to malic acid (2.40 and 4.70 ppm), choline (3.27 ppm), and glucose (5.24 ppm), and the biochemical rationale for this relation is discussed.

KEYWORDS: Proton NMR spectroscopy; harvest; animal feed value; grass; sugars; NDF; iNDF

INTRODUCTION

Metabolic fingerprinting seeks to detect the maximum number of metabolites in a high-throughput analysis. Plants and plant materials contain thousands of low molecular weight metabolites, which are characteristic for the properties of the plant or plant material. Consequently, metabolic profiling is an important tool in plant science. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy has become a powerful technique in the metabolic fingerprinting and profiling of plants (*1*, *2*), which can be ascribed to the many advantages related to the use of ^{1}H NMR. First of all, sample preparation is minimal when compared to other analytical techniques, which provides possibilities for high throughput and makes the technique less discriminatory. Moreover, the reproducibility of NMR is superior compared with many other analytical techniques (*3*−*5*). Finally, the fact that ^{1}H NMR in principle detects all proton-containing low molecular weight metabolites implies that the technique covers a broad range of compound groups.

Seasonal changes in the chemical composition of grass expressed as dry matter, crude protein, and soluble sugar contents have been reported, and these factors are known to affect the value of the grass as animal feed (*6*, *7*) and milk composition in dairy animals (*8*, *9*). However, detailed analyses of cultivar differences and seasonal changes in the metabolite profile of grass are limited. The aims of the present study were to (i) investigate cultivar differences and seasonal changes in the metabolite profile of 18 grass and 3 leguminous cultivars representing a large variety in nutritional value using ^{1}H NMR-based metabolic fingerprinting of aqueous extracts and (ii) explore how these changes are related to the value as animal feed evaluated as concentration of sugars, neutral detergent fibers (NDF), indigestible neutral detergent fibers (iNDF), and in vitro organic matter digestibility (IVOMD).

MATERIALS AND METHODS

Plant Material and Sampling. The experiment was conducted at Bredeløkke, Denmark (55° 20′ N, 12° 23′ E), on a fine Cambisol soil (FAO) containing 23% coarse sand, 39% fine sand, 17% silt, 19% clay, and 1.7% humus, pH 6.8. Twenty-one different forage species representing 13 perennial ryegrasses, 2 festuloliums, 2 hybrid ryegrasses, 1 cocksfoot, 1 lucerne, 1 white clover, and 1 red clover (**Table 1**) were sown in plots (8.0 m × 1.5 m) on September 4, 2007, at a sowing density of recommended values (DLF-Trifolium). Plots were drilled lengthwise with 10 drills per plot 120 mm apart and fertilized at seed sowing with 250 kg ha^{-1}

*Corresponding author [telephone (+45) 89 99 33 44; fax (+45) 89 99 34 94; e-mail HanneC.Bertram@agrsci.dk].

 Published on Web 03/12/2010

Table 1. Forage Types Included in the Investigation

variety name	type	earliness
Kimber	PRG 2n	early
Neptun	PRG 4n	early
Perun	Festulolium 4n	inter
Hykor	Festulolium 6n	inter
Tetratop	hybrid 4n	inter
Butara 1	PRG 2n	inter
Indiana	PRG 2n	inter
Glenstal	PRG 4n	inter
Amos	red clover	inter
Storm	hybrid 4n	late
Asturion	PRG 2n	late
Cancan	PRG 2n	late
Eiffel	PRG 2n	late
Foxtrot	PRG 2n	late
Kentaur	PRG 4n	late
Loporello	PRG 4n	late
Polim	PRG 4n	late
Turandot	PRG 4n	late
Donata	Cocksfoot 4n	inter/late
Riesling	white clover	
Daisy	Lucerne	

(21:3:10:4 $N/P_2O_5/K_2O/SO_3$). During the spring and early summer of 2008 the plots were fertilized using 500, 400, and 350 kg ha^{-1} of the same fertilizer and once with 160 kg ha^{-1} K_2SO_4, equaling a total N treatment of 315 kg ha^{-1} $year^{-1}$. Data obtained in this investigation correspond to measurements based on cuts in the first year (2008) after sowing. The plots were cut on June 2, July 2, and July 29, and samples for NMR measurements were based on the first and third cuts (spring growth and second regrowth, respectively). All cuts were carried out at 6 cm above ground level with a Haldrup plot harvester (Haldrup, Logstor, Denmark). Fresh samples of herbage (between 2 and 4 kg) were chopped and kept below 8 °C until use.

