Carbohydrate degradation during sample storage for herbage analysis

C. Matthew[1], B.W. Howard[1], A.R. Drysdale[2], C. Duranton[3], M.A. Osborne[1], and S.S. Bhatia[1]

[1]Institute of Agriculture and Environment, PN433, Massey University,Private Bag 11-222, Palmerston North 4442, New Zealand
[2] Analytical Research Laboratories, Ravensdown, P.O. Box 989, Napier, New Zealand,
[3]ISARA-Lyon, 23 Rue Jean Baldassini, 69364 Lyon, France

Abstract

Near infrared spectroscopy (NIRS) analysis of herbage is rapidly gaining industry acceptance as a tool to assist farmers to assess feed value, but questions remain about sample handling protocols to minimise sample deterioration prior to analysis. Two experiments with a total of 110 samples were conducted to evaluate factors affecting the composition of herbage soluble sugars and starch (SSS) analysis by NIRS. A primary objective was to quantify the effect of storage time and storage conditions on rate of SSS loss by respiration between cutting of samples in the field and oven drying in the laboratory. In this experiment, which investigated the rate of respiratory loss during sample storage in airtight bags respiration loss of SSS during storage was found to be four times less than would have been predicted from published respiration rates of ryegrass herbage, and the rate of loss was not affected by storage temperature. A hypothesis for further confirmation is that when herbage samples are stored in airtight bags, the rate of respiration is oxygen limited. If these results are confirmed, a likely practical recommendation would be that overnight transit to the laboratory between field harvesting and drying of samples should not affect herbage quality in airtight bags provided excess air is squeezed out before closing to minimise oxidative degradation. NIRS calibrations of changes in herbage during storage indicated a potential for predicting the time elapsed between cutting and drying, which could allow a correction to be made to determine the true value of feed at the time of sampling.

Additional keywords: Soluble sugars and starch (SSS), metabolisable energy (ME) near infrared spectroscopy (NIRS), anthrone method, herbage respiration, oxygen-limited, perennial ryegrass

Introduction

A valuable technology to assist farmers and consultants to manage feed better and evolve farm systems that deliver high quality feed for improved animal performance is near infrared reflectance spectroscopy (NIRS). With adequate calibration and validation, NIRS predictions can be obtained cheaply for any forage quality characteristic. In current industry usage, a farmer or consultant can obtain a rapid and comparatively inexpensive

estimation of a range of forage quality traits.

Development of NIRS analysis of forage materials began in the 1970s with the use of filtered infrared sources to detect specific compounds, and evolved over a decade to scanning monochromators which effectively detect absorption of light at specific wavelengths by specific chemical bonds. Signals from the sample can then be statistically compared to data from samples of known composition. J Shenk at Pennsylvania State University was a notable pioneer of the technology (Clark, 1989). Data transformation routinely uses log(1/reflectance) and second derivative of spectral data, with new innovations in fast fourier transformation to optomise spectral differences between samples. Modern NIRS machines have been developed with increased precision and have become a widely accepted alternative to wet chemistry. Implementation of NIRS in New Zealand is described by Corson *et al.*, (1999), and the technique is routinely used by the main commercial forage analysis laboratories.

However, despite the sophistication and growing adoption of the technology, there is still very little information published on sensitivity of results to sample storage time. In particular it is known that cell metabolism continues in live herbage after sample collection and that carbohydrates within cells are consumed to sustain respiration. Carbohydrate levels are related to herbage metabolisable energy (ME) (Armstrong *et al.*, 1964) and therefore there is a possibility of underestimation of feed value from poor sampling and storage protocols. This is a significant issue for NIRS analysis of herbage as a majority of samples would be subject to an overnight courier delivery, at ambient temperature in a plastic bag, before reaching the laboratory for processing. There is the potential for additional delays between collection and despatch to the lab. Factors such as the time of day may also affect the soluble carbohydrate levels (Cajarville *et al.*, 2015). Sugar depletion may occur through respiration after sampling (Penning de Vries *et al.*, 1979), daily variation related to levels of cloud cover (Fulkerson and Donaghy, 2001) and nocturnal translocation (Cajarville *et al.*, 2015). NIRS prediction of soluble sugars and starches (SSS) may assist in interpretation of poor analytical results if best practice sampling procedures have not been followed to minimise degradative losses.

