Oecologia (2008) 156:107–116
DOI 10.1007/s00442-008-0960-y

PLANT-ANIMAL INTERACTIONS - METHODS

A simple, integrative assay to quantify nutritional quality of browses for herbivores

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Received: 20 April 2007 / Accepted: 19 December 2007 / Published online: 21 February 2008

Abstract Many regard the concentrations of nitrogen (N), tannins and plant cell wall constituents (fibre) as key indicators of food quality and habitat suitability for browsing herbivores; yet there is no method for measuring their combined effects. We have developed a simple in vitro assay for measuring the effects of tannins and fibre on N availability in browse. We determined the effects of tannins by measuring the polyethylene glycol (PEG)-binding capacity (PEG-BC) of *Eucalyptus* leaf samples, followed by a two-stage in vitro digestion with pepsin and cellulase to determine the digestibility of dry matter and N. There was a significant relationship between concentrations of digestible N and the PEG-BC of the leaves. Furthermore, adding PEG significantly improved the digestibility of N. Our results concur with in vivo observations from several mammalian species. This suggests that our method is effective for measuring the nutritional quality of browse and the benefits of adding PEG, providing some index of the detrimental effects of tannins. We further simplified the assay by removing the PEG step, allowing us to quickly analyse samples in bulk. Nevertheless, this simplified method is still not practical for analysing the many samples necessary to compare the nutritional values of different tracts of forest. We used near-infrared reflectance spectroscopy to produce calibration equations and predicted total and digestible N in 322 trees at eleven sites. Both within and between sites, we found a wide variation in concentrations of digestible N but a much lower variation in total N, with either no relationship or poor relationships between the two measures. This confirms the variability in the nutritional quality of eucalypt forests, which may explain the distribution and abundance of mammalian herbivores. Thus, our assay provides a useful tool for understanding how food resources influence herbivore populations at different scales.

Keywords Tannins · Nitrogen availability · PEG · *Eucalyptus* · Folivory

Communicated by Jörg Ganzhorn.

Electronic supplementary material The online version of this article (doi:10.1007/s00442-008-0960-y) contains supplementary material, which is available to authorized users.

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Introduction

The nutritional quality of food for browsing herbivores may constrain both habitat use and reproductive fitness (e.g. Batzli 1986; Moore and Foley 2005). However, measuring food quality depends on the ability to easily assess the proportion of food that can be digested and metabolised by animals (Foley et al. 1999). Plant secondary metabolites are ubiquitous in browse and can reduce the digestibility of energy and protein (Iason 2005).

Condensed tannins, common in many leaves, bind to proteins and reduce their availability to foragers (Hagerman et al. 1992; Jones and Mangan 1977; Robbins et al. 1987). For example, *Eucalyptus* leaves typically contain only 0.8–2% N on a dry matter (DM) basis (Moore et al. 2004a), but they also contain more than 12% condensed tannin (e.g. Fox and Macauley 1977), which may have profound effects on the nutrition of *Eucalyptus* folivores, even determining whether they can exist in particular habitats (Cork and Catling 1996). Despite the prevalence of tannins in browses, no simple method exists for assessing their effects on the nitrogen digestibility of forages consumed by mammalian herbivores.

Existing methods for analysing tannins have several shortcomings. First, many methods measure total phenolics or tannins, but do not measure their biological effects. For example, colorimetric assays, such as vanillin and HCl/butanol reactions, are nonspecific, difficult to replicate consistently and cannot predict the nutritional responses of animals to tannins (Hagerman and Butler 1989; Mueller-Harvey 2006). Second, methods that do attempt to measure the protein-precipitating capacity of tannins, such as BSA-binding, are difficult or expensive. Third, contamination of the cell wall constituent (fibre) residues by tannins means that the extraction of tannins is incomplete (Makkar and Singh 1991; McArthur 1988). Regardless, a bigger difficulty has been to integrate the results in order to interpret their ecological significance, since animals do not eat tannins, protein or fibre in isolation. An exception was the work of Robbins et al. (1987), who used intensive in vivo digestion trials to validate an equation using protein-precipitating capacity, along with neutral detergent fibre analysis, to predict the protein and energy digestibility of forages for deer.

Polyethylene glycol (PEG), a nonabsorbable tannin-blocking agent, together with in vitro digestion, provides a potential assay for ranking the effects of tannins on the N digestibility of plants. Previous studies have shown that animals eat more and digest more of tannin-containing foods when they ingest PEG (Decandia et al. 2000; Foley and Hume 1987; Gilboa et al. 2000; Marsh et al. 2003a; Marsh et al. 2003b; Min et al. 1999; Silanikove et al. 1994, 1996a). Silanikove et al. (1996b) developed an assay using PEG that predicts the effects of tannins on the microbial metabolism and the availability of N from feeds, which has been widely applied in agriculture (Getachew et al. 2000; Jones and Palmer 2000; Makkar 2003; Palmer and Jones 2000; Silanikove et al. 2001). Here we describe and test our modification of Silanikove et al.'s technique, designed to quickly and easily evaluate the effects of tannins on the N digestibility of foliage, for use in ecological studies.