Determination of Water-Soluble Carbohydrates. Samples were dried in a Heto FD3 freeze-dryer (Heto-Holten, Denmark) for 48 h, ground, and passed through a 0.5 mm filter in an MF 10 Basis mill (IKA Werke, Germany). Subsequently, a sample of 500 mg of material was extracted in 25 mL of 100 mM acetate buffer (pH 5.0) at 65 °C for 60 min and centrifuged for 10 min at 7000*g*, and subsequently 2 mL of supernatant was hydrolyzed by the addition of 2 mL of 74 mM H_2SO_4 and incubation at 80 °C for 70 min. Assay mixture was prepared by adding 1 mL of triethanolamine buffer (pH 7.6) (14 v/w % triethanolamine hydrochloride and 0.25 v/w % magnesium sulfate, $7H_2O$), 0.1 mL of ATP solution (4.55 v/w % ATP disodium salt hydrate and 5 v/w % sodium hydrogen carbonate), 0.1 mL of 0.94 v/w % β-NADH phosphate disodium salt, and 0.1 mL of extracted or hydrolyzed sample to a total volume of 3.2 mL. The glucose content was determined by measuring NADPH formation (Hitachi U-1100 spectrophotometer, Japan) at 340 nm after incubation with 6.8 units of hexokinase (HK)/3.4 units of glucose-6-phosphate dehydrogenase (G6-PDH) (Roche, Germany) for 15 min (*10*, *11*). Total hexose (glucose plus fructose) was determined by adding 0.7 unit of phosphor glucose isomerase (PGI, Roche, Germany) to the previous reaction, and formation of NADPH was measured after 15 min. The content of glucose and fructose in the extraction sample is defined as glucose and fructose, whereas the increase in content of glucose and fructose after hydrolysis is defined as sucrose and fructan. Each sample was extracted twice, and as a reference for the extraction, hydrolysis, and enzymes used for measurements, a soy sample was included.

Determination of Feed Value Parameters. Samples were dried for 48 h at 60 °C and milled on a 1 mm screen before analysis for neutral detergent fibers (NDF) and in vitro organic matter digestibility (IVOMD) and on 1.5 mm screen for indigestible neutral detergent fibers (iNDF) analysis. Ash-free neutral detergent fiber (NDF) was analyzed using a Fibertec according to the method of Mertens (*12*). iNDF was determined in situ as the NDF residue after 288 h of rumen incubation in 12 μm dacron bags. IVOMD was determined using the two-step rumen fluid method described by Tilley and Terry (*13*).

NMR Measurements. Samples for NMR analyses were snap-frozen in liquid nitrogen, homogenized using a "Minihakker", and stored at −80 °C. Prior to NMR analyses the samples were thawed, and 100 mg of sample was dissolved in D_2O containing sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate (TSP) added as an internal standard. Five replicates of each sample type were prepared. The NMR measurements were performed at 298 K on a Bruker Avance III 600 spectrometer, operating at a ^1H frequency of 600.13 MHz and equipped with a 5 mm ^1H TXI probe (Bruker BioSpin, Rheinstetten, Germany). Standard one-dimensional spectra were acquired using a single 90° pulse experiment with a relaxation decay of 5 s. Water suppression was achieved by irradiating the water peak during the relaxation delay, and 16K data points spanning a spectral width of 12.15 ppm were collected. All spectra were referenced and normalized to the TSP signal at 0 ppm. In addition, to aid spectral assignment 2D $^1H-^1H$ correlation (COSY), 2D $^1H-^1H$ total correlation (TOCSY), 2D $^1H-^1H$ NOESY, and 2D $^1H-^{13}C$ HSCQ spectra were recorded on selected samples. The TOCSY spectra were acquired with a spectral width of 6250 Hz in both dimensions, 4K data points, 512 increments with 32 transients per increment, and an 80 ms spinlock period. The NOESY spectra were acquired with a size and number of data points similar to those of the TOCSY and a mixing time of 600 ms. The HSCQ spectra were acquired with a spectral width of 6250 Hz in the F2 dimension and 21128 Hz in the F1 dimension, a data matrix with a size of 4096 × 512 data points, and 64 transients per increment.

