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Determination of Poly(ethylene glycol)-Binding to Browse Foliage, as an Assay of Tannin, by Near-Infrared Reflectance Spectroscopy

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Nutritionists are interested in functional assays of tannins that do not require time-consuming and expensive extraction, such as the ^{14}C-labeled poly(ethylene glycol) (PEG)-binding (PEG-b) assay. This paper reports the application of near-infrared (NIR) spectroscopy to determine the percentage of PEG binding, in place of the ^{14}C-labeled PEG-b assay of tannin, in Mediterranean woodland vegetation. Calibration was done with 53 samples from 14 species and was validated on 25 samples from 10 species. PEG-b ranged between 1.4 and 20.7% in the samples. The calibration obtained by using the modified partial least-squares (MPLS) method, with all wavelengths in the 1100−2500 nm range combined, and the validation were reasonably linear ($R^2 = 0.96$ and 0.91, respectively). The accuracies, estimated from the standard errors of cross-validation and prediction, were ±1.6 and ±1.7% PEG-b, respectively. The NIRS-aided procedure proposed here can serve as an accurate, inexpensive, time-saving, and environment-friendly functional assay of tannin in Mediterranean browse.

KEYWORDS: NIRS; goats; nutrition; poly(ethylene glycol); pasture

INTRODUCTION

The role of grazing goats and, to a lesser extent, of cattle, in controlling brush encroachment, decreasing fire hazard, and increasing plant biodiversity in Mediterranean woodland and scrubland is acknowledged (*1*). In these environments, browse may represent 60% of goats' diets on a yearly basis (*2*). The foliage of many Mediterranean species contains tannin, which binds with dietary proteins and enzymes in the gastrointestinal tract (*3*) as a means of protection against herbivores. When browse leaves are given as sole feed, goats will consume them in amounts that are inversely correlated with tannin content (*3*−*5*); therefore, accurate analysis of tannin content is a prerequisite for the evaluation of the nutritional adequacy of browse for goats exploiting woodlands and scrublands and for monitoring the impact of grazing animals on these rangelands.

Chemically, tannins are complex phenolics that are broadly subdivided into two groups: hydrolyzable and condensed tannins (*4*). However, protein binding, the main nutritional feature of tannins, occurs with tannins in both categories (*6*); therefore, for nutritionists and ecologists, the protein-binding capacity of plant samples provides a more meaningful evaluation than a strictly chemically oriented analysis, because it reflects directly the antinutritional properties of tannins. Indeed, a recent inventory of chemical assays of tannins compiled by FAO/IAEA (*7*), in addition to addressing traditional chemical analyses for total phenolics, condensed tannins, and gallotannins, places a new emphasis on the determination of protein-precipitable phenolics, filter-paper assay of protein-binding capacity, and a method based on the precipitation of radiolabeled bovine serum albumin. All of these methods require preliminary extraction of tannins with aqueous methanol or aqueous acetone.

On the basis of the assumption that "there is considerable analogy between poly(ethylene glycol) (PEG)−tannin and protein−tannin complexation", Silanikove et al. (*8*) have devised an in situ method for the determination of tannin in plant samples. This method uses ^{14}C-labeled PEG binding (PEG-b) of tannins and does not require their pre-extraction. Since the publication of this method by Silanikove et al. (*8*), the functional approach of measuring PEG-b as an indicator of the effect of tannin on the nutritional value of forages has been adopted in numerous studies (*9*, *10*), and its usefulness has recently been acknowledged in a review on the analysis of condensed tannins (*11*). However, for some laboratories, safety issues and costs of working with and disposal of radioactive materials prevent the application of this method (*11*).