We first measured the PEG-binding capacity (PEG-BC) of a range of different *Eucalyptus* leaves and then measured the effect of PEG on DM and N digestibility in vitro. We also evaluated two simplifications that would allow many samples to be measured quickly and in less specialised laboratories. We assessed whether measuring DM and N digestibility alone, without the need to use radioactive PEG, could provide an even simpler method of quantifying the integrated effects of leaf N, tannins and cell wall constituents. We then used near-infrared reflectance spectroscopy (NIRS) to develop predictive equations for the rapid analysis of the digestibility of leaf samples (Foley et al. 1998). To demonstrate the application of our method, we used NIRS to examine the variability in both total and digestible N content of over 300 eucalypts from eleven sites in northern Australia.

Methods

In vitro determination of PEG-binding capacity, dry matter digestibility and nitrogen digestibility

Sample collection and preparation

We collected mature leaf samples from 77 trees of ten *Eucalyptus* species in the series Siderophloiae (*E. crebra*, *E. drepanophylla*, *E. elegans*, *E. fibrosa* ssp. *nubila*, *E. granitica*, *E. melanophloia*, *E. rhombica*, *E. shirleyi*, *E. siderophloia* and *E. taurina*) at several sites in Queensland, Australia. We froze the samples on pellets of solid CO_2 in the field and later freeze-dried them; or we dried them over silica desiccant in the field. Finally, we ground all samples to pass a 1-mm screen in a Tecator (Höganäs, Sweden) Cyclotec mill.

In vitro determinations

To quantify the protein-binding component of tannins, we measured PEG-BC, largely following the original method of Silanikove et al. (1996b). The in vitro pepsin/cellulase digestion that followed emulates digestion in mammalian herbivores, with pepsin simulating protein digestion and a crude cellulase preparation digesting fibre. We used a modification of the procedures of Choo et al. (1981) and Jones and Palmer (2000). Briefly, for each sample we weighed 800 ± 10 mg of dry leaf into six pre-weighed (tube + lid), dry 50-ml plastic centrifuge tubes (Iwaki, Tokyo, Japan) destined for three treatments. To two of these tubes we added 7.5 ml of a solution containing 33.33 g l^{-1} of PEG 4000 (analytical grade, Sigma, St. Louis, MO, USA) in 0.05 M Tris–BASE buffer (Sigma) spiked with 1.85 MBq ^{14}C-labelled PEG 4000 (GE Healthcare Life Sciences, Uppsala, Sweden), to measure PEG-BC. To a second pair of tubes we added 7.5 ml of a

solution containing 33.33 g l^{-1} PEG 4000 in 0.05 M Tris–BASE buffer. These tubes mimicked the first set of tubes and provided a nonradioactive sample for later laboratory analyses. To the final pair of tubes we added 7.5 ml of 0.05 M Tris–BASE buffer as a control. The pH of every solution was 7.1. We mixed the tubes on a vortex mixer before placing them horizontally in an oven at 37°C for 24 h with occasional shaking. Note that we did not measure PEG-BC in tannin-free plant material to correct for non-specific binding because different substrates give widely differing values (Mlambo and Makkar 2005).

After incubation, we centrifuged all tubes at 4,190×*g* for 10 min, before decanting about 1.5 ml of the supernatant from the tubes spiked with [^{14}C]-labelled PEG into a 2-ml micro tube with a screw cap containing an O-ring seal (Sarstedt, Leicester, UK). This provided ample for any repeat counting. We then weighed (±0.1 mg) about 75 µl into glass scintillation vials containing 10 ml of scintillant (Packard Emulsifier-Safe, Groningen, The Netherlands) and counted them for 10 min or to a precision of 1.5% in a Beckman (Fullerton, CA, USA) LS 6500 scintillation counter. In a similar way, we prepared and counted triplicate 75 µl samples of the working solution and of 0.05 M Tris–BASE buffer to determine, respectively, the amount of radiation added to each tube and the background radiation.