Postprocessing and Multivariate Data Analysis. The spectra were subdivided into 0.007 ppm integral regions and integrated, reducing each spectrum into 1243 independent variables in the regions of 0.5−4.6 and 4.9−10.0 ppm. Further 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, PLS regressions were used for prediction of soluble carbohydrate concentrations, iNDF, NDF, and IVOMD using NMR spectra as *X* variables. During all regressions, Martens' uncertainty test (*14*) was used to eliminate noisy variables, and all models were validated using full cross-validation including the five replicates in the same segment during cross-validation (*15*).

RESULTS

Typical ^1H NMR metabolic fingerprints obtained on water extracts of a grass and a leguminous cultivar are shown in **Figures 1** and **2**. A multitude of signals are detected, and through detailed analysis some of these have been tentatively assigned. The aliphatic region 0.5−3.0 ppm is dominated by signals from amino acids and organic acids. In leguminous cultivars additional singlets at 1.35, 1.65, 1.70, 1.92, and 2.44 ppm are observed, which most probably should be assigned to methyl protons attached to carbonyls or tertiary carbons. In the region 3.0−4.3 ppm numerous peaks from various carbohydrates including fructose are observed, and at 4.63, 5.23, and 5.42 ppm characteristic signals from β-glucose, α-glucose, and sucrose, respectively, appear (**Figure 2**). Visual inspection of the spectra reveals higher intensities of fructose signals in the 3.0−4.3 ppm region in grass cultivars compared to leguminous cultivars. To investigate the detailed differences in the metabolic fingerprints between different cultivars and between the two growths, PCA was carried out. A scatter plot of score one versus score two for mean-centered data (**Figure 3A**) is dominated by a clear clustering into grass and leguminous cultivars, and analysis of the loadings (**Figure 3B**) reveals that the separation into grass and leguminous cultivars can be ascribed to differences in the 3−4.3 ppm region representing various carbohydrates. In addition, a higher intensity of a signal at 3.60 ppm in leguminous cultivars compared with grass cultivars is also crucial in the separation of the different cultivars, and intensities of this signal were determined to examine the quantitative difference between cultivars (**Figure 3C**). On the basis of the NMR 2D experiments the signal could be ascribed to 3-*O*-methyl-β-D-glucose (*16*). In addition, within each of the grass and leguminous cultivar groups there is a separation into first and