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Table 1. PEG Binding (Percent of Dry Matter) of Browse Samples in the Calibration and Validation Sets

	PEG binding		
	n	mean	SD
Calibration Set			
Arbutus unedo L.	1	15.9	
Asphodelus microcarpus L.	2	1.7	
Chamaerops humilis L.	3	4.3	0.7
Crataegus monogyna Jacq.	1	8.7	
Lonicera implexa Aiton	5	1.7	1.2
Mirtus communis L.	4	14.5	1.8
Osyris alba L.	1	10.8	
Pyrus amigdaliformis Will.	4	5.6	0.8
Pistacia lentiscus L.	6	20.7	1.6
Quercus ilex L.	8	7.1	2.9
Q. ilex L., acorns	1	6.3	
Quercus suber L.	6	6.7	1.9
Rhamnus alaternus L.	5	1.4	0.7
Rubus ulmifolius Schott.	4	7.8	2.7
Smilax aspera L.	2	2.1	
Validation Set			
Arbutus unedo L.	1	18.8	
Asphodelus microcarpus L.	1	2.0	
Chamaerops humilis L.	1	4.0	
Mirtus communis L.	3	15.7	3.1
Pistacia lentiscus L.	3	18.9	0.8
Pyrus amygdaliformis Boiss.	3	5.1	0.9
Quercus ilex L.	5	7.6	0.9
Quercus suber L.	4	7.6	0.3
Rhamnus alaternus L.	3	2.6	1.2
Rubus ulmifolius L.	1	8.4	

Analysis of organic component by near-infrared spectroscopy (NIRS) requires no preparation of samples, apart from oven-drying and subsequent grinding, and NIRS calibrations of condensed tannins for individual species such as *Leucaena leucocephala*, *Leucaena diversifolia* (*12*), *Lotus uliginosus* (*13*), and *Sericea lespedeza* (*14*) have been reported. The method has also been applied to other chemical attributes of Mediterranean browse species, apart from tannin content (*15*).

The purpose of the present study was to build a wide, multispecies NIRS calibration of PEG binding for use as a functional assay of tannin in Mediterranean browse species.

MATERIALS AND METHODS

Samples. Two sets of samples were collected in a woodland grazed by goats. The area is close to the Bonassai Experimental farm of the Istituto Zootecnico et Caseario per la Sardegna (41° N latitude) in Italy and was described previously (*16*). The calibration set consisted of 55 samples of browse from 14 species collected from March 1996 to July 1997 (**Table 1**). The validation set consisted of 25 samples of browse from 10 species usually consumed by goats and randomly selected from a collection of 160 samples gathered from November 1996 to July 2000. The samples were transported to the laboratory immediately after collection and dried in a forced-ventilation oven at 52 °C. Dried samples were ground in a mill to pass a 1 mm screen, thoroughly mixed, and stored in a plastic leak-proof bottle with screw closure to prevent any increase in humidity.

PEG Binding to Browse Samples. PEG-b to samples was fully described previously (*8*). In brief, 1 g aliquots of sample dry matter (DM) were mixed with 15 mL of a solution of PEG (MW 4000) in 0.05 M buffer Tris-BASE (Sigma) at pH 7.1 in 50 mL centrifuge tubes. The PEG working solution contained 100 g/L of PEG and 50 μCi/L of ^{14}C PEG 4000 (Amersham). The tubes were then placed horizontally for 24 h, with occasional mixing. After centrifugation for 30 min at 2500*g*, 40 μL of the supernatant was added to 4 mL of liquid scintillator (Insta-Gel II, Packard), and radioactivity was determined in a β counter (Beckman 7800). The radioactivity of 40 μL of the working solution in 4 mL of liquid scintillator served as standard and that of 40 μL of buffer solution in 4 mL of liquid scintillator served as a blank. The calculation of PEG-b was based on the assumption that labeled and nonlabeled PEG, and their respective radioactivities, exhibited the same binding capacities to the samples:

$$\text{PEG-b (g/100 g, DM basis)} = (C_{st} - C_{bl}) - (C_{sm} - C_{bl}) \times A_{PEG}/(C_{st} - C_{bl}) \times SW$$

C_{st}, C_{bl}, and C_{sm} are the ^{14}C counts of the standard, blank, and sample, respectively, A_{PEG} is the amount of PEG in the test tube, and SW is the dry weight of the plant tissue.