After removing the subsample of supernatant for counting, we washed the samples to remove any remaining PEG or buffer. This involved pouring the supernatant from all tubes before adding 25 ml of distilled water, mixing thoroughly on a vortex mixer, centrifuging as described above, and repeating the process. We then started the in vitro protein digestion by adding 35 ml of solution containing 2 g 1:10,000 pepsin (Bacto, Liverpool, Australia) in 1 l 0.1 N HCl (pH 1.0) to each tube, mixing thoroughly, then incubating them horizontally in an oven at 37°C. After 24 h, we washed the samples as described earlier, before starting the cellulase digestion. In this step we added 35 ml of a solution containing 6.25 g cellulase (Onazuka 3S, Lab-Chem, Pittsburgh, PA, USA), 6.8 g sodium acetate and 2.9 ml glacial acetic acid per litre (pH 4.8) to each tube. After 48 h of incubation at 37°C, we washed the residues twice. After drying the tubes and their contents to a constant mass in an oven at 50°C, we weighed them to determine the dry residue before discarding the tubes containing radioactive material. We then determined the N content of the residues from the four remaining tubes for each sample.

Nitrogen analysis

We determined the concentration of N in leaves on duplicate samples (250 ± 5 mg) of material using the semi-micro-Kjeldahl technique with a Tecator 2012 digester, selenium catalyst and a Vapodest 5 analyser (Gerhardt, Germany). Concentrations of N in the residues were determined singly for each tube, as the residue mass was generally insufficient (approx. 400 mg) to allow duplicate analyses. We dried a separate sample (1 g ± 5 mg) of freeze-dried, ground foliage to constant mass at 60°C to determine residual DM.

Calculations

$$\text{PEG - BC (g per 100 g DM)} = 100 \times \frac{(C_{st} - C_{sn}) \times A_{PEG}}{(C_{st} - \text{Sw})}$$

where C_{st} are the ^{14}C counts, corrected for background counts, added to the tube, and C_{sn} are the ^{14}C counts, corrected for background, in the supernatant at the end of the incubation. A_{PEG} is the amount of PEG (g) added to the tube and Sw is the dry weight (g) of the plant tissue.

$$\text{DM digestibility } (\%) = 100 \times \frac{\text{Sw} - (\text{Sr} - \text{PEGb})}{\text{Sw}}$$

where Sw and Sr are the dry masses (g) of the sample and the sample residue respectively, and, because PEG is indigestible, PEGb is the mass (g) of PEG bound.

$$\text{N digestibility (\% DM)} = 100 \times \frac{(N_{leaf} - N_{res})}{N_{leaf}}$$

where N_{leaf} and N_{res} are the amounts of N (mg of DM) in the original leaf and in the residue, with the mass of the latter corrected for the mass of bound PEG.

The amount of digestible N (% DM) in the leaf was determined by multiplying the N digestibility of each sample (%) by the concentration of N in the original leaf sample (g per 100 g DM).

We analysed the relationships between PEG-BC, DM digestibility and N digestibility using simple linear regression in GenStat (9th edition).

A simplified method to determine digestibility without using PEG

Many researchers may only be interested in overall digestibility, rather than specifically ascribing effects to tannins, so we tested whether an in vitro DM and N digestibility alone, without the addition of PEG, could provide an even simpler method of quantifying the integrated effects of leaf N, tannins and cell wall constituents. This simplification also reduces problems associated with performing the assay in plastic tubes, such as the time it takes to wash the samples and the risk of losing some sample when decanting the supernatant. To study this, we digested a subset of the original samples in bulk and

compared the results to those achieved in "In vitro determination of PEG-binding capacity, dry matter digestibility and nitrogen digestibility". Of the samples already analysed, we selected the 22 with the lowest coefficients of variation between replicates. In duplicate, we weighed 800 ± 10 mg of dry, ground leaf sample into an oven-dry pre-weighed filter bag (ANKOM F57; ANKOM Technology, Macedon, NY, USA) and heat-sealed the bag. We then placed all bags in a 4-l conical flask, to which we added pepsin solution (see section "In vitro determinations") at a volume of 35 ml per bag, and then placed the flask on a magnetic stirrer in an oven at 37°C for 24 h. After incubation, we washed the samples five times in distilled water and replaced the pepsin solution with cellulase solution (35 ml per bag), as before. We then placed the samples back in the oven on the stirrer for 48 h. At the end of this incubation, we washed the filter bags ten times, dried them to constant mass at 60°C, and then reweighed them to determine the amount of leaf digested. We analysed the concentration of N in the original leaf and in the residue, as described in "In vitro determination of PEG-binding capacity, dry matter digestibility and nitrogen digestibility".

We tested the relationship between the in vitro DM digestibility and N digestibility values from the full assay in the centrifuge tubes and this simplified method using major axis regression in the program SMATR (Falster et al. 2006).