Best practice involves immediate freezing of fresh in liquid nitrogen, storage at -10°C and freeze drying, but this protocol is mostly impractical when sampling on farm. A set of guidelines for sample handling from the field through to the laboratory was reported by Dewhurst *et al.* (2008). However, this protocol does not cite published data on sample deterioration after collection. Nor were the authors able to locate any published data on relative quantitative losses for pasture species between sample collection and delivery to the laboratory.

Therefore, the primary aim of this study was to quantify the rate of decline in carbohydrate levels of perennial ryegrass pasture samples during the time between cutting and oven drying. A second aim was to test whether a NIRS machine could produce a calibration to determine the time elapsed between field cutting and laboratory drying of samples. This work was seen as a first step towards possible future development of a correction of NIRS-SSS results for the effect of duration of sample storage time.

Materials and Methods

Sample collection and processing

Two experiments were carried out; a pilot experiment (Experiment 1) with herbage collected on 9th February 2015, and a main experiment (Experiment 2) with herbage collected on 26th June 2015. For both experiments herbage was collected from a sward of Bealey perennial ryegrass sown without clover as a buffer zone adjacent to a research trial, at Massey University No. 4 Dairy, Palmerston North. The field was grazed by dairy cows at intervals defined by the seasonal rotation on the dairy farm and was visually estimated at 2,600 kg DM/ha when herbage was collected. Herbage (approx. 10 kg DM) was cut to 25 mm above ground level using an electric shearing handpiece, and taken immediately in large plastic bins to the laboratory, mixed, and non-ryegrass herbage picked out by hand, to obtain a quantity of bulk herbage from which botanically identical samples could be drawn. This procedure was carried out twice on each of the two sampling days; 10 min after daybreak (6.00 am and 6.50 am in pilot and main experiments, respectively) and in mid-afternoon (2.50 pm in both experiments), to largely capture diurnal variation in plant sugar levels.

In Experiment 1, immediately on reaching the laboratory, one sample was placed in a high-air-volume hot air draft oven set at 80°C, and a second sample loaded into a freeze drier with a refrigeration unit to cool samples. Further samples were stored in the laboratory in zip-lock bags at ambient temperature and pairs of samples placed in the oven and freeze drier at intervals as set out in Table 1a. In Experiment 2, ryegrass samples were collected from the field at daybreak and mid-afternoon as before. However, in this experiment all samples were oven dried, and samples subjected to contrasting warm or cool storage conditions: 25°C, stable ambient temperature in the oven drying room or 4°C, or a walk-in refrigerator, respectively. A total of 42 storage regimes were tested (Table 1b), with duplicates in each case giving 84 samples in all.

NIRS analysis

All samples (n=110) were ground to a fine powder in a 'Cyclone 1093' mill at Massey University, loaded into 20 mm diameter flat bottomed glass vials and scanned for near infrared reflectance spectra (Bruker MPA NIRS with 3 nm wavelength steps from 760-2600 nm) at Ravensdown ARL laboratories, Awatoto. Soluble sugar and starch and in-vitro digestibility (IVD) were determined from commercial (ARL) calibrations. ME was determined as IVD × 0.146, following standard practice at ARL laboratories.

A NIRS calibration for determination of sample storage time was derived and tested using oven-dried samples from experiment 1 (n=14) and Experiment 2 (n=82). The sample set was arbitrarily divided into a calibration set of 76 samples and a validation set of 20 samples chosen to represent the range of storage times. NIR spectral data was pre-processed by calculation of first derivatives followed by partial least squares validation using Bruker OPUS software.