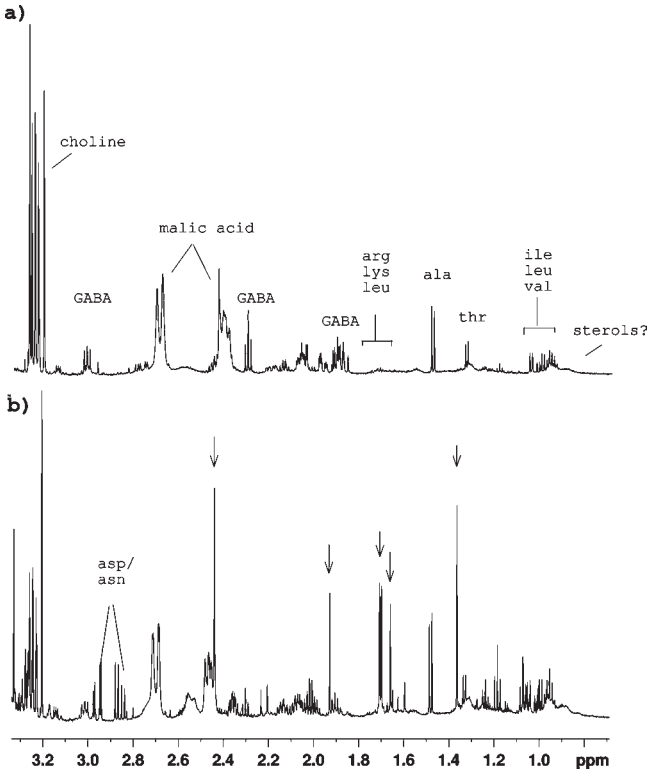

Figure 1. Expansion of a representative ^{1}H NMR spectrum obtained on extracts of (**a**) Foxtrot (late perennial ryegrass) and (**b**) Riesling (white clover). Arrows indicate peaks only found in leguminous cultivars, which tentatively have been assigned to methyl protons attached to carbonyls or tertiary carbons. arg, arginine; ala, alanine; asp, aspartate; asn, asparagine; GABA, γ-aminobutyric acid; ile, isoleucin; leu, leucine; lys, lysine; thr, threonine; val, valine.

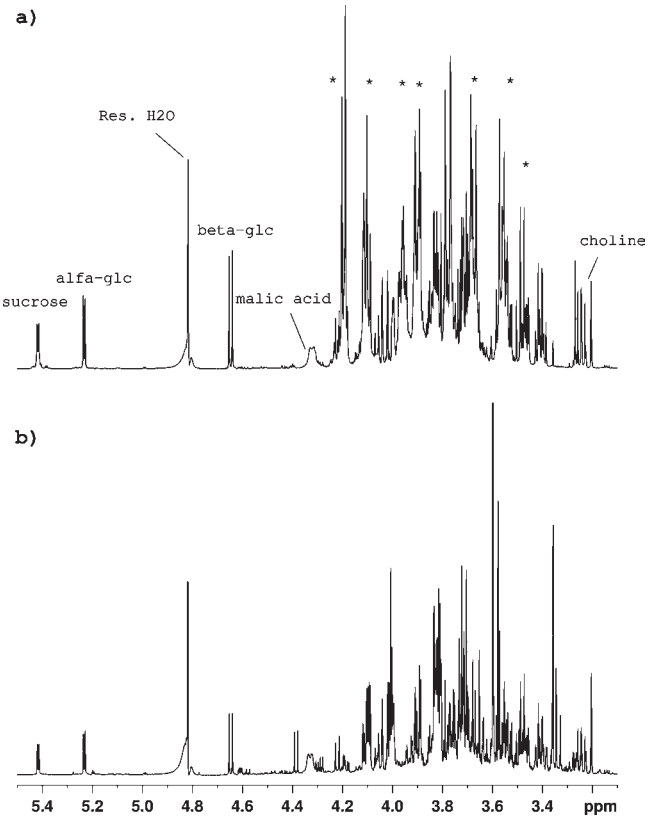

Figure 2. Expansion of a representative ^{1}H NMR spectrum obtained on extracts of (**a**) Foxtrot (late grass) and (**b**) Riesling (white clover). alfa-glc, α-glucose; beta-glc, β-glucose; Res. H2O, residual H_2O. Asterisks ($*$) show peaks assigned to fructose.