Using near-infrared reflectance spectroscopy to predict digestibility in the presence and absence of PEG

To apply our method to large numbers of samples, we used NIRS to predict the eight chemical traits measured in the laboratory assays, including DM digestibility; N digestibility and digestible N concentration in the presence and absence of PEG; PEG-BC; and N concentration. For most traits, we used the results from the 77 trees sampled for the in vitro assay, while the equation for PEG-BC came from a larger calibration set encompassing a wide range of eucalypt species.

We recorded the reflectance spectrum of each sample between 400 and 2,498 nm using an NIRSystems 6500 scanning spectrophotometer with spinning cup attachment (Foss Silver Spring, MD, USA), following the procedure described by Moore et al. (2004b), and developed calibrations using the software WinISI 3, version 1.50E (Infrasoft International, Port Matilda, PA, USA). Briefly, we modelled the relationship between the trait of interest and the spectral characteristics of ground foliage by modified partial least squares regression (Shenk and Westerhaus 1991), following the standard principles described by the American Society for Testing and Materials (ASTM 1995). We performed two cycles of outlier elimination to remove samples with '*H*' values greater than 10.00, '*F*' values greater than 8.00 and '*T*' values greater than 2.5 (Shenk and Westerhaus 1991). For each measure we selected the combination of wavelengths, mathematical derivation and smoothing of the spectra and particle size correction that produced the best model. This was the model that minimised the standard error of cross-validation (SECV) but maximised the standardised SECV—the ratio of the standard deviation of the sample set to the SECV and the coefficient of determination (r^2) between the spectra and the analytical values (the r^2 of calibration) (Table 1).

Variation in the total and digestible nitrogen contents of foliage across a landscape

To demonstrate the application of our assay, we used our NIRS models to predict the concentrations of total N and digestible N in mature foliage from five *Eucalyptus* species (*E. crebra*, *E. drepanophylla*, *E. exilipes*, *E. granitica* and *E. shirleyi*) of the series Siderophloia. We sampled 322 trees at 11 sites in savanna woodland in northern Queensland (Table 2). Samples were prepared as described in "In vitro determination of PEG-binding capacity, dry matter digestibility and nitrogen digestibility".

Statistical analysis

We examined the relationship between available and total N both within and between sites, with simple linear regression, using total N as the independent variable. Secondly, we used an *F* test to determine whether the digestible N content of leaves varied more than the concentration of total N (F = sum of squares for digestible nitrogen/sum of squares for total nitrogen). For both the regression and the comparison of variation, we used mean site data for the between-site comparison.

Results

In vitro determination of PEG-binding capacity, dry matter digestibility and nitrogen digestibility

There was no relationship between PEG-BC and DM digestibility of eucalypt foliage either in the presence ($r^2 = 0.02$, $F_{(1,75)} = 1.51$, $P = 0.22$) or absence of PEG ($r^2 = 0.03$, $F_{(1,75)} = 2.48$, $P = 0.12$). However, adding PEG did cause a small but significant improvement to DM digestibility (paired t-test, $t_{76} = 1.75$, $P = 0.04$, mean difference ± SE = 1.68 ± 0.96). In contrast to the results for DM digestibility, there was a strong negative

Table 1 Near-infrared reflectance spectroscopy modified partial least squares regression models for predicting foliar concentrations of polyethylene glycol-binding component (PEG-BC), nitrogen (N), dry matter digestibility (DMD), nitrogen digestibility and digestible nitrogen

Constituent	Calibration samples[a]			r^2	SECV[b]	SECV/SD	Math[c]	Scatter correction[d]
	n	Mean	SD					
PEG-BC	106	10.29	N/A	0.95	1.18	N/A	2,8,8,1	SNV + D
N	72	1.07	0.16	0.95	0.04	4.0	2,8,8,1	SNV + D
DMD + PEG	72	45.15	5.60	0.84	2.26	2.5	2,8,8,1	SNV + D
DMD	75	44.36	9.44	0.98	2.80	3.4	2,4,4,1	SNV + D
Digestible N + PEG	74	0.78	0.19	0.96	0.06	3.2	2,8,8,1	SNV
Digestible N	73	0.26	0.28	0.98	0.10	2.8	2,4,4,1	SNV + D
N digestibility + PEG	74	71.47	10	0.92	4.19	2.4	2,6,4,1	SNV
N digestibility	72	22.93	25.94	0.99	8.42	3.1	2,4,4,1	SNV + D

The annotation + PEG indicates treatments with the addition of PEG. We used the full spectrum (408–2,492 nm) for all constituents

[a] PEG-BC is measured as g/100 g dry mass; all other measures are presented as percentage of dry mass. n, mean and range refer to the calibration set

[b] Standard error of cross-validation

[c] *Math* describes the mathematical treatment applied to the spectra [captured log(1/reflectance)]. The first two numbers describe the derivative used, while the third and fourth numbers indicate the degrees of primary and secondary smoothing performed on the derivative. Thus 2,4,4,1 indicates that the second derivative was calculated with a gap size of 4 nm and that a maximal primary smooth (4) but no secondary smooth (1) was used