The software produced more than one equation, the best of which was then applied to the independent validation set. The correlation between actual and NIRS-estimated storage time, standard error of calibration and standard error of prediction were reported by the software. The latter two statistics are not presented here.

Table 1: Sample storage times and conditions used for collection of 26 samples in Experiment 1 on 9 February 2015 and 84 samples in Experiment 2 on 26 June 2015.

a) Ex. 1	Time sampled	Drying method	Storage times (h)
	Daybreak	Oven	0, 1, 3, 6, 24
		Freeze Dry	0, 1, 6, 9, 12, 24, 30
	Mid afternoon	Oven	0, 1, 3, 6, 17, 24
		Freeze Dry	0, 1, 3, 6, 9, 12, 24, 30
b) Ex.2	Time sampled	Storage conditions	Storage times (h)
	Daybreak	Cool	1, 2, 4, 6, 9, 12, 15, 24, 30, 48
		Ambient	0, 1, 2, 4, 6, 9, 12, 15, 24, 30, 48
	Mid afternoon	Cool	1, 2, 4, 6, 9, 12, 15, 24, 30, 48
		Ambient	0, 1, 2, 4, 6, 9, 12, 15, 24, 30, 48

Carbohydrate analysis

An established 'anthrone' method, used elsewhere for perennial ryegrass, was employed here to determine total sugar composition (Liu *et al.* 2015). The anthrone analysis was performed in two steps, an extraction followed by a colorimetric assay. A 25mg subsample of the dried, ground herbage was extracted in 1ml of 80% ethanol to solubilise low molecular weight (LMW) water soluble carbohydrates. The mix was then homogenised using a vortex mixer, shaken in a water bath at 65°C for 30 minutes, centrifuged for 15 minutes at 10,000 rpm, and the supernatant pipetted into an Eppendorf tube. This procedure was repeated on the residue and the second supernatant combined with the first. Next, the residue was extracted twice as before but with Milli-Q water instead of ethanol, to solubilise high molecular weight (HMW) water soluble carbohydrates. The Eppendorf tubes containing LMW and HMW extracts were stored at -4°C until analysis.

The colorimetric analysis was based on the reaction of anthrone reagent with sugars under acidic conditions, which leads to a blue-green colouration. Dilution series of sucrose and inulin standards were prepared for analysis of concentrations of LMW and HMW carbohydrates, respectively. The samples and standards were diluted in a microwell plate, incubated at 65°C for 25 minutes, and absorbance was measured at 620 nm on a plate reader spectrophotometer (Perkin Elmer Victor X). Each sample was measured three times and each standard measured twice, and results averaged. Sugar concentrations were calculated using the known standards and reagent blanks.

Statistical analysis

The relationships between anthrone-determined LMW + HMW sugars as % DM and NIRS-SSS and between NIRS-ME and NIRS-SSS as % DM for morning- and afternoon-collected samples were determined using 'linear functional relationship regression' (also known as 'model 2' regression) in Genstat version 16.1 (VSN International Ltd, UK). This

regression model adjusts the slope estimate for the effect of error in the X variable.

A regression analysis was performed on data from Experiment 1 (n=26) examining decline in NIRS-SSS (% total dry weight) per hour of sample storage time. Additional terms were added in a multiple regression model in Minitab 10.51 (Minitab Inc. 3081 Enterprise Drive, State College, PA 16801 USA) to account for variation in NIR-SSS between morning or afternoon harvests, with drying method (freeze drying or oven drying). The model therefore provided four coefficients that estimate the SSS% of the herbage at harvest (intercept), the rate of loss per hour during storage, the shift of intercept between morning and afternoon samplings, and the shift of intercept for any loss of SSS that may occur during oven drying, when compared to freeze drying.