Table 2. Accuracy of Prediction by NIR Reflectance Spectroscopy of PEG Binding in Mediterranean Browse Samples, Using MPLS Calibration and the Calibration/Validation or Cross-Validation Procedures (SE Units Are Percentage on Dry Matter Basis)

calibration		validation			
SE calibration	R^2	SE prediction	R^2	slope	bias
1.2	0.96	1.7	0.91	0.91	0.14

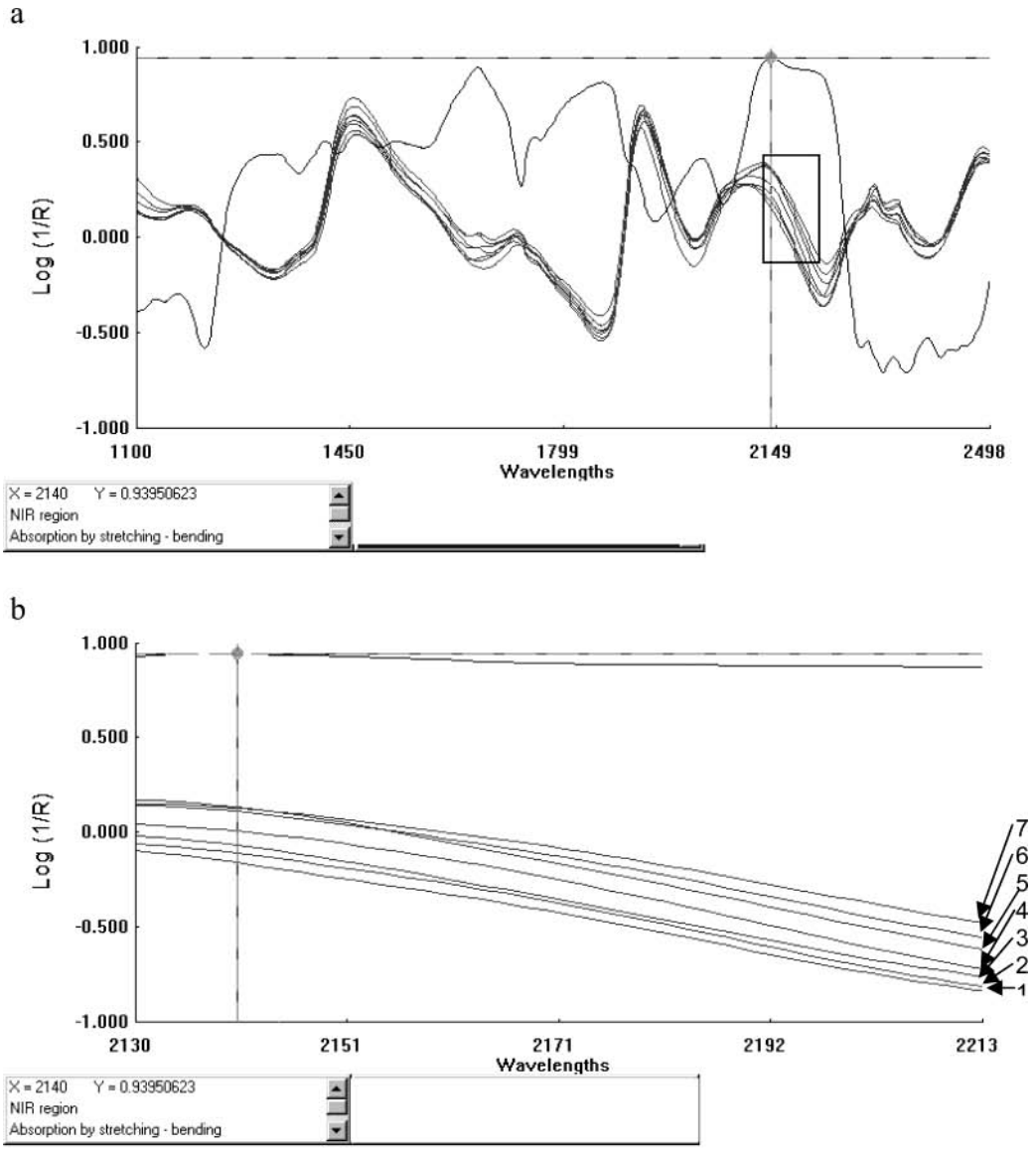
cross-validation		
SE calibration	SE cross-validation	R^2
1.2	1.6	0.96