[d] SNV and detrend transformations are described by Barnes et al. (1989). SNV is the standard normal variate and D refers to detrend

Table 2 Summary of sites for the collection of samples for comparing the nutritional quality of *Eucalyptus* foliage

Site number	Site name	Location	Sample size	Species	Drying method
1	Blackbraes National Park	19.5228°S, 144.1849°E	28	*E. crebra*	Silica desiccant
2	Blackbraes National Park	19.5050°S, 144.1511°E	28	*E. crebra*	Silica desiccant
3	Blackbraes National Park	19.5444°S, 143.951°7E	28	*E. crebra* and *E. exilipes*	Silica desiccant
4	Hidden Valley	18.9720°S, 146.0104°E	28	*E. crebra*	Freeze-dried
5	Mount Fox	18.8232°S, 145.7900°E	30	*E. crebra*	Freeze-dried
6	Tabletop Station	19.3959°S, 146.4654°E	30	*E. drepanophylla*	Freeze-dried
7	Tabletop Station	19.3550°S, 146.4217°E	30	*E. drepanophylla*	Freeze-dried
8	Taravale	12.1909°S, 146.0516°E	30	*E. crebra*	Silica desiccant
9	Taravale	19.1152°S, 146.1051°E	24	*E. crebra*	Freeze-dried
10	Taravale	19.1269°S, 146.0792°E	35	*E. granitica*	Freeze-dried
11	Taravale	19.1269°S, 146.0792°E	30	*E. shirleyi*	Freeze-dried

relationship between PEG-BC and N digestibility ($r^2 = 0.36$, $F_{(1,75)} = 42.80$, $P < 0.001$) (Fig. 1a). The addition of PEG largely negated this relationship ($r^2 = 0.14$, $F_{(1,75)} = 12.61$, $P = 0.001$) and increased N digestibility markedly (paired *t*-test, $t_{76} = 22.62$, $P < 0.001$, mean difference ± SE = 48.70 ± 2.15). The difference between slopes of the regressions between nitrogen digestibility and PEG-BC with and without PEG (Fig. 1a) indicates an interaction between PEG-BC and potential improvement in N digestibility. Samples with greater PEG-BC (higher concentrations of tannins) had considerably more scope for improved N digestibility when PEG blocked the tannins ($r^2 = 0.39$, $F_{(1,75)} = 48.54$, $P < 0.001$) (Fig. 1b), leading to a significant negative relationship between the concentration of digestible N and the PEG-BC of foliage ($r^2 = 0.35$, $F_{(1,75)} = 41.18$, $P < 0.001$) (Fig. 2). A notable result is the preponderance of negative values for digestible N among samples with high PEG-BC.

A simplified method to determine digestibility without using PEG

Major axis regressions revealed highly significant relationships between samples digested in ANKOM bags and those digested in tubes for both DM digestibility ($r^2 = 0.88$, $P < 0.001$, $n = 22$) and N digestibility ($r^2 = 0.88$, $P < 0.001$, $n = 22$). For DM digestibility, the slope

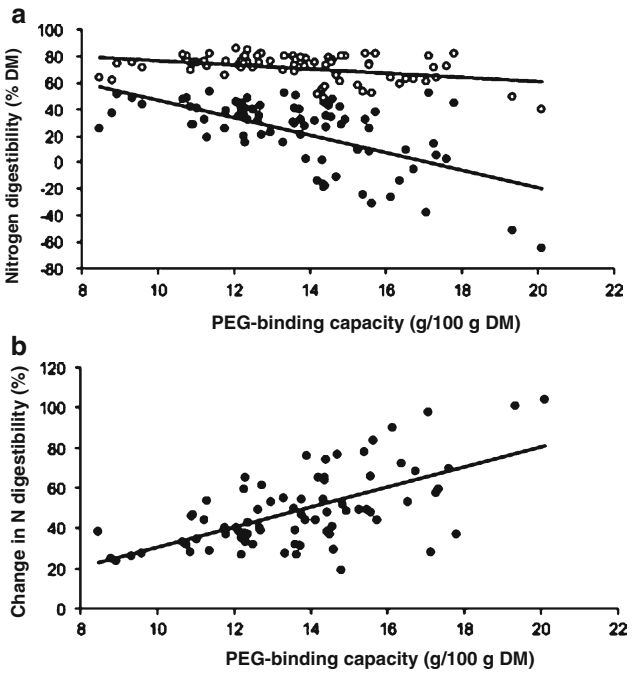

Fig. 1 **a** Relationship between the PEG binding capacity and nitrogen digestibility of *Eucalyptus* foliage, both in the presence ($r^2 = 0.14$, $P = 0.001$, $n = 77$, *open circles*) and the absence ($r^2 = 0.36$, $P < 0.001$, $n = 77$, *filled circles*) of PEG. **b** Relationship between the PEG-binding capacity of leaves and the change in nitrogen digestibility due to the effects of PEG ($r^2 = 0.39$, $P < 0.001$, $n = 77$)