For a combined data set of 95 oven-dried samples from both experiments, Pearson correlation coefficients were calculated between the 5 measured herbage traits: LMW-SSS, HMW-SSS, total SSS, NIRS-SSS, and ME. A regression analysis of NIRS-SSS similar to that described above for Experiment 1 was conducted, but with additional terms to detect: i) differences in slope (as well as intercept) between samples collected in the morning and afternoon, and ii) differences in slope only for cold- and warm-stored samples. As the cold- and warm-stored samples were drawn from the same initial bulk herbage sample they should have had same starting composition. Where there was no statistically significant difference in slope or intercept for model terms, a reduced model was run with those terms omitted. Residuals from the regression analyses were checked for homogeneity of variance using the Minitab version 10.51 %Normplot command and Anderson-Darling test for normality.

Results

In Experiment 1 the NIRS-SSS% of FD herbage samples at the morning sampling was 10.21%, the SSS loss per hour during storage was 0.043% (meaning approximately 25 h for a 1% loss of SSS), the herbage SSS increase from morning to afternoon was 2.76%, and the SSS loss associated with oven drying compared to FD was 3.26% (Table 2). For the combined data set from Experiments 1 and 2, the correlation between NIRS-SSS and (LMW+HMW) determined by the anthrone method was 0.856 (Figure 1, Table 3), with a similarly high correlation with LMW, but a weaker correlation with HMW sugars (Table 2). A superficially weak correlation between NIRS-SSS and NIRS-ME was an artefact of NIRS-ME being lower by 0.67 MJME/kg DM for samples harvested in the afternoon compared to the morning. When analysed within morning and within afternoon data sets, the two traits showed a highly significant correlation (Figure 2).

Table 2: Results for regression analysis of factors affecting NIRS-determined concentration of soluble sugars and starches (NIRS-SSS%) in herbage at harvest and loss per hour of storage in Experiment 1.

Factor	Coefficient	SE	*P*
Intercept (morning SSS%, FD sample)	10.21	0.357	<0.001
Rate of SSS% loss per hour	0.043	0.0176	0.026
Afternoon increase of SSS%	2.76	0.360	<0.001
SSS% loss in oven drying	3.26	0.352	<0.001
R-squared	0.879	-	<0.001

Table 3: Pearson correlations between herbage traits measured by anthrone wet chemistry analysis for low molecular weight sugars (LMW%), high molecular weight sugars (HMW), and total sugars (LMW+HMW%) expressed as % of dry weight and 'soluble sugars and starch' (NIRS-SSS%) and ME MJ/kg DM), determined by near infrared spectroscopy.

	LMW%	HMW%	LMW+HMW%	NIRS-ME (MJ/ kg DM)
HMW%	0.394			
LMW+HMW%	0.961	0.631		
NIRS-ME (MJ/kg DM)	0.054	-0.477	-0.103	
NIRS-SSS%	0.866	0.415	0.856	0.116[a]

[a] Correlation coefficient is reduced by a lower value of NIRS-ME for afternoon than morning samples; correlation is higher within morning and afternoon data sets: see Figure 2.

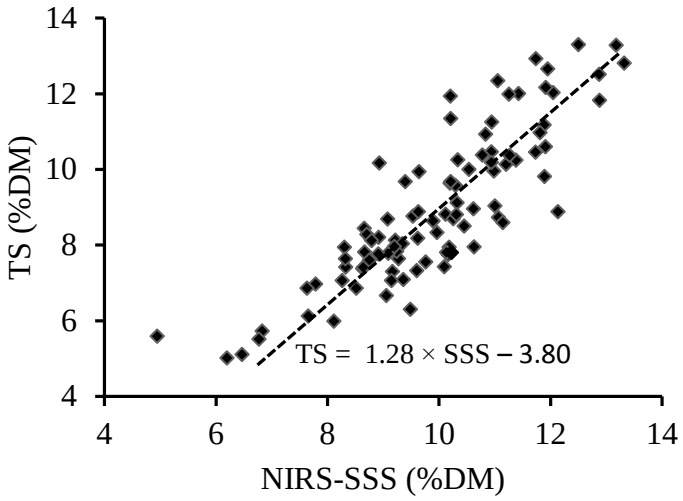

Figure 1: Relationship between total of low and high molecular weight sugars determined by the anthrone method (TS, %DM) and soluble sugars and starch, determined by near infrared spectroscopy (SSS, %DM) and, as determined for the same samples by the anthrone method. The trend line was determined by 'model 2' regression in Genstat.