NIRS Spectra and Calibration. Ground samples (~3−5 g) were scanned using a Foss NIRSystems 5000 reflectance apparatus (Foss Tecator, Hoganas, Sweden) with data collected in 2 nm wavelength steps from 1108 to 2492 nm. Reflectance was recorded as log(1/*R*), where *R* represents reflected energy. Each spectrum was paired with its PEG-b reference value in a calibration file, by using the appropriate WinISI procedures (*17*). The modified partial least-squares (MPLS) method, with all wavelengths in the 1108−2492 nm range, in increments of 4 nm, combined, was used to establish a relationship between the first derivative of log(1/*R*) and PEG-b, with a subtraction gap and smoothing segment of four data points ("1, 4, 4, 1" procedure) by means of WinISI software (*17*). Corrections of NIR spectra for particle size were applied by using the standard normal variance (SNV) and detrend procedure (*18*). The spectral data were analyzed to detect outliers, expressed by the *H* and *T* values (*19*). *H*, the Mahalanobis distance, was calculated by means of the WinISI "Score" procedure, from a multidimensional vector representing the distance between a test sample and the centroid of the elliptical distribution of all samples. *T*, which represents the distance between actual and predicted values, was calculated as part of the calibration process. Cutoff values were 1.5 and 2.5 for *T* and *H*, respectively (*20*). The linearity of the relationship between the spectral measurements and the PEG-b values was expressed by the R^2 of correlation between actual and predicted values of PEG-b. The calibration accuracy was evaluated in two ways: (i) by cross-validation, that is, by dividing the whole set of samples into six subsets and calibrating PEG in five of them and using the sixth for validating the equation, and (ii) by using for validation a set of samples collected independently from the calibration set, that is, at different locations and times.

Correlations between the first derivative of log(1/*R*) at increments of 2 nm and PEG-b were computed by means of the WinISI procedures (*17*) to identify NIR segments that best accounted for the chemical nature of the calibration.

RESULTS AND DISCUSSION

On a dry matter basis, PEG-b ranged between 1.4 and 20.7% and between 2.0 and 18.9% in the calibration and validation sets of samples, respectively (**Table 1**). Two outliers were removed from the calibration set of samples, one because of an *H* value and one because of a *T* value higher than the predetermined limits, that is, 6.71 and 2.95, respectively. All 25 browse samples were used for validation, as planned. Linearity ($R^2 = 0.96$, **Table 2**) was high in the calibration set



1	*A. microcarpus*	2.0
2	*P. amygdaloformis*	4.1
3	*Q. ilex*	6.6
4	*C. monogynus*	8.7
5	*M. communis*	15.2
6	*A. unedo*	15.9
7	*P.lentiscus*	18.0

Figure 1. Spectra of log(1/*R*) transformed by means of the standard normal variance and de-trend procedure of seven browse species differing in PEG binding and the correlation between log(1/*R*) and PEG binding (**a**). When spectra were focused (**b**) at 2140 nm—the wavelength of highest correlation—the browse species were correctly ranked with respect to PEG-binding values (percent of DM), shown under the graphs.

of PEG-b; it was lower in the validation set ($R^2 = 0.91$), but still acceptable for prediction purposes. For comparison, R^2 values for NIRS calibrations of total phenolics and condensed tannins in our laboratory (not shown) are not greater than 0.80 for this type of vegetation. The published R^2 values for NIRS calibrations of tannin in *L. leucocephala* (*12*), *L. uliginosus* (*13*), and *S. lespedeza* (*14*) were 0.84, 0.88, and 0.91, respectively, and those for validation were 0.85, 0.83, and 0.90, in the same order. The accuracy assessed by SECV of predicting PEG-b in browse, without extraction, was 1.6%, on DM basis with the cross-validation procedure, and 1.7% with SEP, in the calibration−validation procedure, similar to those reported elsewhere for condensed tannin after extraction (*13*, *14*).