of the regression was not significantly different from 1 (slope = 1.12, 95% C.I. = 0.95–1.34, $F = 2.06$, $P = 0.17$) and the intercept was not significantly different from zero (intercept = −3.86, 95% C.I. = −13.16–5.44, $t = -0.87$, $P = 0.40$). For N digestibility, however, the slope of the regression was significantly greater than 1 (slope = 1.47, 95% C.I. = 1.24–1.76, $F = 21.97$, $P < 0.001$), and the intercept less than 0 (intercept = −14.85, 95% C.I. = −24.97–−4.73, $t = -3.06$, $P = 0.006$).

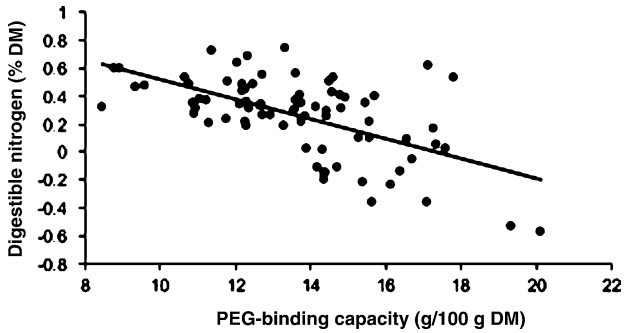

Fig. 2 Relationship between the PEG-binding capacity and the digestible nitrogen concentration of *Eucalyptus* foliage ($r^2 = 0.35$, $P < 0.001$, $n = 77$)

Using near-infrared reflectance spectroscopy to predict digestibility in the presence and absence of PEG

We successfully generated NIRS calibrations for all measures of interest. In most cases the r^2 of calibration exceeded 0.95 with a standardised SECV of greater than 3 (Table 1). The exception was the model for the DM digestibility of samples with PEG, which gave an r^2 of 0.84 and a standardised SECV of 2.5.

Variation in the total and digestible nitrogen content of foliage across a landscape

The values for digestible N in the 322 leaf samples collected across northern Australia were far more variable than were those for total N (Fig. 3). The concentrations of digestible N were significantly more variable than were those for total N both between sites ($F_{(10,10)} = 6.72$, $P < 0.005$), and usually within sites (8 of 11 sites). A result of this variability is that there was either no relationship or a poor relationship between the concentrations of digestible N and total N. There was no relationship between total and digestible N across sites ($r^2 = 0.006$, $F_{(1,9)} = 0.059$, $P = 0.81$) and often no relationship between these measures within sites (7 of 11 sites) (Table 3).

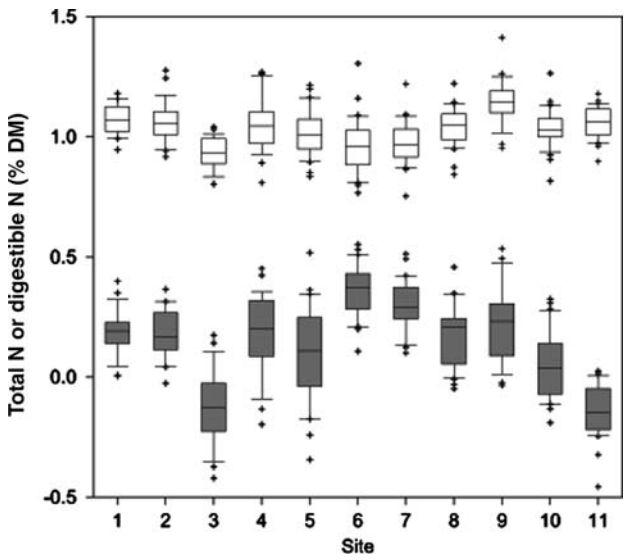

Fig. 3 *Box and whisker plots* showing the concentrations of total leaf nitrogen (%DM) (*open boxes*) and digestible nitrogen (%DM) (*filled boxes*) in *Eucalyptus* foliage at 11 sites. The *boxes* incorporate the 25–75% quartiles and the median while the *whiskers* cover the range of values

Table 3 Comparisons of the variability of digestible nitrogen and total nitrogen and the relationships between the two variables both within sites and between sites