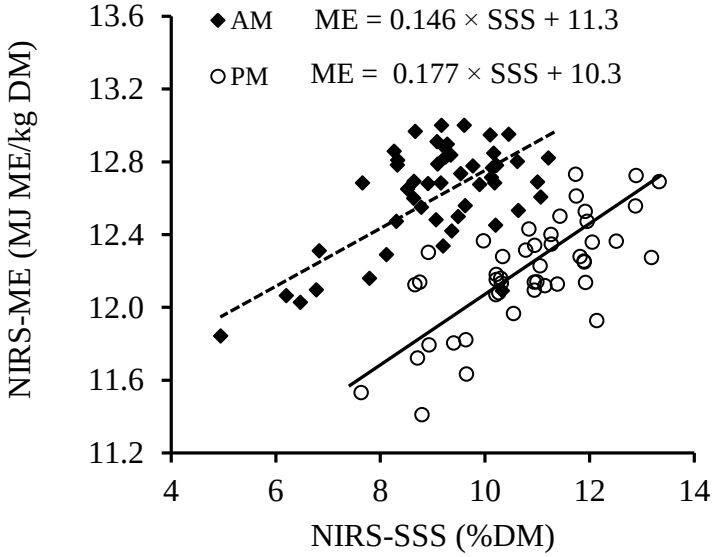

Figure 2: Relationship between herbage metabolisable energy and soluble sugars and starch, as determined by near infrared spectroscopy (NIRS-ME and NIRS-SSS, respectively). Note that NIRS-ME was lower for afternoon-collected samples. Trend lines were determined by 'model 2' regression in Genstat.

For the combined NIRS-SSS data for oven dried samples from both experiments, the average decline per hr in NIRS-SSS during storage was 0.047 percentage units, indicating a little over 20 hr for the reported value of SSS to fall by one % unit. The rate of loss was not significantly affected by storage temperature, nor by time of day of sample collection (Figure 3). For data from the anthrone analysis, loss rates during

storage of 0.050%/hr and 0.060%/hr were observed for LMW and LMW+HMW fractions, respectively, for afternoon collected samples. However, for morning collected samples LMW sugar loss was statistically undetectable and LMW+HMW loss of 0.022%/hr just attained statistical significance at the 5% probability threshold (Table 4).

For the best equation developed from the calibration set of 76 samples for prediction of storage time for the test set of 20 samples, the correlation between predicted and actual values was statistically significant ($P < 0.05$) but was low: 0.456.

Table 4: Parameter estimates for a combined data set from Experiment 1 and Experiment 2 for multiple regression analysis describing changes in wet chemistry traits and NIRS analysis traits. LMW = low molecular weight sugars % DM; HMW = high molecular weight sugars % DM; LMW+HMW = total sugars % DM; NIRS-ME = NIRS-determined metabolisable energy MJ/kg DM; NIRS-SSS = NIRS-determined soluble sugars and starch (% DM).

	Herbage trait				
Parameter estimates	LMW	HMW	LMW+HMW	NIRS-ME	NIRS-SSS
Intercept[1]	6.47	1.35	8.24	12.7	10.1
Change/hour[2]	NS	NS	-0.022	NS	-0.047
Afternoon[3]	2.59	0.81	3.26	-0.36	1.63
Loss rate (pm)[4]	0.050	NS	-0.038	NS	NS
Loss rate (warm)[5]	NS	NS	NS	-0.007	NS
Expt. 1. Dif[6]	-2.93	0.56	-2.41	-0.60	-2.67
R-squared	0.740	0.561	0.740	0.684	0.738
P (normality)[7]	0.052	0.013[8]	0.005[8]	0.757	0.620

[1]Value at harvest for a sample collected in the morning.