second harvest (data not shown). For a more detailed analysis of the grass cultivars only, PCA was carried out leaving out the leguminous cultivars (**Figure 4**). A separation of the two growths is observed, and even though not fully aligned with the first principal component, the separation is for the most part along the first principal component (**Figure 4A**). Analysis of the loadings (**Figure 4B**) reveals that the separation into the two growths can be ascribed to a lower intensity of the sugar region at ~3−4 ppm in samples from the second regrowth compared with the spring growth. In addition, specific signals at 2.39, 2.69, and 4.33 ppm, which are assigned to malic acid, and signals at 3.24, 5.23, and 5.42 ppm, which are assigned to β-glucose, α-glucose, and sucrose, respectively, also contribute to the separation between growths, as these signals are higher in intensity at spring growth compared with second regrowth. For a further elucidation of the change in the metabolite fingerprint between the two growths for the individual cultivars, the scores of the first principal component for the two growths were compared, and difference values were calculated, which shows that the degree of change in the score between harvest times varies for the different cultivars (**Figure 5**). The cultivars named Tetratop, Storm, and Perun represent the cultivars with the largest change in first principal component score values between the two growths, whereas Cancan and Asturion are the cultivars with the smallest change in score values between growths (**Figure 5**).

To investigate the capability of using the NMR data for determination of concentrations of soluble carbohydrate contents, PLS regressions were performed. The ^{1}H NMR spectra independent variables were used as predictors in the regression models to predict contents of glucose, sucrose, total amount of water-soluble carbohydrates (WSC), NDF, iNDF, and IVOMD determined biochemically. The PLS regressions to NDF, iNDF, and IVOMD were performed on grass cultivars, omitting legumes. The results of these regression models are summarized in **Table 2**, and a plot of iNDF predicted from NMR versus measured iNDF is shown in **Figure 6**. Acceptable errors of prediction were obtained with correlations between 0.75 and 0.95. To elucidate the NMR variables responsible for the correlation found between the NMR metabolite profile and iNDF, the regression coefficients were explored (**Figure 6**). The regression coefficients reveal that malic acid (2.40 and 2.70 ppm), choline (3.27 ppm), α-glucose (5.24 ppm), and sucrose (5.42 ppm) together with several signals in the 3−4 ppm sugar region are positively correlated with a high iNDF value, whereas alanine (1.48 ppm), an unidentified signal at 2.44 ppm, and several signals in the 3−4 ppm sugar region are negatively correlated with a high iNDF value (**Figure 6**).

DISCUSSION

Although the chemical composition of grass is known to depend on cultivar and season (*8*, *17*), specific knowledge about cultivar variations and seasonal changes in the metabolite profile of grass is limited. Obtaining knowledge about these cultivar variations and seasonal changes in the metabolite profile of grass could be useful for understanding the value of the grasses as animal feed. In the present study ^{1}H NMR-based metabolic fingerprinting was applied on aqueous extracts of 18 grass and 3 leguminous cultivars harvested during spring growth and after the second regrowth. A clear difference between the metabolite profile of the grass and that of leguminous cultivars was observed, which mainly could be ascribed to a higher intensity of a signal