Site	Variability		Regression		
	F_{df}	P	r^2	F_{df}	P
BB1	$F_{(27,27)} = 2.40$	0.01	0.03	$F_{(1,26)} = 0.70$	0.40
BB2	$F_{(27,27)} = 1.45$	NS	0.001	$F_{(1,26)} = 0.03$	0.85
BB4	$F_{(27,27)} = 5.45$	<0.001	0.001	$F_{(1,26)} = 0.05$	0.83
HV1	$F_{(27,27)} = 1.99$	<0.05	0.09	$F_{(1,26)} = 2.58$	0.12
MF1	$F_{(29,29)} = 4.30$	<0.001	0.20	$F_{(1,28)} = 6.99$	0.01
TT1	$F_{(29,29)} = 0.88$	NS	0.33	$F_{(1,28)} = 13.96$	<0.001
TT2	$F_{(29,29)} = 1.31$	NS	0.099	$F_{(1,28)} = 3.08$	0.09
TV3	$F_{(30,30)} = 2.19$	<0.05	0.068	$F_{(1,29)} = 2.10$	0.16
TVc	$F_{(23,23)} = 2.81$	<0.005	0.34	$F_{(1,22)} = 11.60$	0.002
TVg	$F_{(34,34)} = 2.80$	<0.005	0.06	$F_{(1,33)} = 2.09$	0.16
TVs	$F_{(29,29)} = 2.95$	<0.005	0.23	$F_{(1,28)} = 8.50$	0.007
Between sites	$F_{(10,10)} = 6.72$	<0.005	0.006	$F_{(1,9)} = 0.059$	0.81

Discussion

Our three-step method of measuring N in leaf samples before and after incubating them with or without PEG and digesting them with pepsin and cellulase provides a simple method for evaluating how tannins influence the nutritional value of forages. This method provides measures of in vitro DM digestibility, in vitro N digestibility and the influence of tannins on these measures.

As expected, the main finding of our study was that a simple in vitro assay revealed a strong negative relationship between PEG-BC and the in vitro digestible N content of eucalypt leaves. In contrast, the addition of PEG slightly but significantly increased the in vitro DM digestibility of *Eucalyptus* foliage, but this was not related to PEG-BC. These results were consistent with other in vitro studies (Jones and Palmer 2000), although no simple relationship between PEG and DM digestibility has been apparent in vivo (Dalla Pozza 1993; Foley and Hume 1987; Marsh et al. 2003a; McArthur and Sanson 1991; Silanikove et al. 1996a). This variation likely reflects differences both in the types and concentrations of tannins and cell wall constituents in the plants, and physiological differences among the animals, such as gut structure, adaptations to a tannin-rich diet, nature of the microbial flora and food intake and passage rate through the gut.

A surprising result was the negative in vitro N digestibility values for samples with high PEG-BC, as the true digestibility should be 0 if tannins bind all of the N in the leaf. A simple explanation is that tannins also bind some of the added pepsin or cellulase, so additional N from the enzyme is present in the tube at the end of the digestion. The consequence is that there is more N in the residue than in the original leaf sample, giving an apparent negative digestibility. The tannin-enzyme complex appears insoluble, so the amount bound likely depends on the number of free tannin-binding sites. Negative apparent digestibilities are frequently observed in other in vitro (Getachew et al. 2000; Makkar 2005) and in vivo (Degen et al. 1995; Shimada and Saitoh 2003) studies of tannin-rich plants when tannins bind to endogenous N. Thus, the negative digestible N values observed here are not just an artefact of the in vitro assay. Our criticism of most methods for analysing tannins is that they provide a concentration, often in arbitrary units such as quebracho equivalents, without explaining how the food may affect an animal. Our method still provides information about concentration, measured as PEG-BC, but it also provides more relevant information about the potential effects of tannins on animal nutrition.

One result that is difficult to explain is the slight negative relationship between N digestibility and PEG-BC in samples incubated with PEG. We certainly added enough PEG to the samples, because the highest PEG-BC (ca. 20 g/100 g DM) consumes only 40% of the PEG added to the tube. One possibility is that there is a positive relationship between the N content of the cell wall material and PEG-BC, but confirming this requires additional laboratory work. Similarly, the addition of PEG does not result in complete digestibility of the N because a portion of the N in the leaf remains incorporated in the cell wall matrix (Van Soest 1994). In vivo studies of arboreal folivores feeding on *Eucalyptus* have shown that between 8 and 22% of the total leaf N remains bound in the cell wall after passage through the gut (Hume 1999). Thus, apart from tannins, the N content and digestibility of plant cell walls are additional factors that limit the availability of N. By measuring only digestibility and ignoring the concentrations of components such as tannins and fibre, our assay circumvents problems of interactions between these components during analysis and with interpreting the results.