[2]Parameter estimate denoting change per hour during storage.

[3]Parameter estimate indicating average difference of values for herbage harvested in the morning or afternoon.

[4]Parameter estimate indicating differential rate of change of herbage trait harvested the afternoon compared to morning. Negative value denotes faster rate of loss.

[5]Parameter estimate indicating differential rate of change for warm-stored compared to cold-stored samples, negative denotes value to be subtracted from change/hour.

[6]Parameter estimate indicating the season effect (average difference of values in Expt. 1 and Expt. 2.).

[7]*P* value of Anderson-Darling test for non-normality of error distribution, obtained using %Normplot command in Minitab Version 10.51.

[8]Normal probability plot indicates positive kurtosis but no outlier points or skewed error distribution.

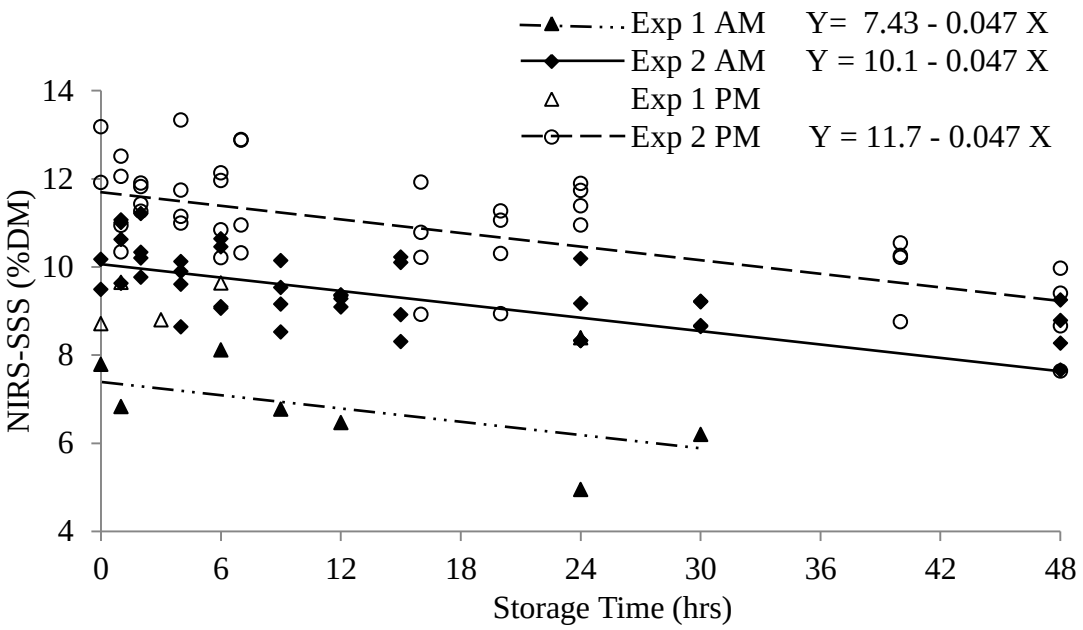

Figure 3: Loss of NIRS-determined herbage soluble sugars and starch NIRS-SSS, with storage time in pooled data for Experiments 1 and 2.

Discussion

The strong correlation (R=0.856, Figure 1) between NIRS-SSS data and herbage concentration of LMW+HMW sugars as measured by the anthrone method indicates that the established NIRS predictions for total soluble sugars in pasture were satisfactory and is, therefore, reassuring for users of NIRS technology. Values for LMW+HMW sugars were typically fractionally lower than those predicted by NIRS (NIRS-SSS) but were considered acceptable. A linear fit forced through the origin indicated that results of the anthrone method were lower by a factor of 1.09 than those obtained by the NIRS-SSS method. Possible explanations for this include that the commercial calibration equation was developed with different chemical extraction protocols from those used in the anthrone method, that systematic errors were introduced though sample preparation differences, or that there were seasonal or other differences between samples used in calibration and those in the prediction sets, in the commercial calibrations.