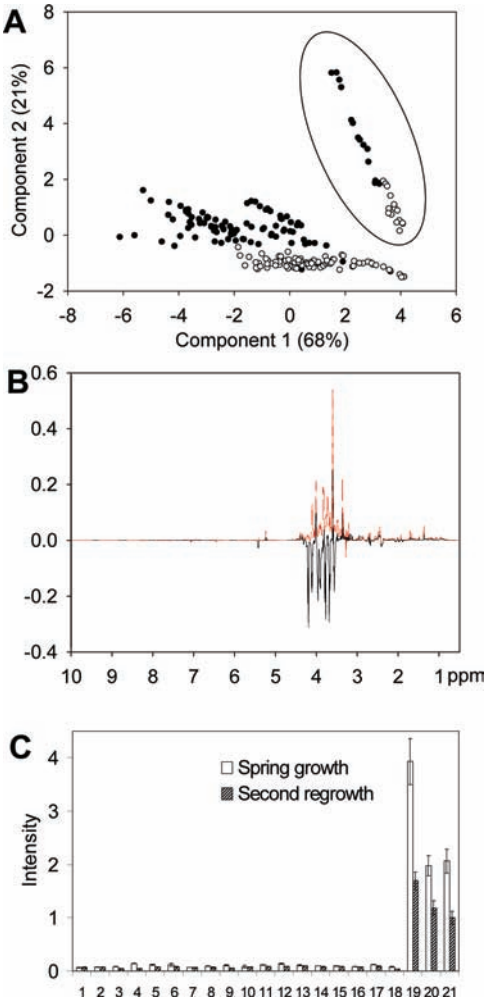

Figure 3. (**A**) PCA score plot showing the two first principal components in a PCA model including both grass and leguminous cultivars. Solid symbols represent samples from spring growth, whereas open symbols represent samples from second regrowth. The ellipse at the right shows all leguminous samples. (**B**) Loading line plot showing the two first principal components: first component, black line; second component, red line. (**C**). Intensity of the 3.60 ppm signal for the different cultivars: 1, Kimber; 2, Neptun; 3, Perun; 4, Hykor; 5, Tetratop; 6, Butara1; 7, Indiana; 8, Glenstal; 9, Amos; 10, Storm; 11, Asturion; 12, Cancan; 13, Eiffel; 14, Foxtrot; 15, Kentaur; 16, Loporello; 17, Polim; 18, Turandot; 19, Donata; 20, Riesling; 21, Daisy. Riesling, Daisy, and Amos are leguminous cultivars.

assigned to 3-*O*-methyl-β-D-glucose (3.60 ppm) in leguminous cultivars. Previous studies have demonstrated that total content of water-soluble carbohydrates (WSC) is lower in leguminous cultivars than in grass cultivars (*8*). The present study revealed that this difference in WSC specifically can be ascribed to a lower content of fructose in leguminous cultivars, as visual inspection of the NMR spectra revealed higher intensities of fructose signals in grass cultivars compared to leguminous cultivars. The differences in WSC between different cultivars were also displayed as effects on signals in the 3−4 ppm region in the PCA loadings. However, the 3−4 ppm region is a very crowded sugar region, making it difficult to assign these variables in the PCA loadings. Although the present study demonstrates the feasibility of ^{1}H NMR spectroscopy to characterize the metabolite profile of grass cultivars using a high-throughput approach, further studies elucidating the significance of specific metabolites in the grass feed for milk taste and composition are needed.

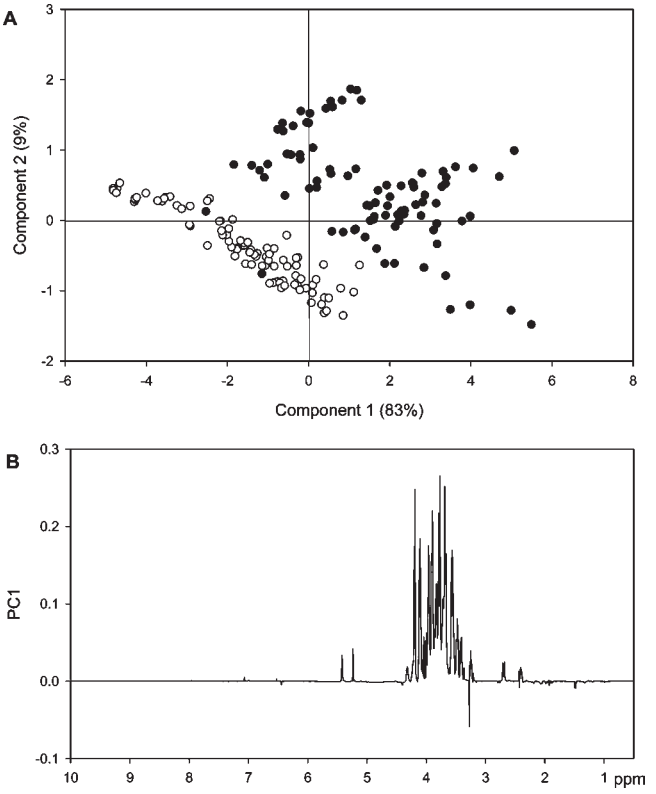

Figure 4. (**A**) PCA score plot showing the two first principal components in a PCA including only grass cultivars. Solid symbols represent samples from spring growth, whereas open symbols represent samples from second regrowth. (**B**) Loading plot of the first principal component.