One aspect of PEG-binding that is not well understood is the chemical nature of the bond (Foley et al. 1999; Foley and Moore 2005). There is good evidence that PEG binds with many tannins and thus prevents the formation of potentially indigestible tannin–protein complexes (Makkar 2003), but we have a much poorer understanding of whether PEG also binds to non-tannin phenolics. An exception is the formylated phloroglucinol compounds (FPCs), another important class of non-tannin phenolic compounds in eucalypts (Eschler et al. 2000). Marsh et al. (2003a) showed that PEG does not bind to FPCs, probably due to the strong hydrogen bonding within the FPC molecule. This suggests that measures of PEG-binding in eucalypts are primarily informing us about the role of tannins and this also means that we can study the effects of both classes of compounds simultaneously.

Our aim was to devise an assay that ranks plants in a way that is relevant to animals, which the in vitro method does. We used mature eucalypt foliage because it is the main food resource of several marsupial folivores, such as koalas (Martin and Handasyde 1999), and is available year-round. Our method, however, is perfectly suited to analysing other plant material, including mixtures of plant parts and growth stages from a variety of species. We did not attempt to do an in vivo validation because it was not our aim to mimic an animal's physiology. Furthermore, the appeal of an in vitro procedure is that it circumvents the inherent problems associated with demonstrating the effects of tannins in animals, such as providing sufficient PEG to completely bind tannins, or maintaining generalist herbivores on restricted diets. Another problem with in vivo validation is that it is species-specific. Our aim was not to produce an assay designed for a particular animal–plant system, but instead to develop one with universal appeal.

Our original method used radioactive PEG to measure the effects of tannins, but we realised that there are simpler methods to derive an index of N availability, both with and without PEG. By eliminating the ^{14}C-PEG step we could do the analysis in bulk, using ANKOM filter bags, originally designed for measuring detergent fibre (Vogel et al. 1999). Thus, this simplified method might be appropriate in laboratories where the use of radioactive substances is not possible. We found good rank agreement between the estimates for DM and N digestibility derived from the full assay in plastic tubes and the simpler method using filter bags. Again, it is important to recognise that any in vitro measure is only ever capable of achieving a rank correlation with an in vivo system. Therefore, we conclude that this simplified method can be satisfactorily used as an indicator of nutritional quality, even though it cannot directly ascribe differences in N digestibility unequivocally to tannins. If the interest is in knowing the exact effect of tannins, then researchers should digest a separate set of samples with nonradioactive PEG (see "Electronic supplementary material" for the complete method).

We further enhanced the practicality of the method by developing NIRS models to enable the analysis of very large numbers of samples. NIRS has been previously used in ecology and in agriculture to determine the nutritional quality of feeds (Landau et al. 2004; Landau et al. 2006; McIlwee et al. 2001). By incorporating the novel use of NIRS to predict in vitro digestibility, we have developed an approach that is suited to the scale of ecological studies, as it enables us to analyse the large number of samples needed to examine the chemical diversity faced by herbivores foraging in complex environments.

We demonstrated the ecological importance of our measure by applying these NIRS predictions to eucalypts growing in a range of habitats across a large geographical area. The consistently low concentrations of digestible N in leaves is indicative of the negative effect of tannins. More importantly, the fact that the digestible N concentrations of leaves varied significantly more than the total N concentrations did and the frequent lack of a relationship between the two variables questions measurements of nutritional quality based on total N only. This highlights that predictions concerning the nutritional suitability of foliage for herbivores made on the basis of total N rather than available N may be misleading, although ecologists have done just this for many years (e.g. Braithwaite et al. 1984; Pettorelli et al. 2001; White 1993).

In conclusion, we offer a simple method for describing the nutritional quality of foliage from the animal perspective in small nutritional studies or across landscapes. We argue that the simplicity of our technique, particularly the minimal equipment required, gives it advantages over other methods. The demonstration of a poor relationship between total and available N provides hope that our simple in vitro analysis combined with NIRS may help to better explain variation in the distribution and abundance of browsing mammals such as marsupials and primates.

Acknowledgements We are grateful to Euan Ritchie and Lewis Anderson for help with collecting leaf samples and Frances Marsh and Elesha Curran for assistance in the laboratory. We thank Raymond Jones for valuable advice and discussions and Glenn Iason and two anonymous reviewers for helpful comments on an earlier version of the manuscript. Ray and Betty Fryer, Brian Furber and the Australian Wildlife Conservancy kindly allowed us access to their land. Leaf samples were collected under Queensland EPA permit number—WISP03374205. Funding was provided by grants from the Australian Research Council to WJF and the Australia-Pacific Science Foundation to BDM. JLD was supported by an Australian National University Graduate School Scholarship and thanks Chris Johnson for advice.

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