An incidental finding in this study was that NIRS estimation of in-vitro digestibility (here expressed as ME using the conversion x 0.146) was insensitive to diurnal change in herbage sugar concentration (Figure 2), even though it is generally accepted that higher sugar levels should mean an increase in herbage ME (Armstrong *et al.*, 1964).

Analytical testing laboratories recommend drying herbage at 60°C to minimise loss of SSS on drying as indicated by the difference in this study between SSS results for freeze-dried and oven dried herbage (Table 2). However, in one previous informal test conducted by C. Matthew, rapid drying at 80°C in the high-volume air draft oven used in this study yielded higher SSS levels in the dried samples than drying more slowly at 60°C in a drying oven with lower air flow. Hence, it should not be assumed that drying at a

lower temperature than 80°C would reduce the oven drying loss of SSS as shown in Experiment 1. Both a longer drying time, and high drying temperature, therefore, may cause carbohydrate loss from samples during the drying process.

The overall rate of loss of sugars during storage of 0.047 percentage units per hour was less than expected. Robson (1982) showed respiration rates for perennial ryegrass of 1.0 and 1.6 mg CO_2/hr at 15°C. Allowing for a stoichiometric ratio of 44:30 between CO_2 and CH_2O and an approximate doubling of respiration in ryegrass at 25°C compared to 15°C (Penning de Vries *et al.*, 1979). This means that warm-stored samples may be expected to respire carbohydrate at 2mg/g/hr or 0.2%/hr, or about four times faster than observed in this study. A possible reason for the low respiration rate is that oxygen exchange was reduced by sealing samples in in zip-lock plastic bags resulting in reduced sugar breakdown.

The higher loss rate for the LMW and LMW+HMV sugar components in samples collected in the afternoon (Table 3) may indicate that respiration of sugars is dependent on the concentration in the tissue. Similarly, samples collected in the morning with lower sugar concentrations should therefore have lower respiration. Penning de Vries *et al.* (1979) reported lower respiration at lower sugar concentration, supporting our results. Lower NIRS-SSS levels in Experiment 1 compared to Experiment 2 supports a seasonal trend of lower herbage carbohydrates in warmer growth conditions. This agrees with results of Moller (1997). There was no indication of increased rate of sugar loss from warm-stored samples compared to cold-stored samples. An explanation may be that respiration rate of both cold- and warm-stored samples is regulated by the hypoxic storage environment within the plastic bags.

Further testing of NIRS calibrations is required to confirm a method to predict time elapsed time between cutting and drying. In this experiment morning and afternoon samples with different sugar levels were included purposely to reduce correlation between sugar level and storage time in the data set, so that any calibration for storage time found, would more likely reflect metabolites other than sugars. It is known that glycolysis continues in hypoxic conditions with the production of alcohols as end products (Kato-Naguchi, 1999). Products of glycolysis under hypoxic conditions in storage therefore are an example of a category of metabolites that might provide a biological basis for NIRS estimation of sample storage time. Future NIRS calibrations to estimate thermal time (°C.hr) of sample storage, may prove worthwhile.

Conclusions

Loss of the SSS fraction during sample storage was approximately four times less in this experiment than would have been predicted from published respiration rates for ryegrass herbage. No difference in respiration rate was detected between warm- and cold-stored samples, although samples collected in the afternoon with higher SSS levels tended to lose SSS more rapidly than morning-collected samples. A practical recommendation for sample handling, subject to confirmation by further testing, is that overnight transit to the laboratory of samples in air-tight plastic bags after sample collection should not reduce SSS levels more than 1% from levels present at cutting. Storage temperature was also found not to be

critical in this experiment. NIRS calibrations showed that it may be possible to develop prediction equations to estimate time elapsed between cutting and drying, and development of such a calibration could assist in interpretation of analytical results in situations where samples take 1-2 days to reach the laboratory.