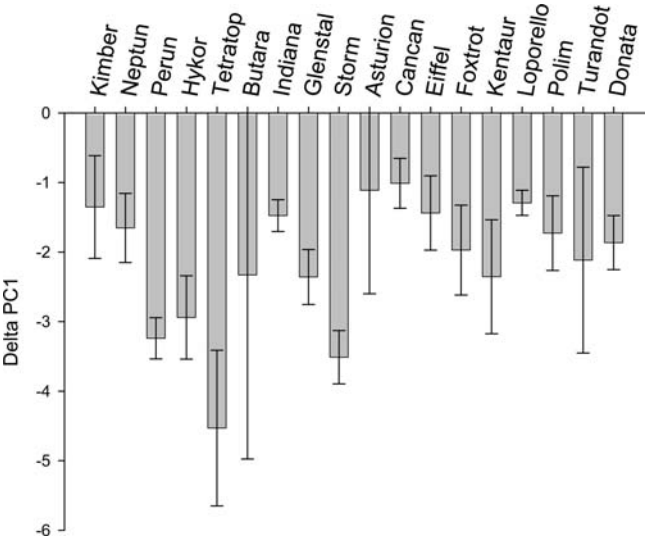

Figure 5. Differences between the two growths in mean scores of the first principal component. Bars show standard deviations calculated from five replicates.

For several plants it is of great interest to harvest them when their content of metabolites of interest is reaching an acceptable level. The present study revealed that ^{1}H NMR spectroscopy was able to detect a clear effect of growth on the metabolite profile of water extracts of grass and to evaluate which cultivars changed most considerably. Accordingly, the applied approach may be useful for elucidating if plants have the right maturity for harvest.

Conventional analytical techniques and animal trials are traditionally used to evaluate the animal feed value of grass and

Table 2. Performances of PLS Regression Models with ^{1}H NMR Spectra as *X* Variable and Soluble Carbohydrate Concentrations as *y* Variable[a]

metabolite	*N*	RMSECV[b]	#PLS[c]	R^d	relative RMSECV[e]	reference value interval
fructose	21	1.07	4	0.95	15%	0.6−10.4
glucose	21	0.60	2	0.92	17%	0.5−6.7
WSC	21	4.93	1	0.80	22%	2.9−32.2
iNDF	18	1.07	3	0.90	17%	3.6−11.5
NDF	18	2.03	2	0.82	4%	44.6−59.8
in vitro digestibility (IVOMD)	18	1.96	2	0.75	3%	65.5−76.5

[a] Five replicates of samples for NMR analysis were included in the analysis. [b] Root mean square error of cross validation (units, mmol/kg of plasma). [c] Number of PLS components used. [d] Correlation coefficient. [e] Relative RMSECV calculated as RMSECV/(mean reference value).