When values of log(1/*R*), transformed by SNV and detrend, and their correlation with PEG-b were plotted against wavelength, correct ranking of the species, with respect to PEG-b, was obtained in the NIR regions of highest correlation, such as 2140 nm (**Figure 1**). The correlation between the first derivative of log(1/*R*) and PEG-b was highest ($R = -0.96$) at 2103 nm, featuring C−O and C−O−O stretch, and OH and OHCB bend for both the calibration (**Figure 2a**) and validation (**Figure 2b**) sets of samples. We have reported previously (*21*) that this NIR region is involved in bonding between PEG and quebracho tannin. Although C−O−O binding (depside bond) is characteristic of hydrolyzable tannins (*4*), unfortunately, it cannot be differentiated from C−O by NIRS. Therefore, the chemical

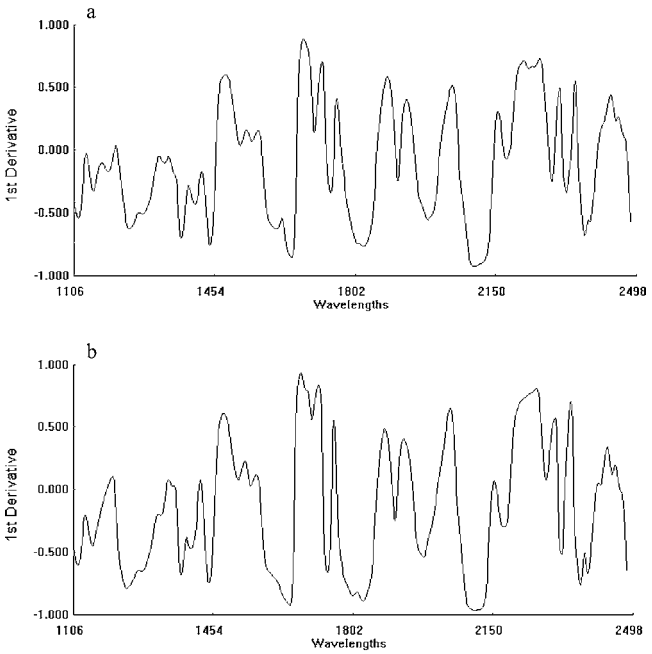


Figure 2. Correlation coefficients (*Y*-axis) between the first-derivative of log(1/*R*) and PEG binding of browse samples of the calibration (**a**; $n = 53$) and validation (**b**; $n = 25$) sets.

moieties that exhibit high correlation with PEG-b cannot be identified as condensed or hydrolyzable tannins. However, despite its chemical interest, such discrimination of tannin structural groups is not crucial, because antinutritional effects are found in both categories, as indicated by the negative linear relation between PEG-b and rumen degradation (*22*).

High correlations between the first derivative of log(1/*R*) and PEG-b were found at 1680−1688 nm (aromatic CH stretch, $R = 0.85$) and 1626 nm (CH and $=CH_2$, $R = -0.71$). A similar analysis of the calibration of condensed tannins in *L. uliginosus* identified segments between 2100 and 2200 nm, between 1660 and1670 nm, and between 1720 and1730 nm as best associated with condensed tannins, showing only partial overlap with our findings in Mediterranean browse. In *S. lespedeza* (*14*), best correlations between log(1/*R*) and individual wavelengths were found at 1664 and 2029 nm.

Even though our calibration of PEG-b in browse is highly linear, its accuracy can be considered low for application in species of low and medium tannin content. By pooling browse samples from the calibration and validation sets ($n = 80$) with samples of herbaceous pasture featuring low PEG-b ($n = 172$, PEG-b < 2.0%), deleting two outliers, and rerunning the calibration process, we achieved higher R^2 (0.98) and lower SE of cross-validation (0.64%). Even though the calibration set in this new calibration of PEG-b cannot be termed "in browse" and is beyond the scope of the present paper, widening the variation in samples and increasing the relative weight of low-tannin samples in the calibration set was a useful way to improve the accuracy of PEG-b analysis by NIRS and, probably, to increase its robustness. It is remarkable that the question of analytical accuracy and the best way of increasing it is addressed in studies on NIRS analysis of tannin (*13*, *14*), whereas most of the chemical−structural methods reviewed in refs *4*, *7*, and *11* address only the linearity of calibration curves and do not include validation.

The data presented here suggest that PEG binding can be evaluated by NIRS with high linearity and sufficient accuracy and can serve as an inexpensive and environment-friendly assay of tannin in Mediterranean browse. In a review on the ecological applications of NIRS (*23*), Foley and coauthors stated that "the ability of NIRS to predict functional aspects of plant secondary metabolites such as astringency or protein-precipitating capacity is unknown". The present study supports their hypothesis that NIRS-aided methods can contribute greatly to the acquisition of a deeper understanding of these functional aspects.

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