Acknowledgements

We thank the TR Ellett Agricultural Trust for provision of a study scholarship to B. Howard, and for supporting the cost of anthrone analyses; Ravensdown Ltd. for provision of NIRS analyses, Ms Hong Xue of AgResearch for advising us on anthrone analysis methodology, Ms Felicity Jackson for use of the Perkin Elmer Victor X plate reader in the Nutrition Laboratory, Massey University, and Professor Tony Parsons for helpful discussions during the preparation of this manuscript.

References

Armstrong, D.G., Blaxter, K.L. and Waite, R. 1964. The evaluation of artificially dried grass as a source of energy for sheep III. The prediction of nutritive value from chemical and biological measurements. *Journal of Agricultural Science (Cambridge)* 62: 417-424.

Bryant, R.H., Parsons, A.J., Rasmussen, S. and Edwards, G.R. 2009. Pasture production and botanical composition of high sugar and control ryegrasses with or without endophyte under irrigation in Canterbury. *Proceedings New Zealand Grassland Association* 71: 177-185.

Cajarville, C., Britos, A., Errandonea, N., Gutiérrez, L., Cozzolino, D. and Repetto, J.L. 2015. Diurnal changes in water-soluble carbohydrate concentration in lucerne and tall fescue in autumn and the effects on in vitro fermentation. *New Zealand Journal of Agricultural Research* 58: 281-291.

Clark, D.H. 1989. History of NIRS analysis of agricultural products. pp. 7-11. *In:* Near infrared spectroscopy (NIRS): analysis of forage quality. Eds Marten, G.C., Shenk, J.S., and Barton II, F.E. Handbook No. 643 United States Department of Agriculture, Springfield.

Corson, D.C., Waghorn, G.C., Ulyatt, M.J. and Lee, J. 1999. NIRS: Forage analysis and livestock feeding. *Proceedings New Zealand Grassland Association* 61: 127-132.

Dewhurst, R.J., Kay, M., White, M., Hill, R., Stiles-Jones, S., Jackson, F. and Knight, T. 2008. Standardised feed analysis for New Zealand's dairy industry. pp. 27-34. Proceedings 6th Dairy3 Conference, 15-16 April, Rotorua, New Zealand.

Fulkerson, W.J. and Donaghy, D.J. 2001. Plant soluble carbohydrate reserves and senescence – key criteria for developing an effective grazing management system for ryegrass-based pastures: a review. *Australian Journal of Experimental Agriculture* 41: 261-275.

Kato-Noguchi, H. 1999. Flooding induced increase in alcohol dehydrogenase activity in timothy and ryegrass seedlings. *Biologia Plantarum* 42: 445-449.

Liu, Q., Jones, C.S., Parsons, A.J., Xue, H. and Rasmussen, S. 2015. Does gibberellin biosynthesis play a critical role in the growth of Lolium perenne? Evidence from a transcriptional analysis of gibberellin and carbohydrate metabolic genes after defoliation. *Frontiers of Plant Science* 6: 944.

Moller, S. 1997. An evaluation of major nutrients in dairy pasture in New Zealand and their effects on milk production and herd reproductive performance. PhD Thesis, Massey University. Retrieved in June 2016 from: http://mro.massey.ac.nz/xmlui/handle/10179/2704

Penning de Vries, F.W.T., Witlage, J.M. and Kremer, D. 1979. Rates of respiration and of increase in structural dry matter in young wheat, ryegrass and maize plants in relation to temperature, to water stress and to their sugar content. *Annals of Botany* 44: 595-609.

Robson, M.J. 1982. The growth and carbon economy of selection lines of *Lolium perenne* cv. S23 with differing rates of dark respiration 1. Grown as simulated swards during a regrowth period. *Annals of Botany* 49: 321-329.