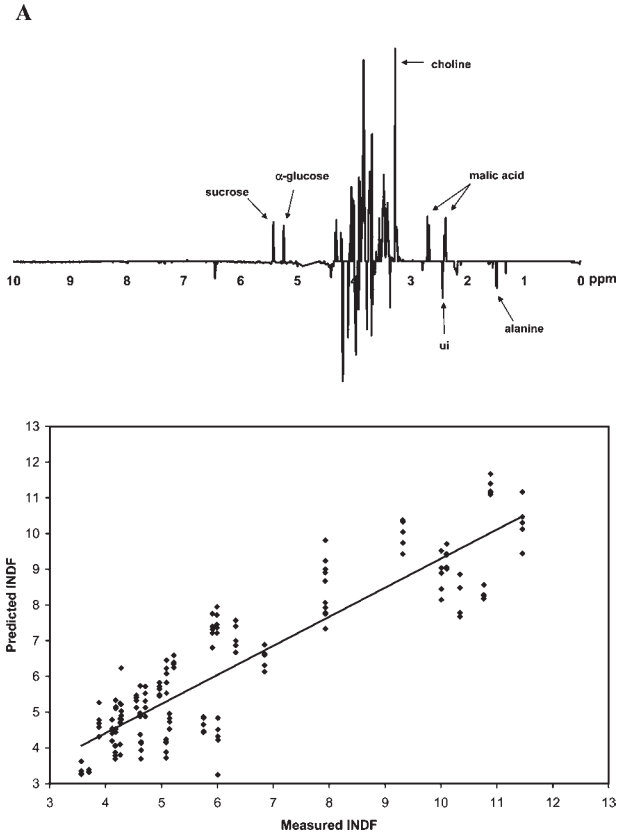

Figure 6. (**A**) Regression coefficients from PLS regression with ^{1}H NMR spectra independent variables used to predict the content of insoluble neutral detergent fibres (iNDF) in grass samples. ui, unidentified. (**B**) INDF predicted from NMR versus measured INDF.

forage materials. However, these traditional analyses are tedious and time-consuming. Moreover, they are selective, and therefore several analyses are usually needed to obtain a complete picture of the metabolic changes. Consequently, the use of near-infrared (NIR) spectroscopy to estimate the composition and feed value of grasses has been introduced, and good correlations have been reported (*17*, *18*). GC-MS-based methods for the analysis of crop and forage plants have also been demonstrated (*19*). However, whereas these methods do provide a complete characterization of fructans, they are demanding with regard to time and labor. In the present study, the use of high-throughput ^{1}H NMR spectroscopy for the prediction of water-soluble carbohydrate concentrations from measurements on water extracts of grass obtained practically without any sample preparation was investigated using PLS regression. The prediction models resulted in correlations between 0.80 and 0.95, which is considered to be promising for future calibrations. Likewise, the traditional analyses determining iNDF, NDF, and IVOMD, which are parameters that characterize the value of the grass for animal feed, are both laborious and time-consuming. The present study demonstrated correlations between NMR metabolite profiles obtained on water extracts of grass and iNDF and NDF as well as IVOMD, which are comparable to values obtained using NIR (*17*, *18*). Consequently, the applied NMR approach is useful for a rapid estimation of these parameters as an alternative to the NIR method commonly used in practice. The regression coefficients from the PLS regression model revealed that the relationship between the NMR metabolite profile and iNDF could be ascribed to a positive correlation between iNDF and signals assigned to malic acid and choline. Malic acid is known to vary according to maturity (*20*), which may explain the correlation to iNDF. The positive correlation to choline should probably be ascribed to the fact that choline represents a fiber constituent. In addition to the positive correlations between iNDF and specific signals for α-glucose and sucrose, both negative and positive correlations to signals in the 3−4 ppm sugar region were found in the regression coefficients. These findings indicate that the composition of the water-soluble carbohydrates somehow is related to iNDF. Further studies are needed to unravel the biochemical relations explaining this discovery.

In conclusion, the present study demonstrated that proton nuclear magnetic resonance-based metabolic fingerprinting of grass extracts is a potential method for tracking differences and changes related to cultivar and season. In addition, correlations between the NMR fingerprints and the value of the grasses as animal feed evaluated as concentration of sugars, neutral detergent fibres, indigestible neutral detergent fibres, and in vitro organic matter digestibility was demonstrated, indicating that NMR of water extracts might be a useful high-throughput method for evaluating the quality of the grass as animal feed.

ACKNOWLEDGMENT

We thank Dr. Gitte G. Rasmussen for her assistance with sampling for analysis of water-soluble carbohydrates and Marianne Rasmussen and Nina Eggers for their assistance with NMR measurements.

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Received for review December 7, 2009. Revised manuscript received March 3, 2010. Accepted March 4, 2010. The Danish Ministry of Food, Agriculture and Fisheries, Fødevareforskningsprogrammet 2007, is acknowledged for funding through the project "Sund og velsmagende mælk baseret på græsmarksafgrøder", and The Danish Research Council FTP is acknowledged for funding through the project "Advances in food quality and nutrition research through implementation of metabolomic technologies" (274-09-0107).